Universidade de Lisboa

Faculdade de Farmácia

TARGETING MIR-124 IN MOTOR NEURONS AS A THERAPEUTIC STRATEGY TO PREVENT NEURODEGENERATION AND GLIAL ACTIVATION IN ALS

Daniela Catarina Ribeiro Vizinha

Dissertation supervised by Professor Dora Maria Tuna de Oliveira Brites and co-supervised by Doctor Ana Rita Mendonça Vaz Botelho

Master Course in Biopharmaceutical Sciences

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Para a minha família

"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more so that we may fear less"
Marie Curie

RESUMO

A Esclerose Lateral Amiotrófica (ELA) é uma doença neurodegenerativa fatal que evolui de forma progressiva e que é caracterizada pela perda de neurónios motores (NMs) do córtex motor, tronco encefálico e medula espinhal (ME). Apesar de ser estudada há vários anos a ELA é uma doença ainda sem cura. Apenas existem duas terapias disponíveis para o seu tratamento, mas com pouco impacto na progressão da doença e na saúde dos doentes. Inicialmente pensava-se ser uma doença unicamente do NM, mas atualmente sabe-se que a ELA envolve também alterações nas células gliais. Estas apresentam diversas disfuncionalidades consideradas aberrantes, perdendo a sua função de suporte aos neurónios e muito contribuindo para a neurodegeneração e progressão da doença. Desconhece-se, contudo, quais os mecanismos de comunicação célula-célula que se encontram alterados e os mediadores envolvidos, quer solúveis, quer os contidos em vesículas extracelulares, como os microRNAs (miRNAs).

Os miRNAs não codificantes têm como função suprimir a expressão de genes através da sua ligação a mRNAs. Nos últimos anos, os miRNAs têm sido indicados como possíveis biomarcadores para o diagnóstico da doença e como alvos terapêuticos na ELA. Determinados miRNAs foram já encontrados como estando diferencialmente expressos, entre os quais alguns com reconhecida associação com vias conhecidas ou sugeridas como estando envolvidas na patogénese da ELA. A sua ação pode ser dirigida a um tecido, ou a um tipo de célula, sendo também elementos ativos na comunicação celular, sendo que afetam a expressão génica tanto das células de onde derivam, como das células onde vão atuar. O miRNA (miR)-124, um dos mais abundantes no sistema nervoso central (SNC) e normalmente associado à diferenciação neuronal, foi recentemente identificado como estando elevado no líquido cefalorraquidiano de doentes com ELA, bem como sobre-expresso em ratinhos com a mutação G93A no gene da SOD1 (mSOD1) nos locais de visível neurodegeneração, tendo sido por nós observado na linha celular de NMs NSC-34 com a mesma mutação, uma das mais abundantes nos casos familiares de ELA. Curiosamente, verificámos que a sua expressão estava aumentada em exossomas libertados desses NMs, os quais evidenciaram produzir ativação de diversos subtipos de microglia. Em condições fisiológicas o miR-124 encontra-se envolvido na maturação neuronal, bem como na organização do citoesqueleto celular, comprimento das neurites e processos associados à autofagia da célula. Uma desregulação na expressão de miR-124 tem sido encontrada em diversas doenças do SNC e associada a neurodegeneração, *stress*, desregulação neuro-imune e tumores cerebrais, entre outras.

Neste trabalho o nosso objetivo foi avaliar de que modo é que a inibição ou a sobre-expressão de miR-124 se refletia na estrutura e função dos NMs, bem como na imunorregulação da microglia e dos astrócitos. Para isso, usámos uma linha de NMs expressando SOD1 humana, quer não mutada (*wild type*, WT), quer com mutação G93A (mSOD1), de modo a mimetizar a acumulação desta proteína e a degeneração neuronal observadas na ALS. Para avaliar de que modo o secretoma de NMs WT, após tratamento com pre-miR-124, e o de NMs mSOD1 modulados com anti-miR-124, influenciava a reatividade da microglia após 4 h de incubação e a dos astrócitos após 24 h de tratamento, usámos a linha de células microgliais de ratinho N9 e os astrócitos isolados da ME de ratinhos transgénicos SOD1G93A, com 8 dias de idade. Qualquer uma das modulações não teve reflexo na viabilidade dos NMs e a acumulação de mSOD1 foi evitada com o anti-miR-124. Este mostrou também prevenir o aumento de expressão génica e proteica da *High Mobility Group Box 1* (HMGB1) e da proteína *inducible Nitric Oxide Synthase* (iNOS), levando ainda a uma diminuição da expressão génica da S100 *Calcium-binding Protein B* (S100B) nos neurónios mutados. A sobre-expressão do miR-124 nos NMs WT apenas se traduziu num aumento de S100B mRNA. A nível dos miRNAs, o anti-miR-124 nos NMs mSOD1 foi capaz de diminuir a sobre-expressão de miR-125b e aumentar o miR-21 e o miR-146a, que se encontravam reduzidos. Curiosamente, o pre-miR-124 nos NMs WT conduziu ao perfil evidenciado pelas células mutadas, ou seja, a um aumento de miR-125b e a uma diminuição de miR-21 e de miR-146a, comprovando a sua contribuição em tal desregulação. Importante foi ainda observar que o anti-miR-124 reduziu a expressão do marcador pré-sináptico sinaptofisina e da proteína motora dineína, ambas elevadas nos NMs mSOD1 e WT tratados com pré-miR124, para valores próximos das células WT, ficando claro que o pre-miR-124 está por detrás de tais aumentos. Pelo contrário, este mostrou estar ligado à diminuição do marcador pós-sináptico *Postsynaptic Density Protein* 95 (PSD-95) e da proteína motora cinesina, já que o anti-miR-124 levou a um marcado aumento da expressão génica destas proteínas. Podemos então concluir que o anti-miR-124 favorece o transporte anterógrado (do soma para a extremidade do axónio), deficiente nas células mutadas, nas quais a elevação da dineína privilegia o transporte retrógado (de volta ao soma) associado à elevação da expressão de miR-124. Esta, mostrou estar também interligada com o menor número de ramificações e de neurites primárias, bem como do seu comprimento, as quais foram corrigidas para valores próximos dos do controlo pelo anti-miR-124 nos NMs mutados, assim indicando a sua influência no citoesqueleto celular, morfologia e funcionalidade dos NMs. De facto, o anti-miR-124 foi capaz de recuperar a viabilidade mitocondrial nos NMs mSOD1, cuja diminuição poderá estar relacionada com o aumento de expressão de miR-124, bem como ainda de favorecer a expressão de Mitofusina (que induz fusão da mitocôndria), a qual se encontrava diminuída nas células mutadas, bem como diminuir a de DRP1 (associada à fissão

da mitocôndria) que estava aumentada nas mesmas. Mais uma vez a sobre-expressão de miR-124 mostrou ser responsável pelo aumento de DRP1 observado nas células mutadas, se bem que não evidenciou associação com a Mitofusina. Tais modificações são sugestivas da existência de stress oxidativo associado à sobre-expressão de miR-124 e que o anti-miR-124 poderá atuar de forma benéfica por favorecer mecanismos anti-oxidantes e protetores da mitocôndria.

Relativamente à ação imunoreguladora, verificámos que tanto o aumento da área, perímetro e diâmetro de *Ferrett's*, como a diminuição de circularidade da microglia após tratamento com o secretoma dos NMs mutados, não se detetavam quando a célula era tratada com o secretoma proveniente dos modulados com o anti-miR-124, sendo que tais parâmetros mostraram correlação direta com a sobre-expressão deste miRNA. De realçar, que a capacidade fagocítica da microglia revelou ser afetada quando exposta ao secretoma dos NMs WT tratados com pré-miR-124, ou ao proveniente dos mSOD1 não modulados ou modulados com anti-miR-124, indicando que este não é suficiente para repor a capacidade fagocítica da microglia. Contudo, foi de novo muito evidente a eficácia da utilização do anti-miR-124 nos NMs mutados quando o seu secretoma se mostrou eficaz na diminuição da expressão génica de iNOS, arginase, *Tumor Necrosis Factor alpha* (TNF-α) e *Interleukin 1 beta (IL-1β)*, todas elas aumentadas tanto na microglia tratada com o secretoma proveniente dos NMs mSOD1, como na tratada com o de NMs WT modulados com pre-miR-124. Ainda, conseguiu diminuir tanto a expressão de HMGB1, muito aumentada pelo secretoma de NMs mSOD1, como elevar a expressão do recetor da manose, o CD206, associado à endocitose e fagocitose nos macrófagos. Curiosamente, encontrámos aqui também uma correlação, desta vez indirecta, com a sobre-expressão neuronal do miR-124, cujo secretoma decresce a expressão de CD206. Quanto ao efeito do secretoma de NMs mSOD1 tratados com anti-miR-124 nos astrócitos WT e mSOD1, verificou-se que ele foi capaz de preservar a expressão do *glutamate transporter* 1 (GLT-1), encontrado diminuído nos astrócitos WT e mSOD1 após incubação com o secretoma dos NMs WT tratados com pre-miR-124 e com o dos mutados não tratados. Efeito semelhante foi obtido para a *Glial Fibrillary Acidic Protein* (GFAP), cuja expressão aumentou relativamente aos astrócitos WT e mSOD1 tratados com o secretoma dos NMs mSOD1, bem como aos WT expostos ao secretoma dos NMs WT tratados com pre-miR-124. Pode-se então pensar que a expressão elevada do miR-124 no NM mutado é, pelo menos em parte, responsável pela produção de um secretoma que determina a diminuição da expressão de GFAP e de GLT-1, uma característica dos astrócitos aberrantes na ALS.

Em suma, os resultados obtidos sugerem que a sobre-expressão do miR-124 nos NMs mSOD1 se encontra envolvido na disfuncionalidade e degeneração destas células e contribui marcadamente para a patogénese e progressão da ALS ao comprometer a homeostasia

celular em resultado de um secretoma com propriedades imunoestimuladoras e indutor de características aberrantes nos astrócitos, quer em células saudáveis como em mutadas. O anti-miR-124 revelou ter efeitos benéficos na prevenção da acumulação de mSOD1 nos NMs, exercendo ação reguladora da transmissão sinática (aumento da árvore neurítica, da cinesina e da PSD-95), anti-oxidante (maior viabilidade mitocondrial, aumento de Mitofusina e diminuição de DRP1) e anti-inflamatória (subida de miR-21 e miR-146a, com diminuição de miR-125b). Para além disso, os NMs mSOD1 modulados com anti-miR-124 revelaram produzir um secretoma que suporta a funcionalidade da microglia (manutenção de uma morfologia normal e expressão de iNOS, Arginase, TNF-α e IL-1β e HMGB1 semelhante à encontrada após incubação com o secretoma de NMs WT), bem como a dos astrócitos (aumenta a expressão de GLT-1 e de GFAP), para além de elevar a expressão do recetor microglial CD206, associado à endocitose e à fagocitose. Desta forma, podemos inferir que a diminuição da expressão de miR-124 para níveis basais nos NMs mutados sustenta a sua funcionalidade e previne a reatividade das células gliais mediada por um secretoma com propriedades imunoestimuladoras, contrariando a neurodegeneração e a progressão da doença.

Em conclusão, este trabalho sugere que o *targeting* do miR-124 especificamente nos NMs mSOD1 poderá ser uma estratégia terapêutica muito promissora e abrangente para o tratamento da ELA, ao permitir não só a recuperação da funcionalidade neuronal, mas também a prevenção da ativação das células gliais, significando a sua atuação em diversos alvos à semelhança do pretendido com a terapia combinada por associação de fármacos.

Palavras-Chave: Degeneração do neurónio motor; Medula Espinhal; Modulação do miR-124; Reatividade glial; Secretoma

ABSTRACT

Amyotrophic Lateral Sclerosis (ALS) is a progressive and fatal neurodegenerative disease characterized by the loss of motor neurons (MNs) from the motor cortex, brainstem and spinal cord (SC). Despite being studied for several years, there are only two available therapies, each one playing a modest impact on disease outcome. Initially, it was thought initially that the disease was only caused by motor neuron degeneration, but today it is known that ALS is a non-cell-autonomous disease involving glial cells, which show several dysfunctionalities considered to be aberrant. Glial cells were shown to lose their neuro-supportive function and to contribute to neurodegeneration and disease progression. However, it is not known which dysregulated mechanisms are affecting cell-to-cell communication, and the molecular mediators of neuronal injury, either existing as soluble factors or mediated by extracellular microvesicles.

MicroRNAs (miRNAs) are non-coding RNAs whose function is to suppress gene expression through their binding to mRNAs. Lately, miRNAs were discovered as diagnostic biomarkers and potential therapeutic targets in ALS. Differentially expressed miRNAs were identified, some with recognized association with known or suggested pathways in ALS pathogenesis. miRNAs can act in a tissue-/cell-type-specific manner and are involved in cellular communication, where they may affect gene expression, either on the cells of origin or in recipient ones.

MiRNA(miR)-124 is one of the most expressed in the central nervous system (CNS) and described as usually associated with neuronal differentiation. It has been found elevated in the cerebrospinal fluid of ALS patients, as well as in the ALS mice with the G93A mutation in the SOD1 gene (mSOD1), namely at active neurodegeneration sites, and identified in our studies as being upregulated in MN NSC-34 cell line with the same mutation. Interestingly, we noticed that miR-124 was upregulated in exosomes derived from such MNs, which showed to induce several activated subtypes once collected by microglia. In physiological conditions, miR-124 is involved in neuronal maturation, as well as in cell cytoskeleton organization, neurite outgrowth and autophagic regulation. Lately, dysregulation of miR-124 has been found in several CNS disorders associated with neurodegeneration, stress, neuro-immune dysregulation, and brain tumors, among others.

Here, we aimed to explore the consequences of miR-124 inhibition and overexpression on the MN function and structure, as well as on microglia and astrocyte immunoregulation. For that, we used the NSC-34-MN-like cell line, either non-mutated (wild type, WT), or expressing the G93A mutation (mSOD1), which is a model that mimics the SOD1 accumulation and the neuronal degeneration observed in ALS. We transfected WT MNs with miR-124 mimic (pre-

miR-124), and mSOD1 MNs with the miR-124 antagonist (anti-miR-124), to assess whether the effects produced by their secretome differently affected glia reactivity. In this sense, the collected secretome was incubated with mice microglial N9 cells for 4 h and with SC astrocytes from WT and mSDO1 8-days-old mice for 24 h. MN viability was not changed by both miR-124 modulations and that using anti-miR-124 prevented SOD1 accumulation. This one also prevented the increase in gene and protein expression of *High Mobility Group Box 1* (HMGB1), as well as of the *inducible Nitric Oxide Synthase* (iNOS) protein, and of *S100 Calcium-binding Protein B* (S100B) mRNA. Overexpression of miR-124 in WT MNs was reflected only in S100B mRNA increase. At miRNAs level, anti-miR-124 in mSOD1 MNs decreased the overexpression of miR-125b and increased that of miR-21 and miR-146a, which were diminished in the disease model. Interestingly, pre-miR-124 in WT MNs led to the profile observed in mutated cells, namely to upregulation of miR-125b and to downregulation of miR-21 and miR-146a, validating miR-124 as a driver of such miRNA dysregulation. Importantly, anti-miR-124 was also capable of diminishing the expression of the pre-synaptic marker synaptophysin and of the retrograde transport marker dynein, both elevated in mSOD1 MNs and in WT-treated pre-miR-124 MNs, toward WT levels, highlighting miR-124 as a major contributor to such increases. In opposite, this miRNA revealed to be associated with the decrease of the post-synaptic marker PSD-95 and the anterograde transport marker kinesin, since anti-miR-124 led to a remarkable increase in the expression of their genes. Thus, we can conclude that anti-miR-124 favors anterograde transport (from soma to the end of the axon), deficient in the mutated cells, in which dynein elevation favors retrograde transport (back to the soma) described to be associated with miR-124 upregulation. Overexpressed miR-124 was also shown to relate with fewer ramifications and number of primary neurites, as well as with their increased length, which were counteracted to values close to control levels with anti-miR-124 in mSOD1 MNs. This indicates the influence of this miRNA in cytoskeleton regulation, cellular morphology, and MN function. In fact, anti-miR-124 was able to recover mitochondrial viability in mSOD1 MNs, whose decrease seems to be related with miR-124 upregulation. It also favored Mitofusin expression (an inducer of mitochondrial fusion), found diminished in mutated cells, and decreased DRP1 (associated with mitochondrial fission), increased in mutated cells. Once again, the overexpression of miR-124 showed to be involved in DRP1 upregulation in the mutated cells. Such alterations suggest the existence of oxidative stress associated with miR-124 overexpression and that anti-miR-124 may have a beneficial action by favoring anti-oxidant mechanisms.

Relatively to the immunoregulatory action exerted by the MN secretome, we verified that the increased area, perimeter and Ferret's diameter, as well the circularity reduction in microglia treated with secretome derived from mutated MNs, was not manifested upon

incubation with the secretome from mSOD1 MNs treated with anti-miR-124. These parameters showed a direct correlation with the miR-124 overexpression. Microglia phagocytic ability was also affected by the secretome from WT MNs treated with pre-miR-124, as well as with that from mSOD1 modulated or not with anti-miR-124, indicating that miR-124 inhibition is not enough to recover such microglial property. However, anti-miR-124 modulation effectiveness was again manifested when the secretome from mSOD1 MNs acquired the capability to decrease the gene expression of iNOS, arginase1, *Tumor Necrosis Factor alpha* (TNF-α) and *Interleukin 1 beta* (IL-1β). All these inflammatory genes were found upregulated in microglia treated with both mSOD1 and pre-miR-124 WT MNs. Additionally, it led to a decrease of HMGB1, highly enhanced after incubation with the secretome from mSOD1 MNs, and to an elevation of the mannose receptor CD206 expression, associated with macrophage endocytic and phagocytic properties. Interestingly, here we found an indirect correlation in that overexpressed miR-124 triggered a CD-206 gene expression decrease. In what concerns the consequences of mSOD1 MN secretome after miR-124 modulation on astrocytes, it was interesting to observe that GLT-1 levels were sustained in this condition, contrasting with the reduction found with the secretome from WT MNs treated with pre-miR-124, as well as with that from mSOD1 MNs. Similar effect was noticed on GFAP levels, whose expression increased relatively to WT and mSOD1 astrocytes treated with the secretome from mSOD1 MNs as well as the WT astrocytes exposed to the secretome from WT MNs modulated with pre-miR-124. Therefore, we can hypothesize that, at least in part, miR-124 elevation in mSOD1 MNs is associated with the production of a secretome determining GFAP and GLT-1 reduced expression, features that relate with the aberrancy of astrocytes in ALS.

Overall, our results indicate that miR-124 overexpression in mSOD1 MNs has a key role in the degeneration and dysfunction of these cells, remarkably contributing to ALS pathogenesis and progression by compromising cellular homeostasis, due to a secretome with immunostimulatory and inductive aberrant features in either WT or mSOD1 astrocytes. Anti-miR-124 showed beneficial effects in preventing SOD1 accumulation in mutated MNs, while exerting synaptic transmission regulation (increase of the neuritic tree, kinesin and PSD-95), anti-oxidant effect (increased mitochondrial viability and Mitofusin and diminished DRP1) and anti-inflammatory action (increase of miR-21 and miR-146a, with decrease of miR-125b). Furthermore, secretome from mSOD1 MNs modulated with anti-miR-124 acquire properties that support microglia functionality (normal morphological characteristics and levels of iNOS, Arginase1, TNF-α, IL-1β and HMGB1 similar to those observed for the secretome from non-modulated WT MNs) and that of astrocytes (increase of GLT-1 and GFAP expression), beyond the elevation of microglial receptor CD206, associated with endocytic and phagocytic abilities. Thus, we can conclude that the decrease of miR-124 expression toward basal levels in mSOD1

MNs backup their capacity to sustain their appropriate function, while preventing a reactive glial response triggered to a secretome with immunostimulatory properties, thus counteracting neurodegeneration and disease progression.

In conclusion, this work supports that the selective targeting of miR-124 in mSOD1 MNs may turn in a promising and broad therapeutic strategy for ALS treatment, by allowing the recovery of MN function and by preventing secretome-mediated glial reactivity, thus acting in multiple targets as usually intended with a combined therapy.

Keywords: Glial reactivity; miR-124 modulation; Motor neuron degeneration; Spinal cord; Secretome

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Abbreviations

ALS	Amyotrophic lateral sclerosis
AMPA	A-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Arg1	Arginase 1
ALDH	Aldehyde dehydrogenase
ATP	Adenosine-5'-triphosphate
BACE1	Beta-secretase 1
BBB	Blood-brain barrier
BSA	Bovine serum albumin
C1q	Complement component 1q
C9orf72	Chromosome 9 open reading frame 72
CD206	Cluster of differentiation 206
cDNA	Complementary DNA
CNS	Central nervous system
CSF	Cerebrospinal fluid
Cx43	Connexin 43
DIV	Days *in vitro*
DNA	Deoxyribonucleic acid
DRP1	Dynamin-related protein 1
EAAT	Excitatory amino acid transporters
ER	Endoplasmic reticulum
ETC	Electron transport chain
FALS	Familial amyotrophic lateral sclerosis
FBS	Fetal bovine serum
FIS1	Mitochondrial fission 1 protein
FTD	Frontotemporal dementia
FUS	Fused in sarcoma
G418	Geneticin 418 sulfate
GFAP	Glial fibrillary acid protein
GLAST	Glutamate aspartate transporter
GLT-1	Glutamate transporter 1
HMGB1	High mobility group box protein 1
HRP	Horseradish peroxidase
Iba1	Ionized calcium binding adaptor molecule 1
IFN	Interferon
IL	Interleukin
IRAK	Interleukin-1 receptor-associated kinase 1
iNOS	Inducible nitric oxide synthase
iPSC	Induced pluripotent stem cell
MAP	Mitogen Activated Protein
Mfn	Mitofusin
MBP	Maltose-binding protein
miRNA/miR	microRNA
MMP	Matrix metalloproteinases
MN	Motor neuron
mRNA	Messenger RNA

mSOD1	Mutated SOD1
mTDP-43	Mutated TDP-43
NADPH	Nicotinamide adenine dinucleotide phosphate
NCM	Neuronal conditionate medium
NEEA	Nonessential amino acids
NF-κB	Nuclear factor kappa B
NG2	Neuron-glia antigen 2
NMJ	Neuromuscular junction
NO	Nitric oxide
PCR	Polymerase chain reaction
PDL	Poly-D-Lysine
PFA	Paraformaldehyde
PNS	Peripheral nervous system
PSD-95	Postsynaptic density protein 95
RNA	Ribonucleic acid
ROCK1	Rho-associated protein kinase 1
ROS	Reactive oxygen species
S100B	S100 calcium-binding protein B
SALS	Sporadic amyotrophic lateral sclerosis
SC	Spinal cord
SDS	Sodium dodecyl sulfate
SOCS1	Suppressor of cytokine signaling 1
SOD1	Superoxide dismutase 1
STAT	Signal transducer and activator of transcription
TDP-43	TAR DNA binding protein 43
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UPR	Unfolded protein response
WT	Wild type

I. Introduction

1. Amyotrophic Lateral Sclerosis (ALS) - more than a motor neuron disease

ALS is a progressive and fatal neurodegenerative disease characterized by the rapid development of progressive muscle weakness and atrophy throughout the body. ALS was first described in 1869 by Charcot and is the third most common neurodegenerative disease, but the most frequent and aggressive motor neuron (MN) disease occurring in adult life (Brooks 1994; Brooks et al. 2000). ALS causes might combine environmental and genetic factors and the global incidence is estimated to be 1.68 (1.50-1.85) cases per 100,000 persons annually (Marin et al. 2017). The mean age for disease onset is around 55 years and most of the patients end up dying, usually due respiratory insufficiency, within 2-4 years after diagnosis. In Portugal, it is estimated that the disease affects over 800 people. Diagnostic is exclusively clinical and is usually based in El Escorial Criteria, published in 1994. However, the diagnosis is not very effective, since the symptoms may be similar to other conditions and patients may present a range of different phenotypes, leading to long diagnostic timelines (Agosta et al. 2015). The disease affects both MNs from the corticospinal tract (upper MNs), brainstem and spinal cord (lower MNs) (Rowland and Shneider 2001) (**Figure I.1.**). ALS onset usually affects an isolated region: speech and swallowing (bulbar onset) or arm and length strength (limb onset) that after spreads to the entire neuroaxis. Approximately half of ALS patients display minor changes in cognition and behavior, while 15% may develop concomitant frontotemporal dementia (FTD), suggesting that ALS is not a disease simply limited to the motor system but also be related with progressively loss of brain network structure (Achi and Rudnicki 2012; Al-Chalabi et al. 2017; Esther et al. 2014).

ALS is a very heterogeneous disease with a complex genetic basis and pathophysiology. Despite being studied for several years, the only available therapies are Riluzole and Radicava, each one playing a modest impact on disease outcome. They act preventing glutamate excitotoxicity (Bensimon et al. 1994) and by relieving oxidative stress effects (Abe et al. 2017), respectively. Much of the early research of the disease focused on a neuron-centric view. Later studies implicated cellular dysfunction of non-neuronal cell types, including astrocytes, oligodendrocytes, and microglia, making the understanding of ALS mechanistic cues very challenging (Brites and Vaz 2014; Peters et al. 2015). Thus, it is essential to dissect the molecular mechanisms underlying MN loss in ALS.

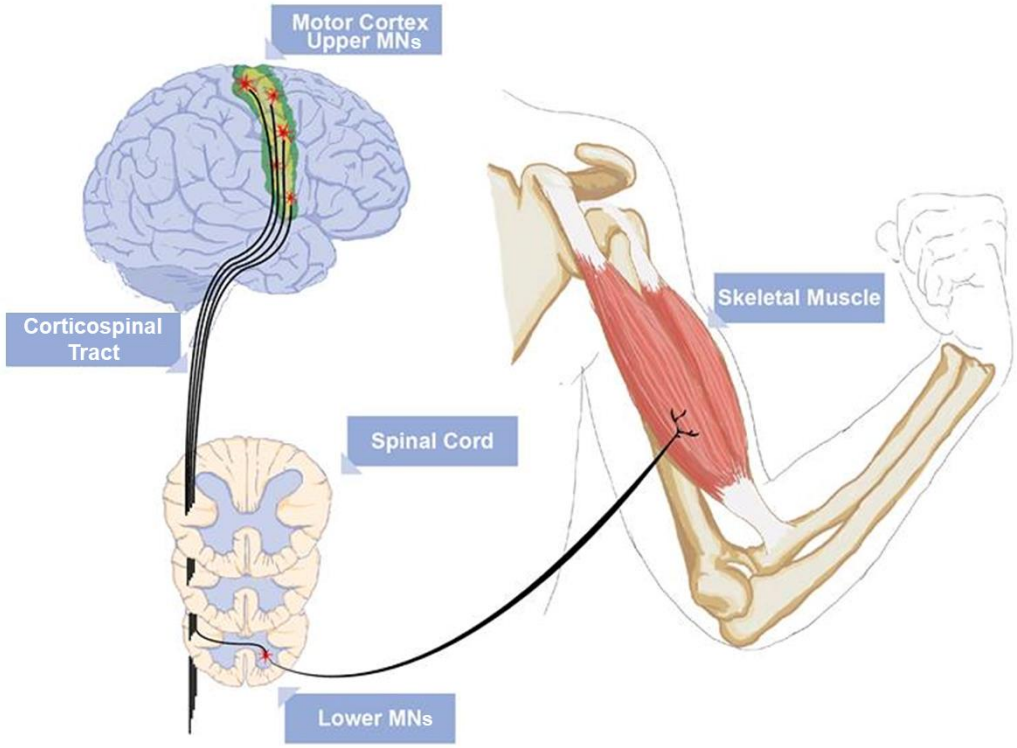

Figure I.1. Amyotrophic Lateral Sclerosis (ALS) is a progressive and fatal neurodegenerative disease characterized by motor neuron (MN) loss. The disease affects both MNs from corticospinal tract (upper MNs) and bulbar and spinal (lower MNs). In the onset, the disease usually affects an isolated region but rapidly spreads to the entire neuroaxis. The main clinical characterization is the rapid development of progressive muscle weakness and atrophy throughout the body, due to muscle denervation.

1.1. ALS genetic basis

As in other neurodegenerative diseases, generally, ALS is divided into sporadic ALS (SALS) or familial ALS (FALS), affecting 90-95% and 5-10% of total cases respectively (Rowland and Shneider 2001). Clinically and pathologically the two forms are very similar and practically indistinguishable. FALS is hereditary and is usually related to autosomal dominant mutations in a heterogeneous set of genes (Belzil and Rouleau 2012; Chen et al. 2013). The disease manifestation is enriched with genetic mutations and in ALS Online Database, 126 genes related to ALS (http://alsod.iop.kcl.ac.uk/) were already identified. Though, the majority does not necessarily give origin to the disease. Interestingly, mutations usually found in FALS have also been identified in SALS patients, suggesting a strong genetic component even in sporadic forms of the disease (Al-Chalabi et al. 2017; Cady et al. 2015). In fact, it is estimate that genetic factors contribute to at least 23% of SALS cases (Keller et al. 2014).

Mutations in superoxide dismutase (SOD1), the first causative gene identified in 1993 (Rosen et al. 1993), are among the mutations most commonly associated with ALS and

represent 20% of FALS patients and about 1-2% of SALS (Ghasemi and Brown 2018; Marangi and Traynor 2015). Its discovery was a breaking point for the disease study since it led to the creation of several cellular and animal models (Pasinelli et al. 2004). SOD1 models closely resemble the pathological features found in human patients. Therefore, they have been widely used to investigate ALS molecular pathogenesis. The mechanism by which mutation in this protein may lead to the disease is unknown but it has been associated with a gain of function with the inducement of oxidative stress, mitochondrial dysfunction excitotoxicity, and toxicity through protein misfolding and aggregation (Pasinelli and Brown 2006). In SALS it was observed misfolded SOD1 in non-mutated cells, suggesting the involvement of post-translational modifications (Forsberg et al. 2010). The identification of hyperphosphorylated, ubiquitylated and cleaved TDP-43 (TAR DNA-binding protein 3) and FUS (fused in sarcoma) in cytoplasmic inclusions associated with TDP-43 and FUS mutations, suggested a common mechanism among the different genetic cases: the protein aggregation (Lagier-Tourenne and Cleveland 2009). These mutations are found in about 4% of FALS cases (Ghasemi and Brown 2018). There is no cause/effect relationship between protein aggregates and disease, but they suggest a dysregulation of the mechanism involved in aggregates regulation. Another important mutation associated with ALS is repeated expansions in chromosome 9 open reading frame 72 (C9orf72). It is the most common mutation in FALS patients (50% of the patients) and is also detected in 5-10% of SALS patients, evidencing the strong genetic component of the disease, even in patients without family history (Al-Chalabi et al. 2017; Ghasemi and Brown 2018). This mutation is also associated with FTD and together with mutations in the RNA-biding TDP-43 and FUS, identify alterations in RNA metabolism as a key event in ALS pathogenesis.

1.2. Molecular mechanisms for motor neuron degeneration

Precisely what makes the MNs to degenerate is the prevailing mystery of ALS, but by studying the genes altered in FALS patients, several mechanisms have been pinpointed in neurons that might be involved in MN degeneration (**Figure I.2**). However, it is not clear which of these altered processes are a result of the disease and which could be a cause, but it is clear that they affect different aspects of the motor neuron function. The lack of knowledge begins with the question of where ALS begins and how the disease spreads. The first two proposals were (1) the dying-forward mechanism, where the insult to MN cell body causes cell dysfunction and leads to NMJ retraction, and (2) the dying-back mechanism, where an insult in the neuron axon causes axonal dysfunction and NMJ retraction (Dadon-Nachum et al. 2011; Vucic et al. 2008). Later, these mechanisms were considered not mutually exclusive (Geevasinga et al. 2016). An interesting fact is how neurodegeneration in ALS is so specific and how different types of MNs are more susceptible to degeneration than others. One of the

MN features that make them vulnerable is their size. MNs are divided in type S (slow) and type F (fast) (Nijssen et al. 2017). S-type MNs are disease-resistant and interestingly have smaller soma sizes when compared with F-type ones, neurons more vulnerable to degeneration (Dukkipati et al. 2018). Moreover, resistant and vulnerable MNs also differentially express several genes, as is the case of metalloproteinase (MMP)-9, only expressed by F-type mutant SOD MNs (Kaplan et al. 2014; Vaz et al. 2015). The existence of MNs populations with different degeneration susceptibilities may help in new studies focusing on the more vulnerable ones.

In the cell body, proteins that are not transported into the nucleus accumulate in the cytoplasm, forming aggregates which may be associated with errors in protein transport. These aggregates are a major hallmark of ALS and can become toxic to the cell in several ways. However, its pathological role and formation mechanisms still need further elucidation. A cell to cell transmission of misfolded protein aggregates in a prion-like mechanism (a protein conformation capable of replicating without a nucleic acid genome) has been directly associated with the generation of novel aggregates and with the propagation of neurodegeneration to the surrounding cells, suggesting a role in ALS dissemination (Al-Chalabi et al. 2017). Associated with protein aggregation is the endoplasmic reticulum (ER) stress, the first compartment in the secretory pathway, responsible for protein synthesis and delivery to their proper target sites. If ER capacity is transcended and the influx is excessive it leads to stress and activates the unfolded protein response (UPR) signaling pathway. If the UPR fails to restore the cell integrity, together with ER stress, induces mechanisms of cellular death and autophagy (Ayers and Cashman 2018). Changes in ER morphology have been observed in ALS patients, mSOD1 mice and it has been found deposits of granular material in SALS patients, indicating accumulation of misfolded proteins associated with ER stress (Jaronen et al. 2014). UPR proteins had also been found increased in ALS patients, indicating again the involvement of ER stress in the disease (Hetz and Saxena 2017).

Autophagy is a major protein degradation pathway that is involved in the clearance of protein aggregates and damaged organelles. Autophagy dysregulation is also an emerging pathogenic hallmark of ALS and most evidence point to an autophagy reduction in diseased neurons. The potential mechanisms through which autophagy can be affected in the disease are by direct dysfunction of proteins involved in protein degradation or through a combined effect of autophagy defects along with other cellular stress that depends on autophagy for their mitigation (Ramesh and Pandey 2017). Defects in different steps of autophagy were also related to neuronal injury in both ALS models and patients, suggesting that it is a neuronal non-cell-autonomous mechanism for neuronal degeneration (Rudnick et al. 2017).

Protein aggregation can also damage mitochondria, the cell power generators that are also related with oxidative stress. MNs have high mitochondrial activity and are exposed to elevated levels of oxidative stress, being more susceptible to energy deficits with the loss of mitochondrial function (Shaw and Eggett 2000). Mitochondria play a vital role in cell survival and metabolism and are well-known producers of ATP. They also have a key role in other essential mechanisms for cells, as calcium homeostasis and apoptosis. In neurons, the calcium buffering has special importance for the modulation of neurotransmitters (Rizzuto et al. 2012). Therefore, the maintenance of correctly localized mitochondria is crucial for neuronal survival and normal function, the reason why it is not surprising that such dysfunction is associated with many neurodegenerative disorders, including ALS. Structurally altered, fragmented and aggregated mitochondria were observed in patients and in different animal and cell ALS models (Delic et al. 2018; Magrane et al. 2014; Onesto et al. 2016; Sasaki and Iwata 2007; Vaz et al. 2015). Mitochondrial damage is reported in ALS early stages, meaning that these morphological alterations probably act as a trigger for cell degeneration (Vande Velde et al. 2011). ALS-associated proteins had also been pointed to induce mitochondrial damage, e.g. mutant SOD1 was found localized in mitochondria intermembrane space where it aggregates reducing the activity of the electron transport chain (ETC) complexes (Ferri et al. 2006; Vijayvergiya et al. 2005). TPD-43 also reportedly accumulates inside mitochondria where it binds to mitochondrial mRNAs associated with the encoding of ETC complexes, impairing their expression and leading to mitochondrial dysfunction (Wang et al. 2016b). C9orf72 was also detected in mitochondrial-enriched fractions (Blokhuis et al. 2016).

Associated with aberrant mitochondrial function and structure is the impairment of mitochondria dynamics, which includes defects in fusion and fission mechanisms, mitophagy and axonal transport. Fusion and fission are dependent on the energetic requirement of the cell and have the function of keeping mitochondria size, shape, and morphology. The unbalance of these mechanisms may result in excessive mitochondrial elongation or fragmentation, respectively. The fission process is mainly regulated by dynamin-related protein 1 (DRP1) (Smirnova et al. 2001) while fusion is regulated by Mitofusin 1 (Mfn1), Mitofusin 2 (Mfn2) and optic atrophy protein 1 (OPA1) (Santel and Fuller 2001). In several ALS models, it has been described a decrease of fusion proteins together with an increase of fission proteins, suggesting a fragmented mitochondrial network (Ferri et al. 2010; Liu et al. 2013; Xu et al. 2010). Recently, it has been reported that the inhibition of DRP1 and its mitochondrial receptor Fis1 may slow the disease progression, meaning that this may be a core mechanism for neurodegeneration (Joshi et al. 2018). The consequence of general increased of mitochondria fission is not clear yet, but is probably associated with smaller and less energetic mitochondria

more prone to reactive oxygen species (ROS) induced damage and less able to fuse back, exacerbating mitochondrial damage (Hoitzing et al. 2015).

As previously referred, mitochondria failure is also related to other MN dysregulated mechanisms in ALS, including the production of ROS. Oxidative stress is originated from an imbalance between ROS production and the system capacity to remove or repair their damage and reduce their levels from the cellular environment. Multiple studies have associated increased levels of oxidative stress with ALS. In post-mortem tissue, elevated carbonyl levels have been found in the spinal cord and motor cortex from SALS patients and increase of 3-nitrotyrosine levels, a marker for peroxynitrite-mediated damage has been found in both SOD1 FALS and SALS patients (Bogdanov et al. 2000; Ihara et al. 2005). Markers for lipid and DNA oxidation are also elevated in spinal cords from ALS patients. More recently, in disease earlier stages, oxidative stress markers and decrease of total antioxidant status (TAS) markers were found in the CSF from patients (Blasco et al. 2017). The increase of oxidative stress is then another key player of neurodegeneration being associated with high production of nitric oxide (NO), pro-inflammatory response and DNA/RNA damage.

The cell transport machinery may also be damaged, affecting RNA, protein and vesicles transport. MNs are highly polarized cells and require transportation of different cargos from the soma to the axon terminal and vice versa. There are two major families of microtubule-based motor proteins. Kinesin mediates anterograde transport from the cell body to the axon, and dynein drives retrograde transport from the distal axon to the cell body (Hirokawa et al. 2010). These proteins transport cargos as mitochondria, lysosomes, signaling endosomes, mRNA and protein complexes. First evidence for axonal transport defects in ALS was obtained in post-mortem tissues, that revealed abnormal neurofilaments, mitochondria and lysosomes accumulation in the proximal axon of MNs. In mSOD1 transgenic mice, decrease of kinesin accumulation in axons, suggest and impairment of axonal transport, especially in the anterograde direction (De Vos et al. 2007; Warita et al. 1999; Zhang et al. 1997). In the case of TDP-43, it is usually found in cytoplasmic granules along axonal length where it is associated with long-distance transport of mRNAs involved in distal neuronal processes (Ishiguro et al. 2016). In ALS mTDP-43 model, these granules are absent or reduced at axons and moving in the retrograde direction, reinforcing the impairment of anterograde transport in the disease (Alami et al. 2014). The proposed molecular mechanisms underlying axonal transport impairment in SALS and FALS include reduction of microtubule stability, mitochondrial damage, and alterations in the phosphorylation state of the motor proteins dynein and kinesin.

Another important contributor in ALS etiology is the synaptic failure. Synaptic terminal degeneration is a pathological hallmark in human ALS progression and apparently occurs

before any apparent MN injury (Moloney et al. 2014). The loss of synaptic communication of MNs is evidenced by the decrease expression of synaptic vesicles proteins, as synaptophysin, in ALS patients and animal models (Zang et al. 2005). The evidence point to a loss of synaptic homeostasis in MNs, involving both lower and upper MNs resulting from a decreased activity of the inhibitory circuits controlling MNs excitability (Casas et al. 2016). Loss of synaptic function has been recently related also with a cognitive decline in ALS (Henstridge et al. 2018).

Excitotoxicity associated with glutamate is another mechanism associated with neuronal injury and appears to also contribute to ALS spread (Menon et al. 2017). Glutamate is synthesized in neuronal presynaptic terminal and goes across the synaptic cleft, activating postsynaptic receptors as α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), triggering action potentials. The membrane depolarization of the postsynaptic MNs leads to the activation of voltage-dependent calcium channels, allowing its entrance in the cell. The concentration of glutamate in the synaptic cleft is then regulated by transporter proteins in glial and neuronal cells, known as excitatory amino acid transporters (EAATs) (Shaw and Eggett 2000). If glutamate levels are not restored it will lead to excitotoxicity, characterized by excessive or prolonged glutamate receptor activation. The unsustainable calcium increase inside the cell leads to generation of ROS, enzymatic and mitochondrial damage, promotion of apoptotic pathways and ultimately to neuronal degeneration (Lin et al. 2012). In ALS, reduced levels of GLT-1 were found in the motor cortex and spinal cord of ALS patients and also in mSOD1 mouse models (Zarei et al. 2015). Loss of GLT-1 has also been observed in astrocytes, the main cells responsible for glutamate uptake from the synaptic clefts. How the decrease in GLT-1 may affect astrocytic reactivity will be discussed in **Section 1.3.3**.

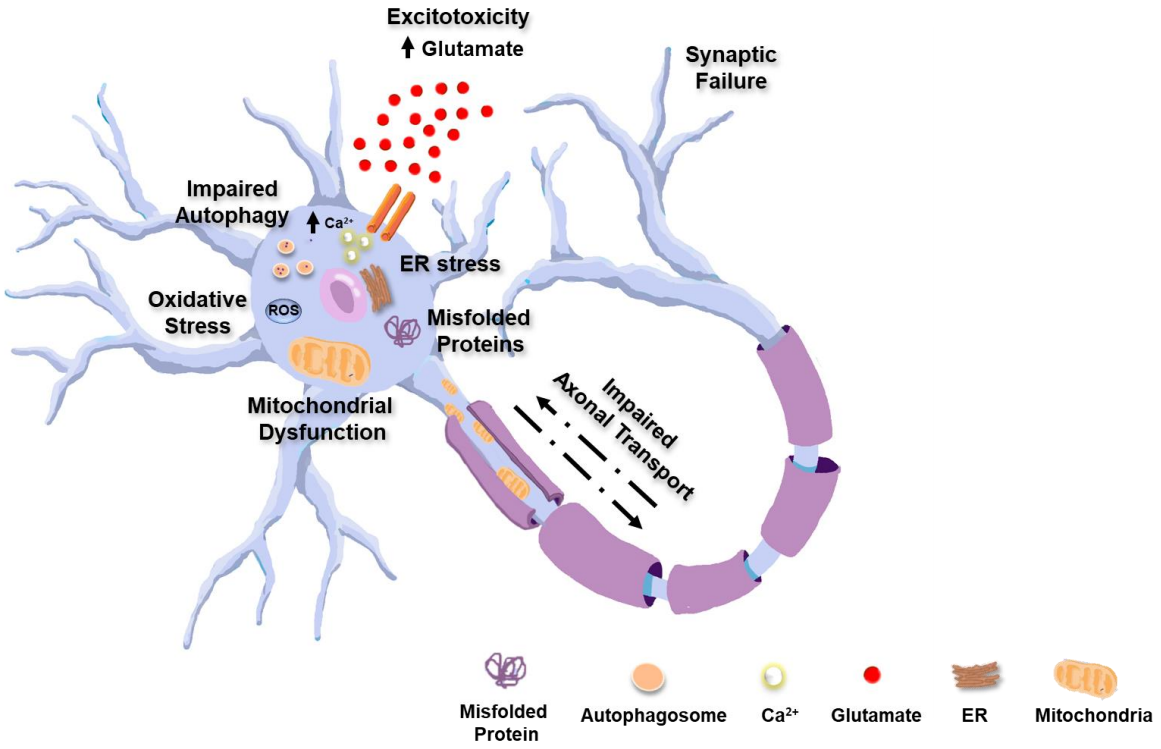

Figure I.2. Molecular interacting mechanisms associated with MN death in ALS. The pathophysiological mechanisms underlying neurodegeneration in ALS appear to be related with a complex interplay between molecular pathways. Protein aggregation of misfolded proteins is a major hallmark of the disease, since it is present in both sporadic and familial patients. Protein aggregates are then associated with the impairment of several mechanisms, as endoplasmic reticulum (ER) stress, mitochondrial dysfunction and autophagy. The impairment of mitochondrial functions is associated with an increase of reactive oxygen species (ROS) inside the cell, adversely affecting axonal transport process. The deficient transport of cargos along the cell by axonal transport ultimately leads to synaptic loss and to depletion of energy support to neuronal extensions, causing axonal loss and denervation of muscle cells. Glutamate-induced excitotoxicity results from the reduced glutamate uptake by astrocytes in the synaptic cleft. This leads to increase influx of Ca^{2+} ions inside neuronal cells.

1.3. The contribution of glial cells reactivity to ALS neurodegeneration

The classic view of neurotoxicity in neurodegenerative diseases is based on the idea that a specific neuronal population is especially vulnerable to cumulative toxic cargo, associated with chronic damage and normal aging, leading to a status that overwhelms neuron's defensive mechanisms, triggering neurodegeneration and neuronal death. Initially, it was thought that these mechanisms would be cell-autonomous and independent of other cell types and interactions. However, in the past decades, cues for non-cell autonomous mechanisms in which neurodegeneration is strongly influenced by toxicity or mutant protein expression in both neuronal and non-neuronal cells have emerged. The major question for glial involvement and contribution to non-cell autonomous mechanisms is whether interactions within and between glial or neuronal cells are necessary and/or have a contribution to the

neurodegenerative process. There are several hypotheses for glia involvement in neurodegeneration. The first keeps neurons as the main source of toxicity, stimulating damage responses from glia that may not be directly damaged. The other hypothesis is that glial cells may be involved as primary toxicity sources, blurring the lines between diseases primarily thought as having a neuronal origin. This involves mutant protein expression in glial cells that could disturb normal glial response exacerbating damage in vulnerable neurons or glial cells as a primary source of neurotoxicity. Communication between the different types of cells have also a crucial role in ALS pathogenesis and disease spreading and can be mediated either by soluble factors or extracellular vesicles, as exosomes.

In CNS injury and disease, a non-specific reactive change of glial cells is usually activated in a process denominated as gliosis. Reactive gliosis is triggered by different and selective ways in neurodegenerative diseases. In ALS, gliosis has been found as an early pathological characteristic in SC and brain and has been associated with intrinsic cellular damage in both neurons and glial cells (Yamanaka et al. 2008). Neurodegenerative insults can also lead to a disruption of neurovascular unit, resulting in the blood-brain barrier (BBB) leak which may serve as a further trigger for reactive gliosis (Stolp and Dziegielewska 2009). In ALS, glial normal function of supporting neurons is often lost and glial cells usually present aberrant reactivity, that contributes to the acceleration of neuronal damage. To predict the harmful potential of glial cells it has to be taken in account that neuroglia populations include cells with very different characteristics and independent embryological origin (Allen and Barres 2009). Glial cells include astrocytes, microglia, oligodendrocytes and Schwann cells.

1.3.1. Oligodendrocytes and Schwann cells

Oligodendrocytes and Schwann cells produce and maintain neuronal myelin sheaths around selected axons, respectively in the CNS and peripheral nervous system (PNS). Another important function of oligodendrocytes is to provide energy supply to neurons by expressing monocarboxylate transporter 1 (MTC1), which provide to neurons substrates like lactate and ketone bodies (Lee et al. 2012). With aging, myelin gets thinner and breakdown, resulting in slower action potentials propagation (Bartzokis 2004).

In both ALS patients and mSOD1 rodent models, oligodendrocytes with abnormal morphology are found and show to degenerate during disease progress (Kang et al. 2013; Philips et al. 2013). However, the overall number of oligodendrocytes does not change. This is associated with activation of NG2+ oligodendrocyte precursor cells, responsible by the compensatory mechanism of cell replacement (Nonneman et al. 2014). However, the new formed oligodendrocytes are immature, dysfunctional and have reduced MTC1 expression levels, indicating an impairment of neuronal metabolic support and insufficiency in generating

myelin basic protein (MBP), thus losing the ability of structurally supporting MNs (Kang et al. 2013; Lee et al. 2012; Philips et al. 2013). A more recent study performed in oligodendrocytes differentiated from induced pluripotent stem cells (iPSCs) generated from human fibroblasts of patients with SALS and carrying different mutations, showed that SOD1 is directly involved in ALS oligodendrocyte pathology (Ferraiuolo et al. 2016).

About Schwann cells, little is known on their involvement in ALS pathology. Curiously, a study performed in mSOD1 mice revealed that the downregulation of mutated protein specifically in Schwann cells was associated with an acceleration in disease progression showing that these cells may act more as neuroprotective during the disease (Lobsiger et al. 2009). The mechanism for this unexpected outcome is unclear, but it is hypothesized to be the result of residual neuroprotective dismutase activity of SOD1 specifically in the Schwann cells,

1.3.2. Microglia

Microglia are CNS resident macrophages, which play protective and supportive roles. Microglia are involved in neuronal synaptic plasticity, dendrite density, detection and elimination of damaged neurons and promotion of neuronal regeneration (Reemst et al. 2016). When CNS homeostasis is compromised microglia change their morphology, gene expression, and functions, entering in an activated phenotype. Activated microglia are usually classified in classical activated (M1) or alternatively activated (M2), following the paradigm used for macrophages. The M1 phenotype is a proinflammatory state that is associated with neurotoxic properties and is typically induced by TLR and interferon-gamma (IFN-γ) signaling pathways (Martinez and Gordon 2014). M1 microglia are characterized by the production of proinflammatory factors, like TNF-α, IL-1β, IL-6, and IL-12 and expression of NADPH oxidase, which generates superoxide and ROS (Cunha et al. 2016). Ultimately, M1 microglia induce inflammation and neurotoxicity. Alternative activation M2 is associated with anti-inflammatory and healing activities of microglia and can be induced by multiple factors, as following the detection of apoptotic cells (Hu et al. 2015). This phenotype promotes the release of anti-inflammatory cytokines as IL-10 and transforming growth factor-beta (TGF-β) and also secretes growth and neurotrophic factors (Tang and Le 2016). Although M1 and M2 categories have been helpful for conceptualizing microglia activities *in vitro* it is now increasingly accepted that this paradigm is inadequate to describe microglia activation *in vivo*, as microglia rarely display a significant bias toward either M1 or M2 phenotype (**Figure I.4.**) (Geloso et al. 2017).

In vivo studies indicate that during ALS progression the number of resident microglia increase and their activation state represent a range between M2 *vs*. M1 phenotypes (Chiu et al. 2013). Our group characterized changes of reactive markers in the SC from mSOD1

transgenic mice in pre-symptomatic and symptomatic stages of the disease. After disease onset, microglia reactivity markers are predominantly in a proinflammatory state, contributing to MN degeneration and as the disease progresses coexist with other less activated phenotypes (Cunha et al. 2017). In line with these results, an increased expression of both iNOS (M1 marker) and arginase 1 (Arg1, M2 marker) showed to be parallel to the general increase of activated microglia in mSOD1 transgenic mice (Lewis et al. 2014).

. Many treatments have been tested on ALS animals with the aim of inhibiting or reducing the pro-inflammatory action of microglia and astrocytes and counteract the progression of the disease (Geloso et al. 2017). Unfortunately, these anti-inflammatory therapies have been only modestly successful. This is due, for example, to the different microglia phenotypes, from surveillant in early stages, to activated states characterized by the expression of harmful and protective genes in different disease stages. Consistently, the inhibition of microglial function seems to be a valid strategy only if the different stages of microglia polarization are considered, specifically targeting only the harmful pathways or potentiating the trophic ones.

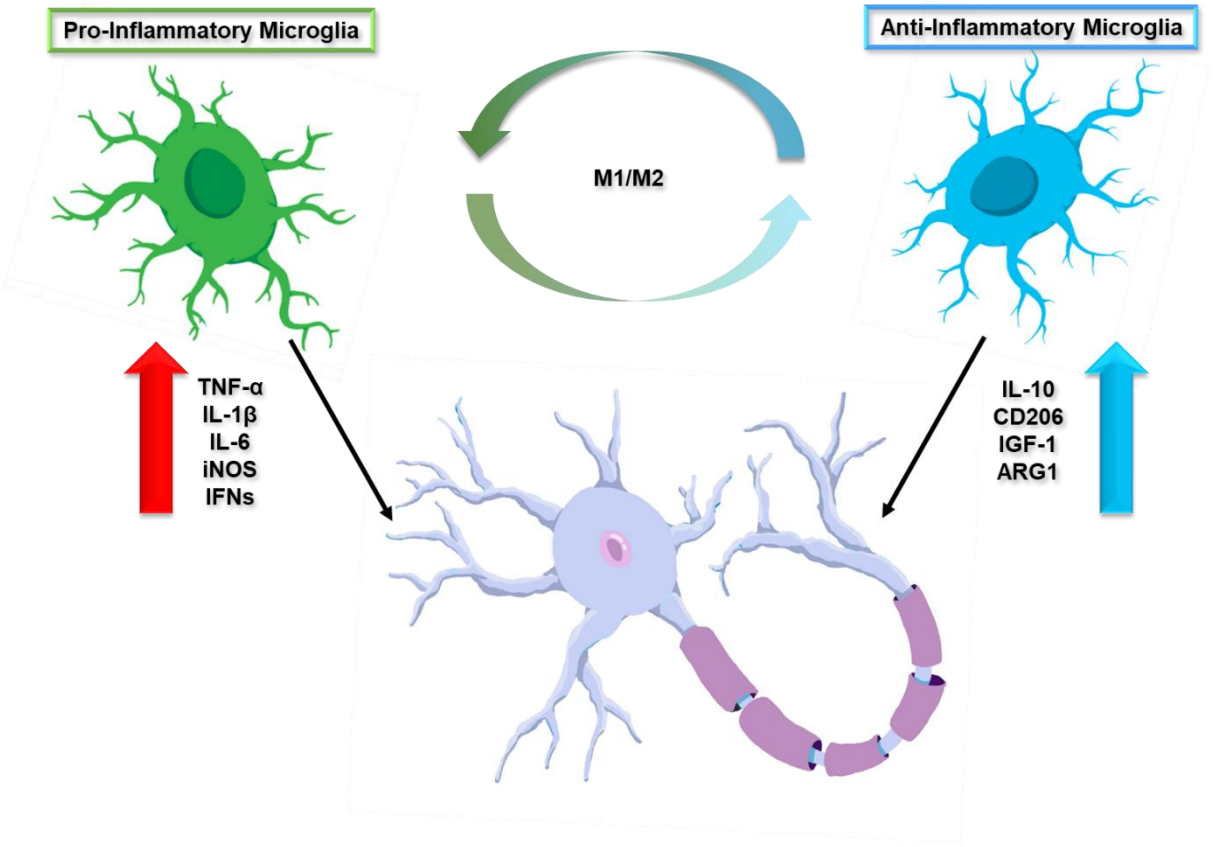

Figure I.3. Microglia contribution to MN death in ALS involves different cell phenotypes. M1 and M2 microglial polarization subtypes were shown to occur during motor neuron (MN) degeneration in amyotrophic lateral sclerosis (ALS). In an early phase the neuroprotective M2 phenotype may play a role in preventing the neuroinflammatory status, favoring tissue repair and neuron survival by producing anti-inflammatory factors, as interleukin 10 (IL-10), the mannose receptor CD206, insulin growth factor-1 (IGF-1) and arginase 1 (Arg1). In a later stage, activated M1 microglia produce pro-inflammatory mediators, including cytokines, as tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), interleukin 6 (IL-6), inducible nitric oxide synthase (iNOS) and interferons (IFNs), which increase inflammation and further sustain M1 polarization, thus contributing to neuronal death.

1.3.3. Astrocytes

Astrocytes are key components of CNS and are present in the brain at similar numbers as neurons (von Bartheld et al. 2016). They play a key role in regulating brain energy supply by increasing blood flow in regions where neurons are active, reason why they are usually positioned between vessels and neuronal cells (Nortley and Attwell 2017). Moreover, astrocytes sustain neuronal ATP production by recycling neurotransmitter glutamate, that would otherwise need to be resynthesized, and do glycogen storage which can be used as energy supply when neurons are active (Bak et al. 2018). Additionally, they maintain neuronal homeostasis by providing them neurotrophic factors and regulating the extracellular concentrations of ions and neurotransmitters at synapse sites, protecting neurons from

overstimulation and ensuring their normal functions (Bylicky et al. 2018). Thanks to their strategic position, in close contact with CNS resident cells and blood vessels, astrocytes play a key role in the control of immune cell trafficking and activation (Ponath et al. 2018). Besides that, they are immune competent cells able to detect danger signals and to respond via cytokines and chemokines secretion and adaptive immune system activation (Colombo and Farina 2016).

Astrocytic populations differ in their proliferation potential (Kriegstein and Alvarez-Buylla 2009) and classically, mature astrocytes can be identified by the expression of the glial fibrillary acid protein (GFAP), calcium-binding protein S100B, glutamate transporter GLT-1, ALDH1L1 among others (Bachoo et al. 2004). However, expression of these markers, as well as astrocyte morphologies vary considerably between cortical regions, suggesting that astrocyte subpopulations can display distinct functions depending on the region of cerebral cortex (Emsley and Macklis 2006; Regan et al. 2007). Astrocyte heterogeneity is also observed in other CNS regions, including spinal cord (Hochstim et al. 2008). The astrocytic response in pathological conditions is also very heterogeneous. When reactive, astrocytes become hypertrophic and more proliferative, leading to reactive gliosis state, releasing pro- or anti-inflammatory cytokines. A more recent study identifies two different subtypes of reactive astrocytes in physiologic and disease context: A1 and A2 (in analogy to microglial M1 and M2 phenotypes) (Clarke et al. 2018). A1 astrocytes are associated with a harmful response and loss of capacity to promote neuronal survival, inducing their death instead. The A2 phenotype is more associated with a protective response, up-regulating many neurotrophic factors (Liddelow et al. 2017). Liddelow *et al.* describe A1 phenotype as being induced by IL-1α, TNFα, and C1q, cytokines specifically released by activated microglia.

In ALS, astroglial activation is associated with MN degeneration by the loss of their ability to exert a protective effect and by the release of harmful factors. In fact, astrocytes carrying mSOD1 mutation are enough to induce MN degeneration, showing that astrocytic mediated neurotoxicity involves not only neuronal dysfunction but also the dysfunction of astrocytes themselves (Papadeas et al. 2011). Astrocytes from SALS and FALS patients with other mutations also showed to be toxic to MNs, suggesting that astrocytes clearly actively participate in ALS pathogenesis. In addition, Díaz-Amarilla *et al.* characterized an ALS specific astrocytic neurotoxic phenotype and called it aberrant phenotype (AbA cells). These cells have a high proliferative capacity and an aberrant phenotypic profile characterized by increased expression of S100B and connexin-43 (Cx43), but lack of glutamate transporter GLT-1 (Diaz-Amarilla et al. 2011). Cx43 is a major astrocyte connexin, and its increase was also detected in iPSCs derived from patients and in tissues from the motor cortex and SC of ALS patients (Almad et al. 2016). In the same study, authors shown that mSOD1 astrocytes exhibited

enhanced gap junction coupling, increased hemichannel mediated activity and elevated calcium levels. Additionally, they found that when in co-culture with MN, the blockage of Cx43 in astrocytes extended the neuronal survival suggesting that the increasing of gap junction communications in ALS astrocytes is closely related with MN loss and may be involved in the disease spreading. The loss of GLT-1 in ALS astrocytes is associated with neuronal hyperexcitability since it leads to excessive glutamate levels in the synaptic cleft resulting in toxicity to neurons through overstimulation (Yamanaka and Komine 2018). Recent findings from our group showed differential expression of astrocytic reactivity markers in different stages of the disease in the spinal cord of mSOD1 mice. Decreased expression of Cx43 in the presymptomatic stage identified impairment of glia-neuron communication as an early event in ALS (Cunha et al. 2017). Another study, performed in mSOD1 mice cortical astrocytes revealed that when co-cultured with MNs reactive astrocytes reduced neuronal neurite length, pre-synaptic protein synaptophysin expression, induced mitochondrial dysfunction, decreased of post-synaptic protein PSD-95, activation of MMP-9 and late apoptosis (Gomes et al. 2018).

Besides the dysregulated mechanisms found in astrocytes, the production of inflammatory mediators by astrocytes also have an impact on neuronal death amplification and disease progression (Yamanaka and Komine 2018). The secretion of inflammatory mediators by astrocytes also affects microglia activation and depending on the disease stage it can have a positive or a negative impact in disease progression (Pehar et al. 2017). Recently, it has been shown that the activation of inflammatory mediator NF-κB in mSOD1 mice astrocytes induces microglial proliferation (Ouali Alami et al. 2018). In presymptomatic stages of the disease it induces a neuroprotective phenotype and delay disease onset while in later stages it enhances a pro-inflammatory microglial activation, leading to an acceleration in disease progression.

Overall, ALS astrocytes lose their homeostatic function failing to MN health. In addition, they secrete numerous toxic factors that affect motor neurons. The active role of astrocytes in ALS disease mechanisms are demonstrated and summarized in **Figure I.3**.

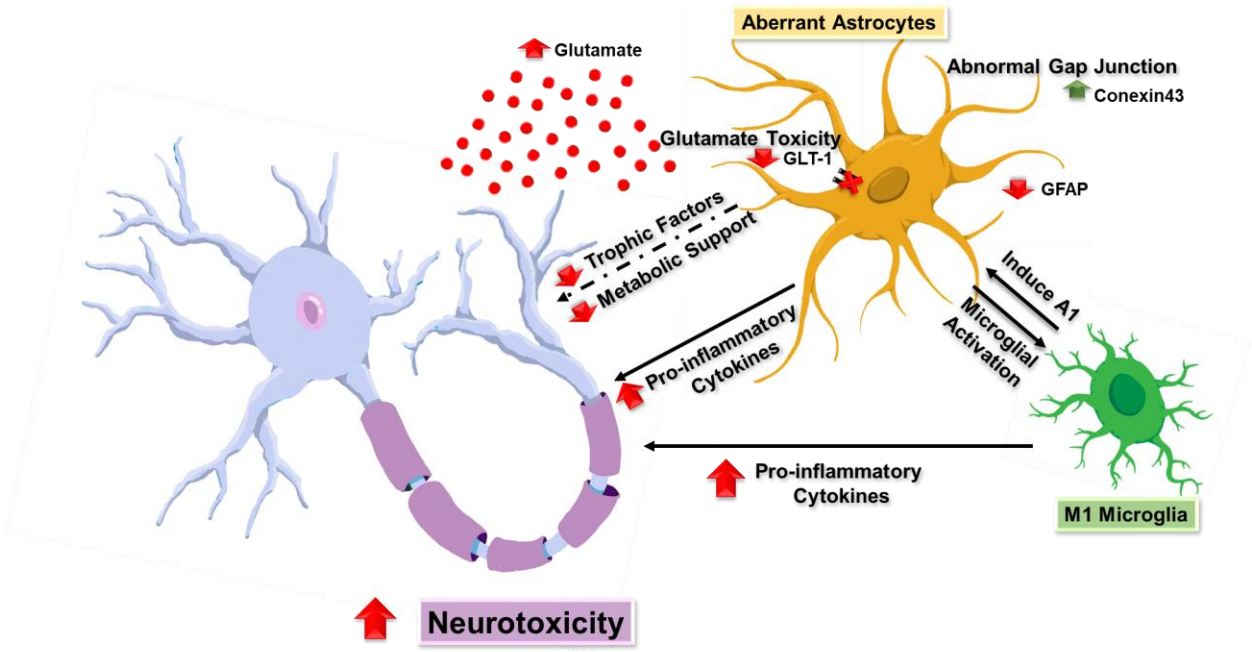

Figure I.4. Dysregulated functions of astrocytes in ALS and contribution to non-cell autonomous neurodegeneration. In physiological conditions, healthy astrocytes support motor neurons providing neurotrophic factors and metabolic support, as well as controlling the glutamate concentration at synapses. In ALS, astrocytes lose their homeostatic function and fail to support MNs and show decreased glial fibrillary acid protein (GFAP) and the decreased expression of glutamate transporter 1 (GLT-1) is associated with glutamate accumulation near MNs, leading to excitotoxicity events. Conexin-43 (Cx43) increase in ALS aberrant astrocytes is associated with enhanced gap junction coupling, increased hemichannel mediated activity and elevated calcium levels. Interactions between astrocytes and microglia are also implicated in non-cell autonomous neurodegeneration, since microglial cytokines may induce a toxic astrocytic phenotype (A1 phenotype). Astrocyte activation also enhances microglial activation to a more pro-inflammatory state (M1 phenotype), exacerbating neuronal damage.

2. MiRNAs in ALS

In recent years, it has been shown that extracellular miRNAs can be mediators of intercellular communication and affect gene expression in the cell of origin and in recipient ones. MiRNAs are the most studied small non-coding RNAs and are typically formed by 18 to 24 nucleotides. They are considered as one of the major post-transcriptional regulators of gene expression through binding to their target mRNAs together by base-pairing and induce either translational repression or mRNA destabilization. It Is estimated that they constitute about 1-5% of the total genome and are involved in most biological pathways and cellular processes, including cell proliferation, apoptosis, cellular development and cellular signaling (Hossain et al. 2012). More than 2000 known human miRNAs have been identified and their biogenesis is regulated by spatial-temporal processes, the reason why any abnormal alteration of their expression is normally associated with pathological conditions. The cellular process of their

biogenesis involves both nuclear and cytoplasmic processes and miRNAs are originated from the large primary (pri) and precursor (pre) transcripts which undergo successive steps of processing until they reach their mature and functional form (**Figure I.5.**) (Graves and Zeng 2012).

In contrast to other RNA species, which are degraded from the extracellular space within a few seconds, some types of miRNAs are remarkably stable and can survive under unfavorable conditions for a long time. The mechanisms underlying the stability of these miRNAs in the RNase-rich environment of blood and other bio-fluids are not well understood. However, two hypotheses have been proposed. The first propose that RNA species present in plasma are protected from degradation probably due to inclusion in lipid or lipoprotein complexes (El-Hefnawy et al. 2004), the second that miRNAs are wrapped with membrane vesicles as exosomes, being protected from the degradation environment (Noferesti et al. 2015). Extracellular miRNAs have been associated with cell-to-cell communication and firstly it has been thought that their transport through cells would be exclusively based on vesicles. However, the theory of vesicle encapsulated miRNA was hugely challenged when was showed that about 90% of extracellular miRNAs are transported associated with Ago protein plasma (Arroyo et al. 2011). *In vitro* and *in vivo* experiments showed that exosomes can be collected by a variety of viable cells and miRNAs can be transferred from one cell to another, acting in the recipient cells by the downregulation of their target genes suggesting that exosomal miRNAs may have a huge impact in mediating intercellular communication. The sensitivity of miRNAs to the cell status makes them good candidates as disease biomarkers, including in neurodegenerative diseases. The importance of miRNAs in ALS was revealed firstly by observation of different miRNAs profiles in ALS patients compared to healthy controls. Analysis on biological samples as muscle tissue, serum, blood, and CSF showed differences in miRNA expression between healthy controls and ALS patients, indicating that their dysregulation may be involved in the disease pathogenesis (Figueroa-Romero et al. 2016; Kovanda et al. 2018).

2.1. MiRNAs are found dysregulated in ALS

Several miRNAs associated with nervous system maintenance and cell death pathways were deregulated on human samples from the spinal cord of ALS patients. Overall, a global reduction of miRNA levels was observed in both FALS and SALS in comparison to healthy controls and other neurodegenerative patients (Rinchetti et al. 2018). Neuroinflammation and the immune system play a key role in ALS disease progression and interestingly miRNAs associated with inflammatory response are usually found dysregulated in ALS models and patients.

MiR-155 promotes inflammation by enhancing the generation of T-cells and macrophages recruitment and is related to the release of inflammatory mediators, being more associated with the microglial M1 phenotype (O'Connell et al. 2010). Among these mediators are iNOS, IL-6 and TNF-α. Furthermore, it binds to suppressor of cytokine signaling 1 (SOCS1) mRNAs, which negatively regulates several inflammatory pathways (Cardoso et al. 2012) and mediates suppression of neural stem cells self-renewal (Obora et al. 2017). In ALS, upregulation of miR-155 was only found during disease progression and end-stages in both animal models and patients samples (Koval et al. 2013). However, in the spinal cord of SOD1 G93A transgenic mice, miR-155 levels were found upregulated on both pre-symptomatic and symptomatic stages (Cunha et al. 2017). In mSOD1 mice, the downregulation of miR-155 led to microglial function restore and increased mice survival (Butovsky et al. 2015) and this miRNA has gained prominence as both therapeutic target and early biomarker of ALS. Despite its expression being more studied in microglia, pro-inflammatory mediators, as IL-1 and IFNγ are associated with miR-155 in astrocytes its inhibition reduces proinflammatory cytokine gene expression suggesting that miR-155 is required for astrocytes polarization into a toxic phenotype (Tarassishin et al. 2011). Recent results from our group shown that miR-155 is also upregulated in SC astrocytes from mSOD1 transgenic mice and its downregulation decreased the expression of pro-inflammatory markers and enhanced astrocytes neuroprotective properties (unpublished data).

Another miRNA associated with inflammatory response in microglia is miR-125b which negatively regulates signal transducer and activator of transcription 3 (STAT3) activating proinflammatory pathways, through NF-κB pathway activation, leading to increased TNF-α expression (Parisi et al. 2013). In mSOD1 mice, a study on the miRNAs expression showed that miR-125b may be involved in microglia proinflammatory signaling (Parisi et al. 2016). The prolonged activation of NF-κB by this miRNA results in a toxic effect on surrounding MNs. Neuroinflammatory miR-155 or mir-125b expressed in microglia are then associated with a more senescent/dysfunctional population of cells and all these phenotypes are believed to have deleterious effects and to accelerate disease propagation. In astrocytes, the miR-125b expression is associated with increased reactivity (Smirnova et al. 2005b).

Also associated with NF-κB pathway activation is miR-146a, which negatively regulates the pathway activation by inhibiting translation of interleukin-1 receptor-associated kinase 1/2 (IRAK1/2) and TNF receptor-associated factor (TRAF6), being more associated with an anti-inflammatory response (Cui et al. 2010). It is mainly produced by astrocytes where it works as a key regulator of inflammatory response through a negative-feedback response of pro-inflammatory signaling (Iyer et al. 2012). Mir-146a is upregulated in various neurological disorders, suggesting a cell compensatory mechanism for pathological inflammation as an

attempt to restore homeostasis. In the case of ALS miR-146a levels were found upregulated in tissue from SALS patients (Campos-Melo et al. 2013), and in the spinal cord of mSOD1 transgenic mice in symptomatic stage (Cunha et al. 2017). However, in astrocytes isolated from the cortex of mSOD1 mice levels, miR-146a is downregulated (Gomes et al. 2018). MiR-21 is another inflammatory miRNA associated not only with microglia but also with astrocytic reactivity and participates in pro-inflammatory signaling by targeting which increases IL-6 and decreases anti-inflammatory IL-10 (Sheedy et al. 2010). In mSOD1 mice, mir-21 levels were found elevated in the spinal cord and in cortex astrocytes accompanied the downregulation of miR-146a in cortex astrocytes (Cunha et al. 2017; Gomes et al. 2018).

miRNAs usually more associated with neurons are also associated with ALS. MiR-124 is one of the most abundant miRNAs in the CNS and its upregulation has been reported in ALS disease end-stages (Marcuzzo et al. 2015). Its contribution to neuronal degeneration will be further discussed in the next topic (**2.2.**). More recently another neuronal miRNA, miR-218, has been found enriched in the end-stage of ALS in a rat model MNs (Hoye et al. 2017). This miRNA was found to be released extracellularly, subsequently to MN loss and it was uptake by astrocytes leading to GLT-1 downregulation (Hoye et al. 2018).

2.2. MiRNA-124: neurotoxic or neuroprotective effect?

As previously referred, miR-124 is one of the most abundant miRNAs in the CNS and suppresses more than 100 different genes. In CNS development miR-124 expression gradually increases and accumulates in parallel with neuronal maturation (Smirnova et al. 2005a). In the adult brain largest neurogenic niche, the subventricular zone (SVZ), miR-124 is a neuronal fate determinant by targeting Sox9 (Akerblom et al. 2012). Besides its effects on neuron fate, miR-124 also contributes to the control of neurite outgrowth during neuronal differentiation by cytoskeleton regulation (Yu et al. 2008). Despite its essential role for neuronal identity acquisition and maintenance, it can contribute to pathological conditions when is aberrantly expressed (Sun et al. 2015) and in the recent years, miR-124 elevated levels had been associated with CNS disorders, such as intracerebral stroke (Wang et al. 2018). Its upregulation had been associated with a neuroprotective effect in cerebral ischemia (Hamzei Taj et al. 2016). MiR-124 is also important in Alzheimer's disease (Mehta et al. 2017) where miR-124 upregulation have a neuroprotective effect by downregulating BACE1 expression, associated with Aβ peptide accumulation, and by decreasing autophagy (Du et al. 2017). In ALS mSOD1 transgenic mice, miR-124 was transported in MNs exosomes and upregulated GLT-1 expression in astrocytes suggesting that miR-124 increase may have a positive effect in GLT-1-based neuroprotection (Morel et al. 2013).

However, other studies indicate that elevated levels of miR-124 are not necessarily associated with neuroprotection. In fact, axonal expression of miR-124 is elevated in muscular disease conditions elevated expression of miR-124 is associated with decreased neurotransmitter release (Zhu et al. 2013). MiR-124 overexpression also negatively impacts MN morphology and mitochondrial activity, where vimentin was identified as a direct target of this miR-124 (Yardeni et al. 2018). Vimentin is more associated with neurons only in neurodevelopment phase, where its expression is necessary for neurite extension. In the adult brain, vimentin expression is usually restricted to endothelial cells and glial cells, mainly in astrocytes. However, expression of vimentin in neuronal cell bodies had been reported in neurodegenerative conditions, such as Alzheimer's disease and in this case, vimentin was expressed in neurons from affected brain regions and in brain tissue subjected to mechanical injury (Levin et al. 2009). In mSOD1 G93A cells, miR-124 was found downregulated in neural stem cells leading to a higher generation of astrocytes (Zhou et al. 2018).

More recently, miR-124 was found upregulated in SALS CSF and, together with miR-9 and miR-125b, was indicated as a potential ALS biomarker for neuronal death (Waller et al. 2017). In ALS model, upregulated miR-124 was found in MNs and their derived exosomes in mSOD1 NSC-34 cell model and the incubation of such exosomes in microglial cells caused increased M1 phenotype polarization (Pinto et al. 2017). However, how these effects were directly caused by miR-124 and how its modulation may affect mSOD1 MNs needs further elucidation to understand how the upregulation may have a neurotoxic or a neuroprotective effect.

2.3. Modulation of miRNAs as a therapeutic approach

The use of miRNA modulation as a disease therapeutic strategy depends firstly on the pattern of their expression in the pathological condition. It may include the use of miRNA precursors of the mature form of miRNA (miRNA mimics or pre-miRNA) in order to upregulate the miRNA levels or miRNA antagonists (anti-miRNA), where the final goal is to downregulate the miRNA level (Christopher et al. 2016). Pre-miRNAs are double-stranded miRNA-like fragments that are used to increase levels of a miRNA, in order to restore its function, is designed to silence genes. They act in the same way as functional miRNAs, blocking the mRNA of the target genes (Wang 2011). An alternative would be to insert the miRNA transcribing DNA sequence in the genome or introduce plasmids. However, in humans, this approach is not viable due to ethical concerns. Anti-miRNAs are short, single-stranded oligonucleotides that target the mature miRNA before it reaches its target mRNA, leading to the breakdown of the endogenous miRNA by forming a duplex structure of the endogenous and exogenous miRNA that is degraded (Lima and Cerqueira 2018). The silencing of the endogenous miRNA by this method is efficient and long-lasting (Baigude et al. 2013). Another

developed strategy for miRNA inhibition is to exploit synthetic sponge mRNA (Ebert et al. 2007). However, this method affects the activity of a whole family of miRNAs. The overall mechanism of miRNAs modulation is represented in Figure I.5.

Before developing a miRNA-based therapy it is important to identify the best miRNA candidates or targets for each disease condition. One of the challenges for RNA-based therapies is the potential degradation of the oligonucleotides by RNases in serum or inside the cells. To avoid degradation inside cells, is possible to alter oligonucleotide chemistry through, for example, methylation. Another strategy is to develop delivery vehicles to encapsulate RNAs, which is also a challenge since it is necessary to design delivery vehicles that confer higher stability to the therapeutic candidate and enable tissue-specific targeting, avoiding potential toxicities and off-target effects. Despite the challenges, in the short time since the discovery of miRNAs, therapeutic approaches to manipulate them have progressed from bench to bedside, whit some successful phase I clinical trials and ongoing phase II trials (Rupaimoole and Slack 2017).

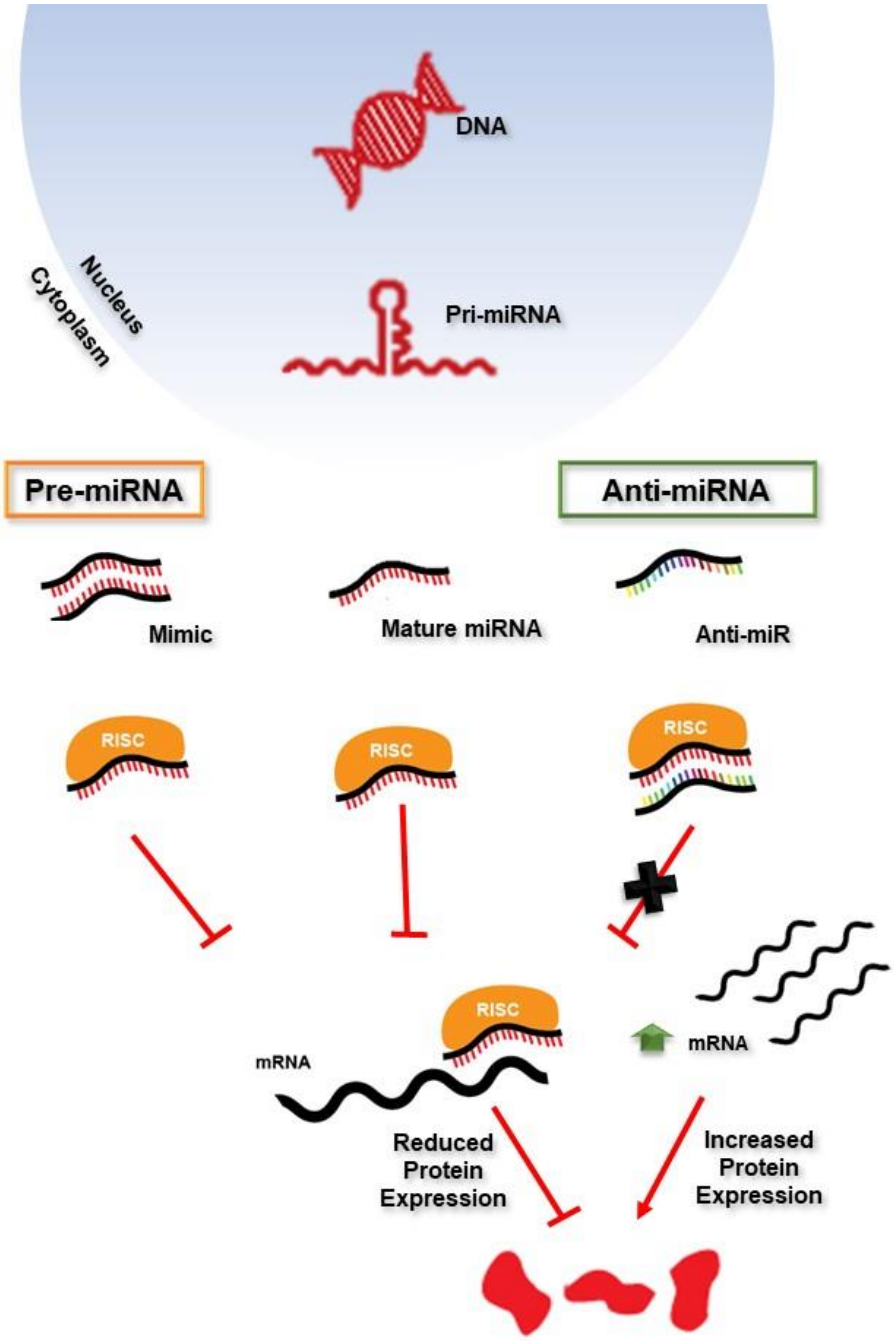

Figure I.5. MiRNA-based strategies in therapeutics. MicroRNAs (miRNAs) are the most studied small non-coding RNAs typically formed by 18 to 24 nucleotides. They are considered as one of the major post-transcriptional regulators of gene expression and act with RNA-induced silencing complex (RISC) by binding to their target messenger RNAs (mRNAs), ultimately regulating protein expression. They can be found in the extracellular space and may have a huge impact in intercellular communication. The sensitivity of miRNAs to the cell status makes them good candidates as disease biomarkers, including in ALS. One strategy is the increase of downregulated miRNAs by using miRNA mimics (pre-miRNAs). Pre-miRNAs are double stranded RNA molecules with the same sequence of the endogenous ones, binding to the same mRNA targets, resulting in protein expression decrease. Another strategy is the use of miRNA antagonists (anti-miRNA) to downregulate miRNAs found upregulated in the disease. Anti-miRNAs are single stranded sequences that bind to the target miRNA, blocking their linkage to mRNAs. In that way target protein levels are increased.

3. Strategies for the study of ALS degeneration

To identify novel therapeutic targets and biomarkers for ALS is essential to have a better understanding of the mechanisms that lead to MN degeneration and in the past years, a very large body of knowledge has been built with the development of several models based on FALS genetic. Model systems vary from cell culture systems to invertebrates, non-mammalian vertebrate's animal models and rodent models, which have been particularly useful to the study of FALS. More recently it had extended to human patient-derived stem cells and the availability of this type of cell models opened new paths for ALS research providing a good balance between the advantages of a cellular model and the approach to the human disease. The development of cross-models approach, in which novel disease mechanisms are identified in simpler systems and later validated in more complex models as well as in human cells combining both in vitro and in vivo models, will maybe improve the chance of success and hopefully allow the identification of novel pathogenic pathways leading to more clinical translational researches.

The elucidation of some aspects of cell-to-cell communication in different conditions may be performed using co-cultures, which are cultures of more than one type of cell, e.g. neuron-astrocyte, neuron-microglia or in a more indirect way by the incubation of one cell type secretome in other cells. From secretome derived from cells is also possible to isolate soluble factors and exosomes, allowing the study of a more specific cellular communication. This type of cell culture assays is needed because it mimics the complexity of cell to cell communication at the same time as they provide control over the different microenvironmental parameters. In ALS, co-culture of mSOD1 astrocytes with cortical MNs showed that when in contact with mutant astrocytes neurons lost their synaptic ability, indicating that astrocytic mutant SOD1 may also potentiate neurotoxicity (Kunze et al. 2013). In this topic, different possibilities of ALS disease models will be further discussed.

3.1. Cell lines

Immortal cell lines are often used in research in the place of primary culture cells and they offer several advantages since they are cost-effective, easy to use and maintain and provide an unlimited supply of material avoiding ethical concerns associated with the use of animals and human tissue. They also provide a pure cell population, providing in that way a consistent sample with reproducible results. However, they are simplified models. In ALS, the most widely used MN cell line to the study of neurotoxicity is NSC-34 cell line. Microglia cell lines, as N9,

may be useful for the study of how neurotoxic neurons and ALS-associated mutations affect surrounding cells.

3.1.1. NSC-34

Cultures of different MN cell lines are used to study the molecular mechanisms of neurotoxicity induced in MNs. The most common cell line used in ALS research is the NSC-34 produced by fusion of MNs from the spinal cord of mice embryos with mice neuroblastoma cells N18TG2. This cell line was first described as having many of the morphological and physiological properties of MN primary cultures, as neurofilaments proteins expression and the expression of synaptic proteins and synapse formation (Charalambous et al. 2013). Despite the extension of stable neurites in these cells they do not express MAP proteins. NSC-34 cells respond to known neurotoxic stimuli which affect voltage-gated ion channels, cytoskeletal organization, and axonal transport. NSC-34 transfected with mutant SOD1 with G93A mutation are used as ALS cell model (Gomes et al. 2008). The mutant cells have less proliferative and differentiation ability and have some of the disrupted mechanisms described in ALS patients. Cells have an accumulation of mutant SOD1 aggregates, mitochondrial impairment, metalloproteinase-9 activation, the release of NO, caspase-9 activation and, more recently overexpression of miR-124 have been described in these cells (Pinto et al. 2017; Vaz et al. 2015). However, these cells retain some characteristics of neuroblastoma cells, which can interfere in cell proliferation and the response to the therapeutic agents (Tovar-y-Romo et al. 2009). It is important for the ALS study in these cells to know that glutamate had no specific effect on differentiated cells (Madji Hounoum et al. 2016).

3.1.2. N9

Microglial primary cultures are time-consuming and immortalized cell lines are the better option to overcome it. One of the most commonly used cell lines is a mouse-derived N9 cell line. N9 cell line was developed by primary microglia cells immortalization with oncogenes of MH2 retrovirus and share many phenotypical features with mouse primary cultures (Righi et al. 1989) but with increased proliferation and cell adherence (Stansley et al. 2012). These cells respond to LPS increasing ROS production which activates pro-inflammatory pathways (Zhao et al. 2011). Our group further characterized LPS-induced N9 microglia and the LPS stimulation lead to an upregulation of M1 markers, enhanced phagocytosis, an increase of the extracellular alarmin HMGB1 and of MMP-9 (Cunha et al. 2016). In addition, N9 cells are also able to release exosomes which recapitulate miRNA cargo of the cells of origin and also shown the capacity to uptake exosomes from NSC-34 neurons (Cunha et al. 2016; Pinto et al. 2017). N9 microglia cell line can also be transduced with lentiviruses to express mutant SOD1 (Dimayuga et al. 2007). Then, we can use these cells in co-cultures of NSC-34 and N9 cell

lines, being a good model to study the mechanisms involved in the dialogue between MNs and microglia in ALS pathogenesis.

3.2. Primary Cultures

Primary SC cultures were established to study morphological, biochemical and electrophysiological characteristics of MNs and can be obtained from both animal cortex and spinal cord and at different stages of the disease. Neurons, astrocytes, and microglia can be extracted, purified and maintained in culture in primary cell cultures. When glial cells are absent of the environment, neurons cultures only last 2 weeks. However, glial cells can be maintained in culture for up to 7 weeks. This type of culture was used to demonstrate that MNs are vulnerable to glutamatergic excitotoxicity through AMPA receptors (Rao et al. 2003). In our group primary cultures have been used to show new insights of the glial involvement in ALS (Gomes et al. 2018).

3.3. Organotypic Cultures

Organotypic cultures involve the preservation of a whole tissue slice which is maintained in *ex vivo* and provide the best way to preserve all the cellular content and cells electrophysiological and biochemical organization. Organotypic slices can be obtained from both embryos and postnatal animals, allowing the analysis of different disease stages. This type of cultures can be maintained for more than 2 months and several types of molecules can be added to the culture medium to modulate neurotoxicity. Organotypic culture is a cellular system that closely reproduces the *in vivo* situation and allows dynamic studies, even if it does not fully recapitulate it. This model allows dynamic studies with many drugs, but, as it happens for *in vitro* studies, it cannot reproduce entirely an *in vivo* system. In the case of ALS, organotypic cultures are important because they are three-dimensional and allow communication between neurons and glial cells in their biochemical and morphological environment.

3.4. Human Pluripotent Stem Cells

The ability to generate iPSCs from differentiated cells has opened new paths for ALS research. By introducing pluripotency genes into human patient-derived cells, it is now possible to generate iPSCs from patients with ALS independently of a known disease-causing mutation. In addition, iPSCs can be differentiated specifically into spinal motor neurons and in the different types of glial cells. An advantage of generating models from patient-derived cells is that they remove the need to overexpress transgenes containing ALS gene mutations and SALS can also be modeled, which is not possible in the other model systems (Kiskinis et al.

2014; Sances et al. 2016). Several aspects of the disease's neuropathology are recapitulated in iPSC-derived motor neurons, such as the aggregation and/or cytoplasmic miss localization of TDP-43, SOD1, FUS, and in the case of C9orf72 repeat expansions, the formation of RNA foci and dipeptide repeat proteins (Matus et al. 2014). Many important functional phenotypes have already been identified in iPSC-derived spinal motor neurons, ranging from increased cell death, neurofilaments disorganization, defects in nucleocytoplasmic transport to changes in excitability (Bilican et al. 2012; Chen et al. 2014; Kiskinis et al. 2014; Zhang et al. 2015). Patient-derived cells have also been used to confirm the importance of non-neuronal cells in human ALS models (Ferraiuolo et al. 2016; Meyer et al. 2014). What makes iPSCs so close to the patient is that they contain the genetic background of an ALS patient, even if the cause of the disease is unknown and allows the study of the different cell types.

4. Aims

The main objectives of this thesis are to explore whether miR-124 upregulation found in ALS MNs is related with beneficia or harmful effects in MN function and survival and influence secretome properties toward microglia activation and astrocyte reactivity, by using pre-miR-124 in WT MNs and anti-miR-124 in SOD1G93A (mSOD1) MNs. MN-like NSC-34 cell line non-mutated or expressing human mSOD1 (mutated MNs), astrocytes from both WT and mSOD1 transgenic mice pups and N9 microglia cell line, will be used as cellular models.

Specific aims are to:

1. Investigate the effects of upregulation of miR-124 in human SOD1 WT MNs (NSC-34/hSOD1wt) and downregulation of miR-124 in mSOD1 MNs (NSC-34/hSOD1G93A) on cell dysfunction and function recovery, respectively, by assessing cell death by necrosis/apoptosis, neurite extension, mitochondria dynamics, synaptic protein expression, and inflammatory-associated miRNAs, relatively to non-transfected WT and mSOD1 cells.
2. Determine the consequences of miR-124 upregulation/downregulation on the MN-derived secretome immunostimulatory/immunomodulatory properties over N9 microglial cells by evaluating cell morphology, phagocytic ability and polarized phenotypes, relatively to non-treated WT cells.
3. Decipher the consequence of miR-124 upregulation/downregulation on the MN-derived secretome influence over astrocyte aberrancy/astrocyte recover competence, respectively, by determining the expression of GFAP and GLT-1 in astrocytes isolated from WT and mSOD1 transgenic mice pups (8-days old animals) after secretome incubation.

II. Materials and methods

1. Materials

1.1. Animals

This study was performed in accordance with the European Community guidelines (Directives 86/609/EU and 2010/63/EU, Recommendation 2007/526/CE, European Convention for the Protection of Vertebrate Animals used for Experimental or Other Scientific Purposes ETS 123/Appendix A) and Portuguese Lawson Animal Care (Decreto-Lei 129/92, Portaria 1005/92, Portaria 466/95, Decreto-Lei 197/96, Portaria 1131/97). All the protocols used in this study were approved by the Portuguese National Authority (General Direction of Veterinary) and the Ethics Committee of the Instituto de Medicina Molecular (iMM) João Lobo Antunes of the Faculty of Medicine, Universidade de Lisboa, Lisbon, Portugal.

Transgenic B6SJL-TgN (SOD1G93A)1Gur/J males (Jackson Laboratory, No. 002726) overexpressing the human SOD1 gene carrying a glycine to alanine point mutation at residue 93 (G93A) (mSOD1) and WT B6SJLF1/J females were purchased from The Jackson Laboratory (Bar Harbor, ME, USA), and were bred at iMM João Lobo Antunes rodent facilities, where a colony was established. Mice were maintained on the B6SJL background by breeding mSOD1 transgenic males with non-transgenic females in a rotational scheme. Transgenic males were crossed with non-transgenic females because transgenic females are infertile. Experiments were conducted in 8-day mice pups. Every effort was made to minimize the number of animals used and their suffering.

1.2. Supplements and Chemicals

Dulbecco's modified Eagle's medium (DMEM) high glucose w/o pyruvate, DMEM-Ham's F-12, fetal bovine serum (FBS), penicillin/streptomycin, L-glutamine, and nonessential amino acids (NEAA) were acquired from Biochrom AG (Berlin, Germany). Hank's balanced salt solution (HBSS) without phenol red (1x), B-27® serum-free supplement (50x) and neurobasal medium were purchased from GIBCO ® (Life Technologies, Inc., Grand Islands, USA). Roswell Park Memorial Institute (RPMI) 1640 medium, trypsin-Ethylenediamine tetraacetic acid (trypsin-EDTA) solution, bovine serum albumin (BSA), Antibiotic-Antimycotic, Tris-base, phenylmethylsulfonyl fluoride (PMSF), Hoechst 33258 dye, fluorescent latex beads 1µm (2.5%) and Poly-D-Lysine (PDL), XtremeGENE™ HP DNA Transfection Reagent, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Geneticin sulfate (G418) was obtained from Calbiochem (Darmstadt, Germany). Triton X-100 and protease inhibitor cocktail tablets

were acquired from Roche Diagnostics (Mannheim, Germany). Nitrocellulose membrane obtained from Amersham Biosciences (Piscataway, NJ, USA). Sodium dodecyl sulfate (SDS) was acquired from VWR-Prolabo. Acrylamide, bis-acrylamide, Tween-20, glycerol and absolute ethanol were obtained from Merck (Darmstadt, Germany). Western Bright Sirius HRP substrate Kit was obtained from Advansta (Menlo Park, CA, USA). Mitotracker® Red CMXRos is from Invitrogen Molecular ProbesTM (Eugene, Oregon, EUA). TripleXtractor, GRS cDNA synthesis kit, and Xpert Fast Sybr Blue were purchased from GRiSP (Porto, Portugal). miRCURY™ RNA isolation kit and miRCURY LNA™ Universal RT microRNA PCR (Universal cDNA Synthesis Kit II and ExiLENT SYBR® Green master mix), as well as the primer mix for miRNAs and SNORD, were acquired from Qiagen (Hilden, Alemanha). Pre-miR™ miRNA precursor 124 (mimic) and Anti-miR™ miRNA Inhibitor 124 were obtained from Ambion and Opti-MEM™ from Life Technologies (Carlsbad, California, EUA). Guava Nexin® Reagent was from Millipore (Burlington, Massachusetts, EUA).

1.3. Primers

The primers used to amplify protein-coding genes was purchased from Thermo Scientific (Waltham, MA, USA), while primer mix used to amplify microRNAs (miR-124, miR-146a, miR-125b, and miR-21) were obtained from Qiagen.

Table II.1. List of primer sequences used in real-time polymerase chain reaction (RT-PCR) to amplify protein-coding genes.

Gene	Forward primer	Reverse primer
β-actin	5'-GCTCCGGCATGTGCAA-3'	5'-AGGATCTTCATGAGGTAGT-3'
HMGB1	5'-CTCAGAGAGGTGGAAGACCATGT-3'	5'-GGGATGTAGGTTTTCATTTCTCTTTC-3'
S100B	5'-GAGAGAGGGTGACAAGCACAA-3'	5'-GGCCATAAACTCCTGGAAGTC-3'
IL-1β	5'-CAGGCTCCGAGATGAACAAC-3'	5'-GGTGGAGAGCTTTCAGCTCATA-3'
TNF-α	5'-TACTGAACTTCGGGGTGATTGGTCC-3'	5'-CAGCCTTGTCCCTTGAAGAGAACC-3'
Dynein	5'-GCCTCAGTCTCTGTCCCATC-3'	5'-AAGTCCTGGGGTAAGGTGCT-3'
KIF5B	5'-GGTCCTACAGTTGCCACCTA-3'	5'-ATTGAAATACGCCAGGCCCA-3'
Synaptophysin	5'-GACGTTGGTAGTGCCTGTGA-3'	5'-GCACAGGAAAGTAGGGGGTC-3'
DLG4	5'-GAGGCTGGCGGCCAGTACACCAG-3'	5'-ACAGAGCAGGCGGTCAG-3'
iNOS	5'-ACCCACATCTGGCAGAATGAG-3'	5'-AGCCATGACCTTTCGCATTAG-3'
Arg-1	5'-CTTGGCTTGCTTCGGAAC-3'	5'-GGAGAAGGCGTTTGCTTAGTT-3'
GFAP	5'-CCAAACTGGCTGATGTCTACC-3'	5'-GCTTCATCTGCCTCCTGTCTA-3'
CD206	5'-GTGGAGTGATGGAACCCCAG-3'	5'-CTGTCCGCCCAGTATCCATC-3'

HMGB1, High Mobility Group Box 1; S100 calcium-binding protein B; IL-1β, Interleukin 1β; TNF-α, Tumor necrosis factor α; KIF5B, Kinesin Family Member 5B; DLG4, Discs Large MAGUK Scaffold Protein 4 (or PSD-95, postsynaptic density protein 95); iNOS, inducible Nitric Oxide Synthase; Arg-1, Arginase 1; GFAP, Glial Fibrillary Acid Protein; CD206, mannose receptor C type 1.

Table II.2. List of primer sequences used in PCR to amplify miRNA.

miRNA	Target sequence
has-miR-124-3p	5'-UAAGGCACGCGGUGAAUGCC-3'
has-miR-146a-5p	5'-UGAGAACUGAAUUCCAUGGGUU-3'
has-miR-21-5p	5'-UAGCUUAUCAGACUGAUGUUGA-3'
has-miR-125b-5p	5'-UCCCUGAGACCCUAACUUGUGA-3'
SNORD110	Reference gene

1.4. Antibodies

The antibodies used in immunocytochemistry (ICC) and Western Blot (WB) assays and the respective source, species and used dilution are shown below.

1.4.1. Primary antibodies

Table II.3. List of primer sequences used in real-time polymerase chain reaction (RT-PCR) to amplify protein-coding genes.

Primary Antibody Against	Source	Specie	Dilution	
			ICC	WB
β-III Tubulin	Merck Millipore, MAB1637	Mouse	1:500	-
Mitofusin 2	AbCam, ab50838	Rabbit	1:50	-
Drp1	AbCam, ab140494	Mouse	1:150	-
Iba-1	Wako, 019-19741	Rabbit	1:250	-
iNOS	BDBiosciences, 610329	Mouse	1:100	1:200
Arg-1	Santa Cruz, sc18355	Goat	1:50	-
GFAP	Sigma-Aldrich, G9269	Rabbit	1:100	-
GLT-1	Santa Cruz, sc-135892	Mouse	1:50	-
β-actin	Sigma, A5441	Mouse	-	1:5000
HMGB1	BioLegend, 651402	Mouse	-	1:200
SOD1	Santa Cruz Biotechnology, sc-11407	Rabbit	-	1:500

DRP1, dynamin-related protein 1; Iba-1, Ionized calcium binding adaptor molecule 1; iNOS, inducible Nitric Oxide Synthase; Arg-1, Arginase 1; GFAP, Glial Fibrillary Acid Protein; GLT-1, glutamate transporter 1; HMGB1, High Mobility Group Box 1; SOD1, Superoxide Dismutase 1;

1.4.2. Secondary antibodies

Table II.4. List of secondary antibodies used in western blot (WB) and immunocytochemistry (ICC), with the respective brand and dilution.

Secondary antibody	Source	Dilution	
		ICC	WB
Alexa 488 anti-mouse	Invitrogen, A10680	1:1000	-
Alexa 488 anti-rabbit	Invitrogen, A11008	1:1000	-
Alexa 494 anti-mouse	Invitrogen, A11005	1:1000	-
Alexa 494 anti-rabbit	Invitrogen, A111012	1:1000	-
FITC anti-mouse	Vector, FI-2001	1:227	-
HRP, anti-mouse	Santa Cruz Biotechnology, sc-2005	-	1:5000
HRP, anti-rabbit	Santa Cruz Biotechnology, sc-2004	-	1:5000

HRP, horseradish peroxidase

1.5. Equipment

To ensure a stable environment for optimal cell growth (37ºC and 5% CO_2), cell cultures were maintained in HERA cell 150 incubators (Thermo Scientific, Waltham, MA, USA). All work involving cell handling was performed in sterile conditions in a Holten Lamin Air HVR 2460 (Allerod, Denmark). Fluorescence images were acquired by using AxioCam HR camera adapted to an AxioScope A1® microscope (Carl Zeiss, Inc., North America) and images were acquired by using Zen 2012 (blue edition, Zeiss) software and quantifications were made using ImageJ (1.50i, National Institutes of Health, USA) software. Guava easyCyte 5HT Base System Flow Cytometer was used for flow cytometry assays. Eppendorf 580R (Eppendorf, Hamburg, Germany) and Sigma 3K30 centrifuges were used for different experimental procedures. Protein and RNA quantification was performed using NanoDrop ND100 Spectrophotometer (NanoDrop Technologies, Wilmington, USA). Protein samples were sonicated in Ultrasonic Processor UP100H (Hielscher-Ultrasound Technology, Teltow, Germany) and the system employed in Western-Blot assays were Mini-PROTEAN Tetra (Bio-Rad). Measurements of Western Blot assays were performed using ChemiDoc™ XRS system from Bio-Rad. For cDNA synthetizes from RNA samples, we used personal Thermocycler (Biometra®, Göttingen, Germany) and determination of mRNA and miRNA expression by quantitative real-time PCR (qRT-PCR) we used a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Life Technologies).

2. Methods

2.1. Cell cultures

2.1.1. NSC-34 cell line

NSC-34 cell line stably transfected with human SOD1, either wild-type or mutated in G93A (NSC-34/hSOD1^{wt} (WT MNs) or NSC-34/hSOD1^{G93A} (mSOD1 MNs), respectively), were grown in DMEM high glucose with glutamine, w/o pyruvate, supplemented with 10% of FBS and 1% of Pen/Strep proliferation media and selection of transfected cells was made with 0.1% G418, as usual in our lab (Vaz et al. 2015). Cell medium was changed every 2 to 3 days. WT MNs were used as a control. For assays, cells were seeded in 6-, 12- or 24-well culture plates at a concentration of 5x10^4 cells/ml and maintained at 37ºC with 5% of CO_2). Culture plates were coated with PDL (10 µg/ml).

After 48 h of proliferation, differentiation was induced by changing medium to DMEM-F12 with 1% of FBS-exosome depleted (removed by differential ultracentrifugation), 1% NEAA, 1% of Pen/Strep and 0.1% of G418, to produce SOD1 accumulation in mSDOD1 cells (Vaz et al. 2015). After 1 day *in vitro* (1 DIV) both WT and mSO1 MNs were transfected with 15 pM of miRNA Pre-miR™ miRNA Precursor 124 (mimic) and Anti-miR™ miRNA Inhibitor 124, respectively, mixed with X-tremeGENE™ HP DNA Transfection Reagent in a proportion of 2:1, and diluted in Opti-MEM™. Cells were left for 12h. Fresh medium was added and transfected cells incubated for more 48 h (4 DIV).

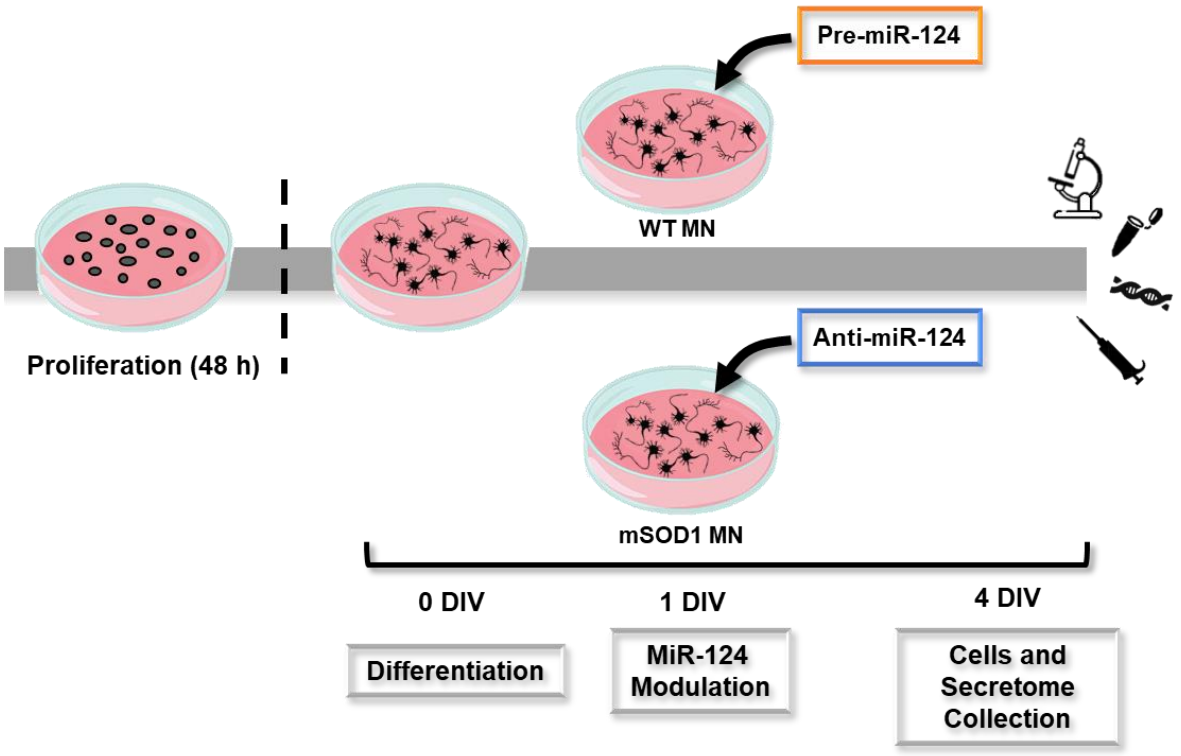

Figure II.1. Schematic timeline representation of miR-124 modulation in NSC-34 cells, either non-mutated (WT) or mutated (NSC-34/hSOD1G93A-mSOD1 MNs). SOD1 WT and mSOD1 MNs proliferated 48 h and then differentiation was induced by changing medium to DMEM-F12. After 1 day in vitro (1 DIV) WT cells were transfected with miR-124 mimic (pre-miR-124) and mSOD1 MNs with anti-miR-124 and cultured for 12 h. Fresh medium was added, and transfected cells were maintained in culture for more 48 h (4 DIV). Cells and secretome were collected. MNs were assessed for function/dysfunction and their secretome [(neuronal conditioned medium (NCM)] was used for incubation in N9 microglia and in cultured astrocytes.

2.1.2. N9 cell line

Cells were cultured in RPMI media supplemented with FBS (10%), L-glutamine (1%) and penicillin/streptomycin (1%) and grown to confluency. For each experience, cells were plated in 6- and 24- well plates, at a concentration of $1x10^5$ cells/ml and maintained at 37ºC and in a humidified atmosphere of 5% CO2 for 24 h. No coating was required, and cells were maintained as usual in our lab (Cunha et al. 2016).

After 1 day in culture, N9 microglia were incubated with the MN secretome [neuronal conditioned medium (NCM)] collected from WT MNs either incubated or not with pre-miR-124 and mSOD1 MNs non-treated or treated with anti-miR-124. Cells were collected after 4 h to mimic a situation of acute response, as previously described in our lab (Pinto et al, 2017).

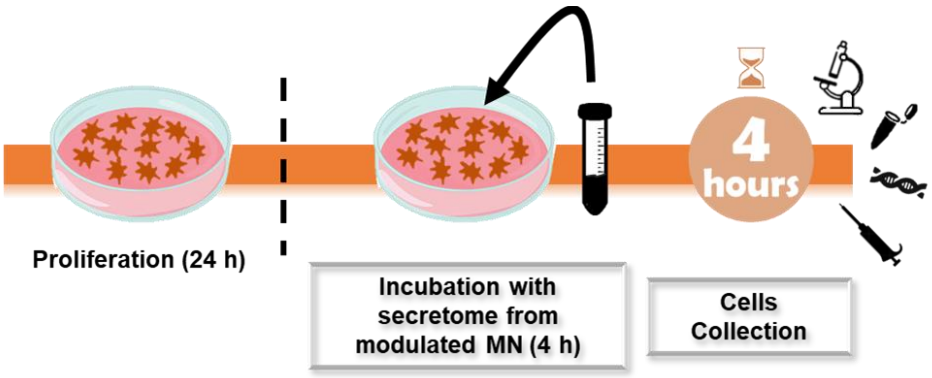

Figure II.2. Schematic timeline representation of N9 microglia culturing and their treatment with MN secretome. N9 microglia cells proliferated 24 h and then were incubated with the motor neuron (MN) secretome/neuronal conditioned medium (NCM) from WT MNs, WT MNs + pre-miR-124, mSOD1 MNs and mSOD1 MNs + anti-miR-124. After 4 h incubation cells were collected for the different assays.

2.1.3. Astrocyte primary cultures

Astrocytes were isolated from the spinal cord of 8-day-old WT and mSOD1 mice, as usual in our group for cortex astrocytes (Gomes et al. 2018) with minor modifications. Cells were plated on tissue culture plates in culture medium supplemented with 11 mM sodium bicarbonate, 38.9 mM glucose, 1% Antibiotic-Antimycotic and 10% FBS, at a concentration of $2.5x10^4$ cells/cm^2. Cells were maintained at 37ºC in a humidified atmosphere of 5% CO_2 until 13 DIV, with culture medium replacement at 7 and 10 DIV. Under these culture system conditions, microglia contamination was less than 5%, which was revealed to not influence astrocyte inflammatory response (Fernandes et al. 2011; Gomes et al. 2018), and that of oligodendrocytes and neurons only accounted for 4% and 1%, respectively.

At 12 DIV WT and mSOD1 astrocytes were incubated with neuronal conditioned medium (NCM) collected from WT and mSDO1 MNs either incubated or not with pre-miR-124 and anti-miR-124, respectively. Cells were collected after 24 h (13 DIV).

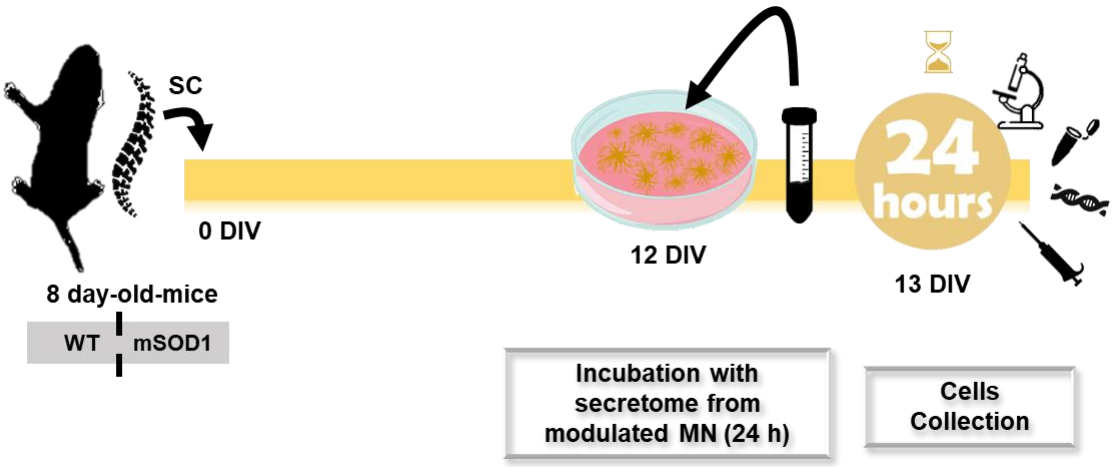

Figure II.3. Schematic timeline representation of isolation and culturing of astrocytes from WT and mSOD1 mice with MN secretome. Astrocytes were isolated from the spinal cord of 8-day-old non-transgenic (WT) or transgenic mice pups expressing human SOD1 with G93A mutation (mSOD1) and cultured for 12 days in vitro (DIV). Then, both WT and mSOD1 cells were incubated with the MN secretome/neuronal conditioned medium (NCM) from WT MNs, WT MNs + pre-miR-124, mSOD1 MNs and mSOD1 MNs + anti-miR-124. After 24 h cells were collected for the different assays.

2.1.4. Quantitative RT-PCR

RNA was isolated from NSC-34 and N9 cell culture using TripleXtractor and quantified using Nanodrop ND-100 Spectrophotometer (Cunha et al. 2017). With the Xpert cDNA Synthesis Mastermix Kit, the RNA samples with a concentration of 1000 ng/µL were transcribed into complementary DNA (cDNA). Then, cDNA was amplified on a QuantStudio 7 Flex Real-Time PCR System, using Xpert Fast SYBR Mastermix (Uni) BLUE, under the following conditions: 50°C for 2 min and 95°C for 2 min, followed by 50 amplification cycles at 94°C for 5 s and 62°C for 30 s). The PCR was performed in 384-well plates, with the set of primers designed for protein-coding genes and cDNA indicated in **Table II.1**. Each sample was measured in duplicate and a non-template control (NTC) was included for each amplified gene. β-actin was used as the endogenous control. For the analysis of miRNA expression, cDNA was produced from 5 ng of the RNA samples, using miRCURY LNA™ Universal RT microRNA PCR. qRT-PCR was performed on a QuantStudio 7 Flex Real-Time PCR System, using PowerUp™ SYBR™ Green Master Mix with pre-designed primers indicated in **Table II. 2**. The PCR conditions were: 10 min at 95°C for polymerase activation/denaturation, 50 amplification cycles at 95°C for 10 s and 60°C for 1 min (ramp-rate of 1.6°/s). Once again, PCR was performed in 384-well plates, with each sample performed in duplicate, and NTC was included for each amplification product. SNORD110 (reference gene) was used as an endogenous control.

2.1.5. Western Blot assay

Western blot analysis was performed as usual in our lab (Fernandes et al. 2006). Total protein from NSC-34 MNs cultures were obtained using TripleXtractor and quantified using Nanodrop ND-100 Spectrophotometer. Equal amounts of protein extracts (50 µg) were separated on a 10 % gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a tris-glycine-SDS running buffer (for iNOS) or in a Tris-Tricine gel (for HMGB1 and SOD1). After electrophoresis, proteins were transferred to a nitrocellulose membrane. Membranes were then blocked for 1 h with 5% non-fat milk in TBS-T at RT. Then, membranes were incubated overnight with the respective primary antibody (**Table II.3**), diluted in TBT-T with 5% of BSA, at 4°C with shaking. After 3 washes with TBS-T, membranes were incubated 1 h at RT with species-specific secondary antibodies (**Table II.4**) conjugated with horseradish peroxidase. The chemiluminescent detection was performed after membrane incubation with Western Bright Sirius HRP and scanning with ChemiDocXRS. The relative intensities of protein bands were analyzed using the Image Lab™ analysis software (Bio-Rad Laboratories, Hercules, CA, USA), normalized to β-actin and expressed as fold *vs*. WT MNs.

2.2. Immunocytochemistry procedure

After the respective cell collection days, NSC-34 MNs, N9 microglia or astrocytes plated on coverslips were fixed with 4 % (w/v) of Paraformaldehyde (PFA). Then, coverslips were incubated 20 min with 0.2% Triton X-100 in PBS for cell permeabilization, 30 min with blocking solution 3% BSA in PBS, and overnight with primary antibodies at 4ºC (**Table II.3**). In the following day, coverslips were washed and incubated for 2h at room temperature (RT) with species-specific fluorescent secondary antibodies (**Table II.4**). Cell nuclei were stained Hoechst 33258 dye. Finally, coverslips were mounted onto uncoated slides using PBS-Glycerol (1:1). Fluorescence was visualized using AxioCam HR camera adapted to an AxioScope A1® microscope and Zen 2012 (blue edition) software. Merge images of UV and fluorescence from at least 10 random fields were acquired with 40x magnification per sample.

2.3. Determination of cell death (Nexin assay)

To analyze cell viability by Nexin (Cunha et al. 2017), both cells and extracellular media were collected to the same tube. Adherent cells were detached with a solution of trypsin 1x for 5 min at 37ºC, after which trypsin action was stopped with FBS. Cells were centrifuged at 700g for 5 min, the supernatant was discharged, and the pellet resuspended in 1% BSA in PBS. The samples were added to 96-well plates with Nexin Reagent® (Annexin V/7AAD) and incubated 20 min at room temperature, protected from the light. Samples were analyzed on Guava easyCyte 5HT Base System Flow Cytometer, and 5000 events per sample were counted.

Three populations of cells were distinguished in this assay: viable cells (annexin V-PE- / 7-AAD-), early-apoptotic cells (annexin V-PE+ / 7-AAD-) and late stages of apoptosis or necrotic cells (annexin V-PE+ / 7-AAD+).

2.4. Mitochondrial viability

To stain viable mitochondria, NSC-34 MNs were incubated for 30 min at 37°C with 500 nM of MitoTracker Red® solution and then fixed with 4% PFA (Vaz et al. 2015). Cell nuclei were stained with Hoechst 33258 dye. Images were acquired as described above for immunocytochemistry.

2.5. N9 microglia phagocytosis assay

To evaluate the phagocytic ability of N9 microglia, cells were incubated with 0.0025% (v/v) fluorescent latex beads with 1 µm of diameter, for 75 min at 37◦C. For immunofluorescence detection, N9 cells were fixed for 20 min with 4% (w/v) paraformaldehyde in PBS and immunocytochemical technique and images acquirement were performed as previously described for **Section II.2.4.**

2.6. Image Analysis

Total fluorescence intensity of Mitotracker, DRP1, Mitofusin 1, GFAP and GLT-1 (**Table II.3**) was quantified by ImageJ software and normalized to the total number of the nucleus. Neurite extension was assessed by the immunofluorescence detection of the cytoskeletal protein βIII-tubulin (**Table II.3**), according to the immunocytochemistry assay described in **Section II.2.4** Using the ImageJ plugin NeuronJ, we manually traced neurites, either primary (emanating directly from the soma), secondary (branching from a primary) or tertiary (branching from a secondary) (Popko et al. 2009). We analyzed neurites number and extension (Silva et al. 2012). The number of microglial cells with ingested beads and total cells were counted using Cell Profiler software (version 2.1.1, NIH) to determine the percentage of phagocytosing cells. Three distinct ranges of values were considered to distinguish populations: cells which phagocyte 0 beads, between 1 and 5 beads and cells that phagocyte 6 or more beads. Analysis of iNOS and arginase1 fluorescence intensity and number of nuclei were automatically quantified using Cell Profiler software (version 2.1.1, NIH). Cells with fluorescence intensity >200 for iNOS and >100 for arginase 1 were considered high to iNOS or to arginase1, respectively.

2.7. Statistical analysis

Results of at least three different experiments were expressed as mean values ± SEM. Results of NSC-34 MNs modulated with pre-miR-124 or anti-miR-124 were represented as fold *vs.* WT MNs. Results of both N9 microglia and astrocytes incubated with neuronal conditioned medium (NCM) were represented as fold *vs.* respective non-incubated cells. Differences between groups were determined by one-way ANOVA followed by multiple comparisons Bonferroni post hoc correction or by two-tailed unpaired Student's *t*-test with Welch's correction, depending on whether variances were equal or different. Statistical analysis was performed using GraphPad PRISM 7.0 (GraphPad Software, San Diego, CA, USA). $p<0.05$ was considered as statistically significant.

III. Results

1. Evaluation of ALS MN function/dysfunction and dependence of miR-124 expression

Mir-124 is one of the most abundant miRNA in the CNS and is intimately related to neuronal differentiation by cytoskeleton regulation and neurite outgrowth (Yu et al. 2008). However, high levels of miR-124 may contribute to pathological conditions within the CNS, turning this miRNA a promising biomarker in the diagnostic of some CNS disorders (Wang et al. 2018). Previously, our group demonstrated that NSC-34 mSOD1 MNs has upregulated levels of miR-124 (Pinto et al. 2017). Thus, here we aimed to understand the role of miR-124 levels in MNs function, as well as in the model that mimic MN degeneration caused by mSOD1. In order to explore how miiR-124 upregulation affects neuronal mechanisms and how its inhibition has a neuroprotective effect, we transfected WT MNs with pre-miR-124 and mSOD1 MNs with anti-miR-124 for 12h. Cells were analyzed 48 h after transfection.

1.1. MiR-124 modulation does not affect MN viability and leads to similar expression in both WT treated with pre-miR-124 and mSOD1 MNs treated with anti-miR-124

We verified the efficiency of transfection by RT-PCR, having confirmed that pre-miR increased miR-124 levels 4.9-fold in WT modulated cells ($p<0.01$ vs. non-treated WT MNs) and anti-miR decreased miR-124 levels 4.2-fold in mSOD1 modulated cells ($p<0.01$ vs. non-treated mSOD1 MNs) (**Figure III.1.A**). To ensure that MN modulation with pre- and anti-miR-124 does not affect cell viability, we collected cells 48 h after transfection and assessed cell death by flow cytometry using Guava Nexin® Reagent. As showed in **Figure III.1.B**, no differences were observed between the cell viability of modulated and non-modulated cells

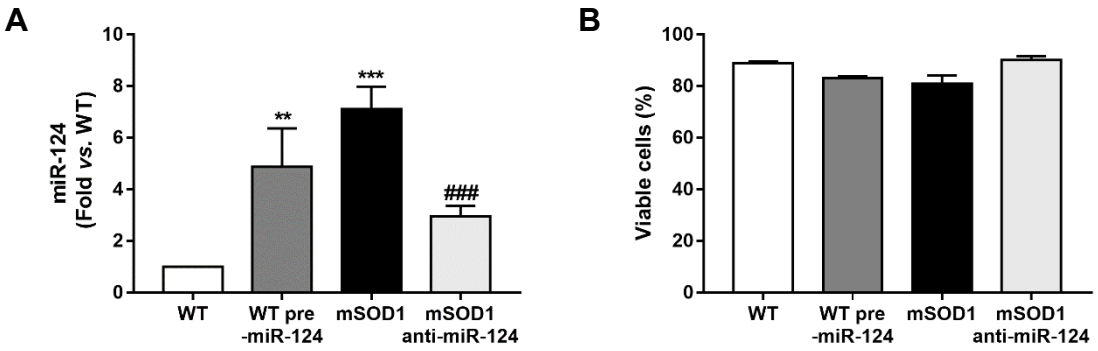

Figure III.1. Anti- and pre-miR-124 are efficiently transfected in MNs without affecting cell viability. After 4 days of differentiation, NSC-34 WT MNs and NSC-34 MNs expressing mSOD1 with G93A mutation (mSOD1) were transfected with pre-miR-124 or anti-miR-124, respectively, for 12 h and analyzed after 48 h. (A) Percentage of viable cells were assessed using Guava Nexin® Reagent and flow cytometry analysis. (B) Levels of microRNA (miRNA)-124 were evaluated by quantitative Real-Time PCR (qRT-PCR). Results are showed in fold *vs*. WT non-transfected cells. SNORD was used as reference gene. Results are mean values ± SEM from at least 3 independent experiments for cell viability and at least 6 independent experiments for miRNA expression. ***p<0.001 and **p<0.01 *vs*. WT MNs; ###p<0.001 *vs*. mSOD1 MNs, one-way ANOVA followed by multiple comparisons Bonferroni post hoc correction.

1.2. Although miR-124 independent, SOD1 accumulation is ameliorated by anti-miR-124 in mSOD1 MNs

We have previously demonstrated that one of the features of this model is the accumulation of SOD1 in mSOD1 MNs (Vaz et al. 2015). Thus, we evaluated how miR-124 modulation affected SOD1 accumulation by Western Blot. As indicated in **Figure III.2**, levels of SOD1 expression did not change in WT pre-miR-124 MNs. However, we observed a reduction of SOD1 accumulation in modulated mSOD1 MNs ($p<0.001$), suggesting that the accumulation of SOD1, despite being miR-124 independent, this miRNA is probably affecting a pathway involved in SOD1 accumulation.

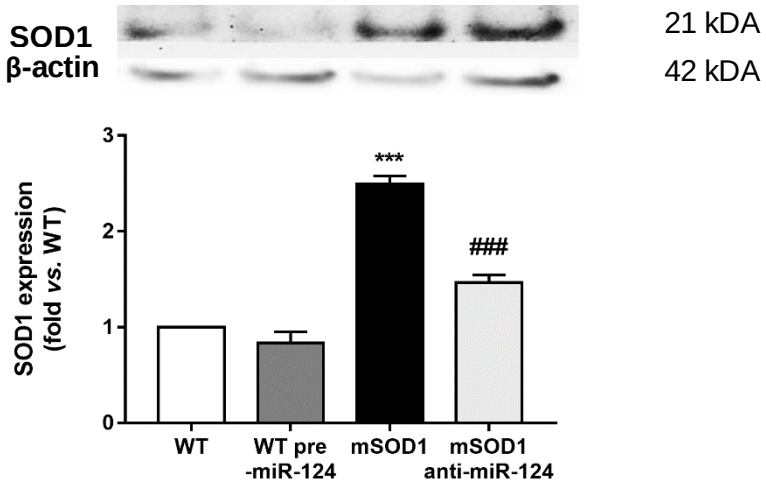

Figure III.2. MiR-124 downregulation in mSOD1 MNs reduces SOD1 protein expression, while pre-miR-124 does not affect SOD1 expression in WT MNs. After 4 days of differentiation, NSC-34 WT MNs and NSC-34 expressing mSOD1 with G93A mutation MNs (mSOD1) were transfected with pre-miR-124 or anti-miR-124, respectively, for 12 h and analyzed after 48 h. Superoxide dismutase 1 (SOD1) expression was assessed by Western blot. Representative results from one blot are shown. β-actin was used as a loading control. Results are mean values ± SEM from at least 4 independent experiments. ***p<0.001 *vs*. WT MNs; ###p<0.001 *vs*. mSOD1 MNs, one-way ANOVA followed by multiple comparisons Bonferroni post hoc correction.

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1.3. Downregulation of miR-124 counteracts the increased levels of alarmins in mSOD1 MNs

Alarmins HMGB1 and S100B are molecules with nuclear functions that are released to the cytoplasm and to the extracellular space in response to cellular damage, functioning as inflammatory mediators (Kato and Svensson 2015). The release of HMGB1 may also derive from the influence of proinflammatory mediators, such as nitric oxide (NO), produced by inducible nitric oxide synthase (iNOS), which besides being mainly associated with oxidative stress, is also considered as a mediator of the inflammatory response (Pisetsky et al. 2008). To assess if miR-124 is related with the expression of alarmins in mSOD1 MNs we evaluated the expression of S100B, HMGB1, and iNOS.

Firstly, we observed that NSC-34 mSOD1 MNs have increased expression of HMGB1 mRNA and protein (**Figure III.3.A-B**, p<0.001), and high iNOS protein expression (**Figure III.3.C**, p<0.001). However, the same was not observed in WT pre-miR-124 MNs, suggesting that HMGB1 and Inos increase in mSOD1 MNs are independent of miR-124 expression. In the case of S100B, WT pre-miR-124 MNs revealed an upregulation of mRNA levels (Figure III.3.D, p<0.001), what was not observed in mSOD1 MNs. Curiously, anti-miR-124 in mSOD1 MNs led to a decrease of all the three inflammatory mediators, decreasing HMGB1 protein and gene

(p<0.001) and iNOS protein (p<0.001) towards WT normal levels. This suggests that miR-124 upregulation in mSOD1 MNs is affecting pathways that favor the alarmin expression, even when they are not directly affected by miR-124 overexpression, as in the case of HMGB1 and iNOS.

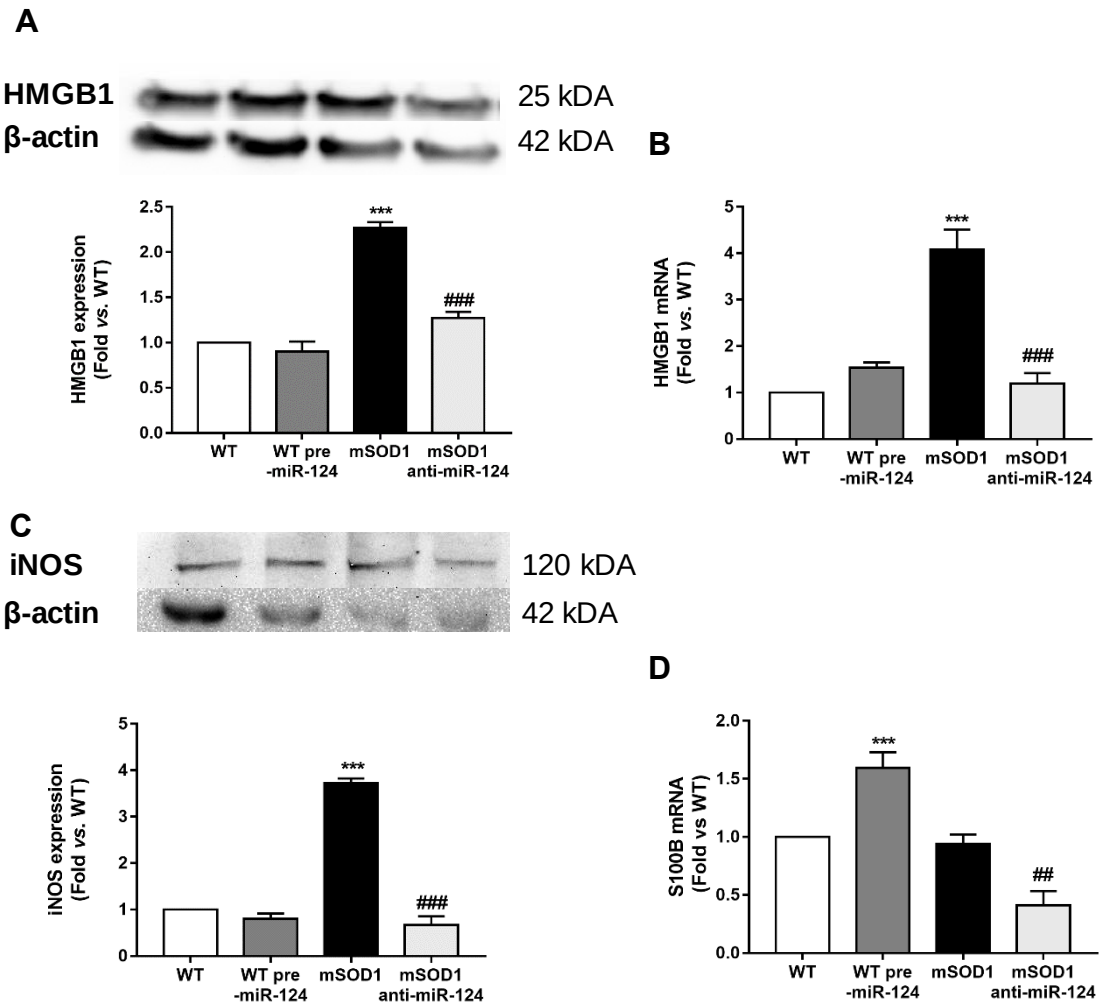


Figure III.3. mSOD1 MNs leads show increased gene and protein levels of HMGB1, as well as of iNOS protein and unchanged S100B mRNA levels, while anti-miR-124 recovers alarmin normal values. After 4 days of differentiation, NSC-34 WT MNs and NSC-34 expressing mSOD1 with G93A mutation MNs (mSOD1) were transfected with pre-miR-124 or anti-miR-124, respectively, for 12 h and analyzed after 48 h. **(A)** High mobility group box 1(HMGB1) and **(C)** inducible nitric oxide synthase (iNOS) expression was assessed by Western blot. Representative results from one blot are shown. β-actin was used as a loading control. Results are shown as mean values ± SEM from at least 4 independent experiments. ***p<0.001 *vs.* WT; ###p<0.001 *vs.* mSOD1 MNs, one-way ANOVA followed by multiple comparisons Bonferroni post hoc correction. **(B)** HMGB1 and **(D)** S100 calcium-binding protein B (S100B) were evaluated by quantitative Real-Time PCR (qRT-PCR). Results are mean values ± SEM from at least 6 independent experiments and expressed as fold change *vs.* WT MNs. ***p<0.001 *vs.* WT; ###p<0.001 and ##p<0.01 *vs.* mSOD1 MNs, one-way ANOVA followed by multiple comparisons Bonferroni post hoc correction.

1.4. Alterations of inflamma-miRNAs in mSOD1 MNs are counteracted by anti-miR-124

As previously described, miRNA dysregulation seems to have a role in ALS pathogenesis, either in familial or in sporadic cases (Rinchetti et al. 2018). As previously referred, other miRNAs rather than miR-124 have been found altered in ALS models. Thus, to evaluate how mSOD1 MNs express differently other miRNAs and if their inflamma-miRNAs profile is dependent of miR-124 expression we assessed, by RT-PCR, the expression of three inflamma-miRNAs: miR-125b, miR-21, and miR-146a.

As indicated in **Figure III.4,** mSOD1 MNs presented a specific miRNAs signature, characterized by increased levels of miR-125b ($p<0.01$) and decreased levels of miR-21 ($p<0.01$) and miR-146a ($p<0.001$), which was reverted towards control levels by anti-miR-124. Curiously, the same pattern was observed in pre-miR-124 WT cells (miR-125b ($p<0.01$), miR-21 ($p<0.001$), miR-146a ($p<0.01$)), suggesting that the observed miRNA profile is associated with miR-124 levels.

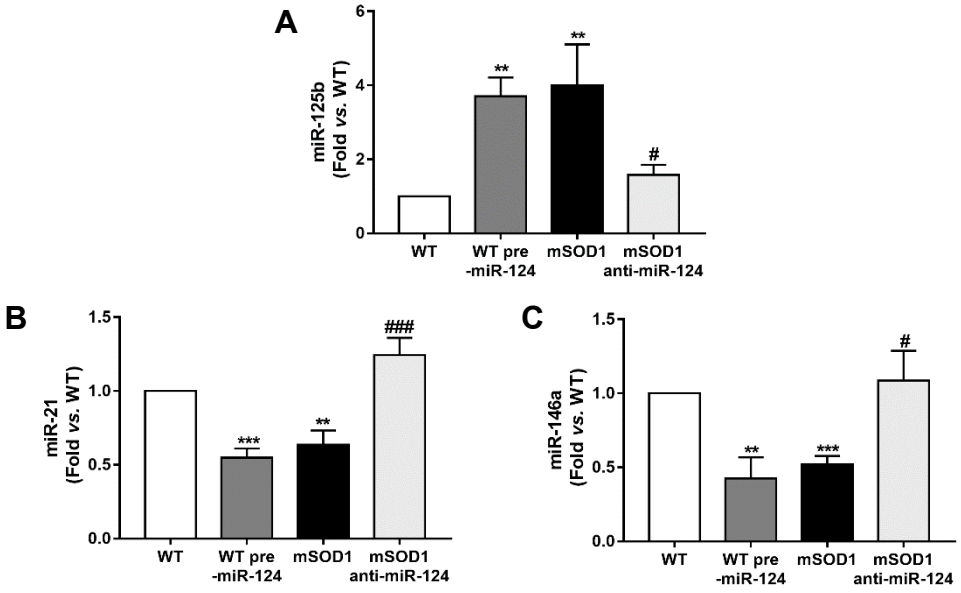


Figure III.4. mSOD1 cells and miR-124 upregulated WT cells show elevated miR-125b, but decreased miR-146a and miR-21, while anti-miR-124 counteracts such effects in mSOD1 MNs. After 4 days of differentiation, NSC-34 WT MNs and NSC-34 expressing mSOD1 with G93A mutation MNs (mSOD1) were transfected with pre-miR-124 or anti-miR-124, respectively, for 12 h and analyzed after 48 h. Levels of **(A)** miRNA-125b **(B)** miRNA-21, **(C)** miRNA-146a were evaluated by quantitative Real-Time PCR (qRT-PCR). SNORD110 was used as reference gene. Results are mean values ± SEM from at least 4 independent experiments and expressed as fold change *vs.* WT MNs, ***$p<0.001$ and **$p<0.01$ *vs*. WT MNs; $^{\#}p<0.05$ and $^{\#\#\#}p<0.001$ *vs*. mSOD1 MNs, one-way ANOVA followed by multiple comparisons Bonferroni post hoc correction.

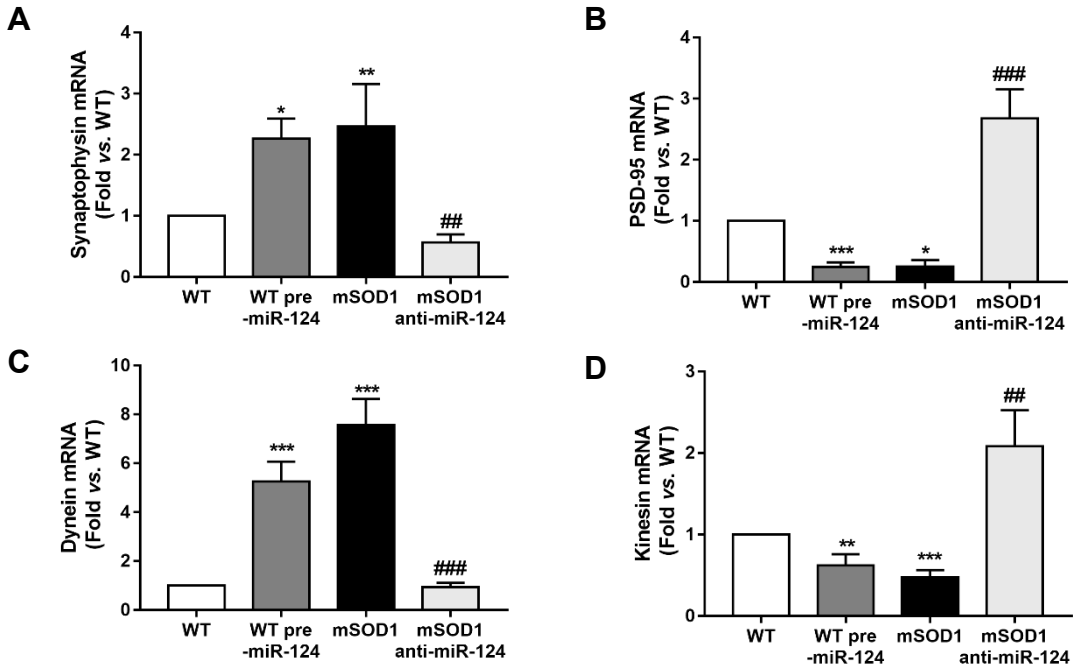
1.5. Axonal function, trans-synaptic signaling, and neuronal arborization are dependent from miR-124 expression levels in MNs

To understand if some of the mechanisms described as impaired in ALS MNs, namely synaptic function, axonal transport and neuronal arborization, were related with miR-124 upregulation, we analyzed the expression of genes involved in the axonal transport and synaptic vesicles markers, as well as neurite number and extension.

Our results demonstrated that mSOD1 MNs has increased mRNA expression of presynaptic synaptophysin (**Fig. III.5.A**, $p<0.01$) and decreased postsynaptic PSD-95 (**Fig. III.5.B**, $p<0.05$), confirming the impaired trans-synaptic signaling in ALS neurons (Zang et al. 2005). Axonal transport was also found unbalanced in mSOD1 MNs, with increased mRNA levels of retrograde transport marker dynein (**Fig. III.5.C**, $p<0.001$) and decreased anterograde kinesin (Hardiman et al. 2017) (Fig. III.5.D, $p<0.001$).

In addition, we observed that mSOD1 MNs have fewer ramifications per cell when compared with WT cells and surprisingly with longer extension (**Figure III.5.E-C**). The same was observed in WT pre-miR-124 MNs and these effects were reversed by anti-miR-124.

Overall, our results demonstrate that in MNs, with high levels of miR-124, there is an attempted synapse promotion, by upregulation of synaptophysin, that is probably not being recognized by the synaptic terminal. In the same way, axonal transport is being favored in a retrograde way, not promoting transport to the synaptic site. Moreover, having decrease neurite number with longer length also difficult synapse, since it requires more energy.



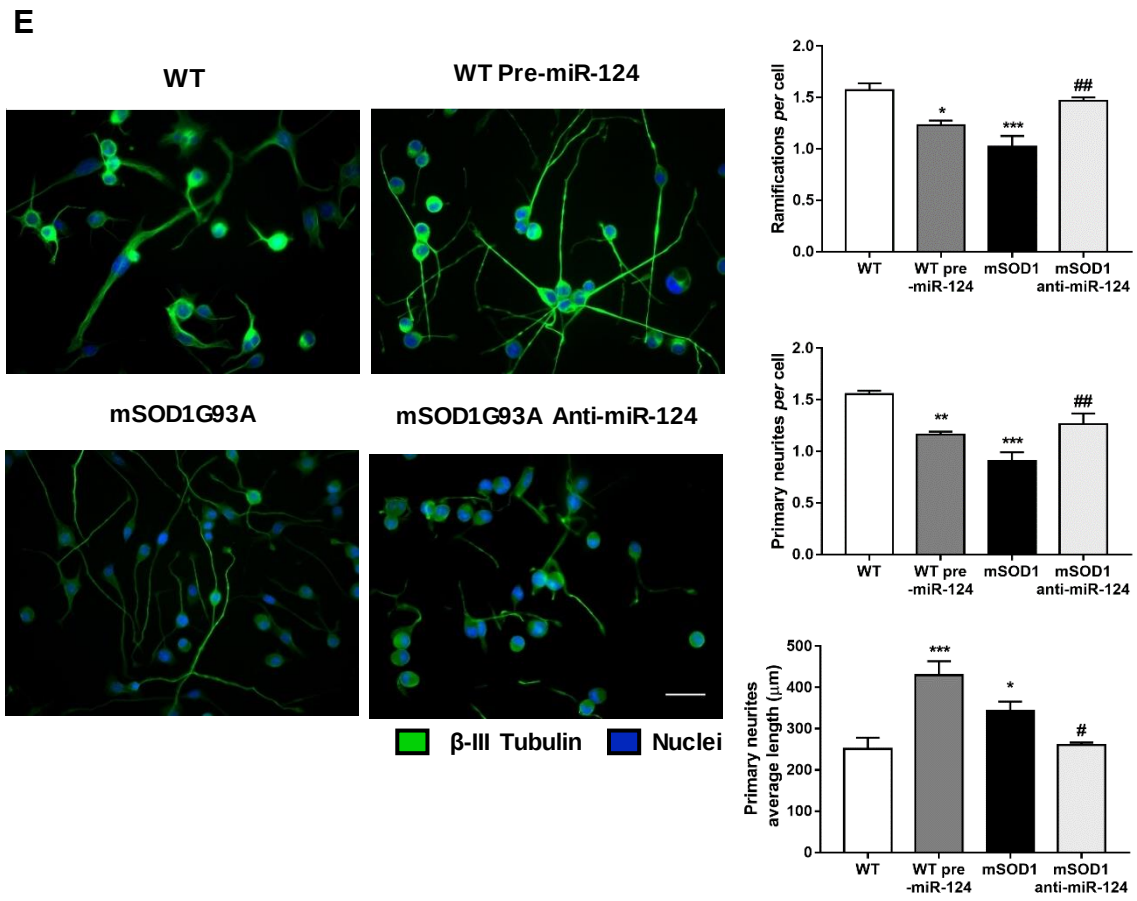


Figure III.5. Anti-miR-124 in mSOD1 MNs leads to the recovery of the synaptic function, axonal transport and neuronal arborization. After 4 days of differentiation, NSC-34 WT MNs and NSC-34 expressing mSOD1 with G93A mutation MNs (mSOD1) were transfected with pre-miR-124 or anti-miR-124, respectively, for 12 h and analyzed after 48 h. mRNA levels of **(A)** Synaptophysin, **(B)** postsynaptic density protein 95 (PSD-95), **(C)** dynein and **(D)** kinesin were evaluated by quantitative Real-Time PCR (qRT-PCR). Results are shown as mean values ± SEM from at least 6 independent experiments and expressed as fold change *vs*. WT MNs. ***p<0.001, **p<0.01 and *p<0.05 *vs*. WT; $^{\#\#\#}$p<0.001 and $^{\#}$p<0.05 *vs*. mSOD1 MNs, one-way ANOVA followed by multiple comparisons Bonferroni post hoc correction. **(E)** MNs were stained with an antibody to β-III tubulin and neurite number and length were obtained using NeuroJ (plug-in from ImageJ software). Representative images of each condition are shown. Results are mean values ± SEM from at least 4 independent experiments ***p<0.001, **p<0.01 and *p<0.05 *vs*. WT; $^{\#\#}$p<0.01 and $^{\#}$p<0.05 *vs*. mSOD1 MNs two-tailed unpaired Student's *t* test with Welch's correction when required. Scale bar: 40 μm.

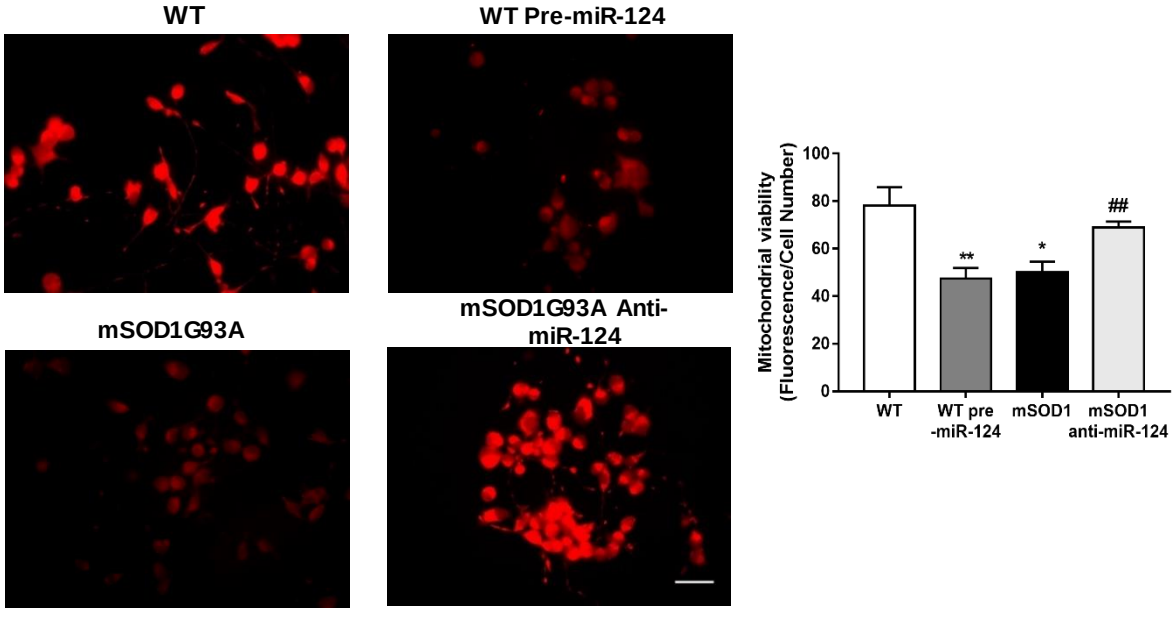
1.6. Anti-miR-124 re-establishes normal mitochondrial function and dynamics by promoting the balance of fusion and fission processes in mSOD1 MNs

Mitochondrial dysfunction in MNs is an event very well described in ALS (Vaz et al. 2015), which may be related with an impairment in axonal transport (Hirokawa et al. 2010), As indicated in Figure III.6.A NSC-34 mSOD1 MNs have decreased mitochondrial viability

(p<0.05). Once again, the same was observed in WT pre-miR-124 MNs (p<0.01) and the decreased mitochondrial function was reverted by anti-miR-124 in mSOD1 cells (p<0.01), suggesting that mitochondrial dysfunction is also dependent on miR-124 levels.

The impairment of mitochondrial dynamics, including fusion and fission mechanisms, is associated with the aberrant mitochondrial function. Such mechanisms have the function of keeping mitochondrial size, shape, and morphology depending on the cell energy requirement (Smirnova et al. 2001). In several ALS models, it has been previously reported a decrease of fusion proteins together with an increase of fission proteins, resulting in a more fragmented mitochondrial network (Liu et al. 2013). To check if miR-124 and mitochondria viability loss were associated with an unbalanced fusion and fission, we evaluated the expression of Mitofusin 1 (fusion protein) and DRP1 (fission protein). In **Figure III.6.B** is demonstrated that mSOD1 MNs had decreased levels of Mitofusin 1 (p<0.01) and increased ones of DRP1 (p<0.05), suggesting that the fission mechanism is favored. Despite no differences were observed in Mitofusin 1 in WT pre-miR-124 MNs, DRP1 expression was also decreased in such condition. In addition, the impairment of these two proteins was counteracted by anti-miR-124 in mSOD1 MNs, suggesting that the decrease of mitochondrial viability in these cells is favoring fission mechanisms, which may be an attempt of the cell to keep mitochondrial network.

A



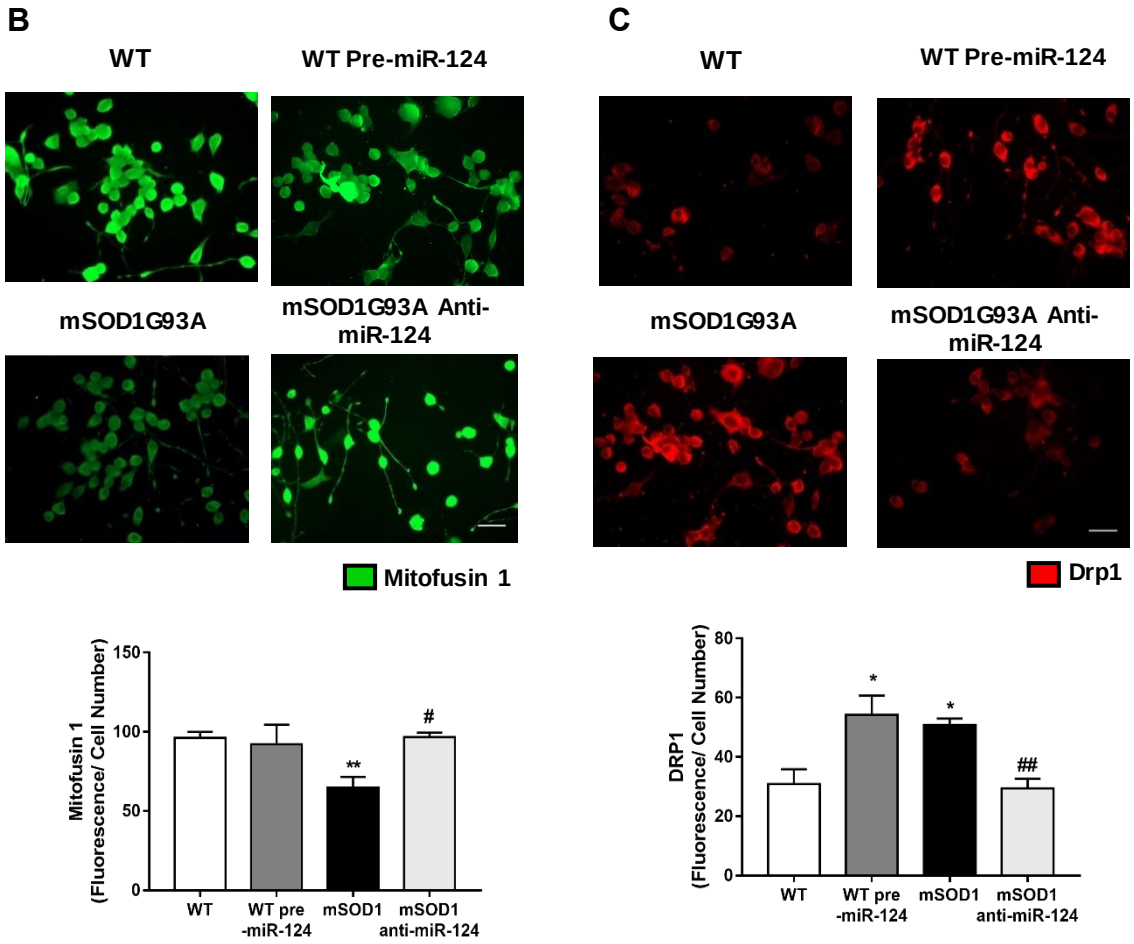


Figure III.6. MiR-124 overexpression is related with the loss of mitochondrial viability observed in mSOD1 MNs and also with mitochondrial fusion/fission impairment. After 4 days of differentiation, NSC-34 WT MNs and NSC-34 expressing mSOD1 with G93A mutation MNs (mSOD1) were transfected with pre-miR-124 or anti-miR-124, respectively, for 12 h and analyzed after 48 h. (A) Mitochondrial viability was measured using MitoTracker™ Red CMXRos staining by immunocytochemistry. Protein levels of **(B)** Mitofusin 1 and **(C)** dynamin-related protein 1 (DRP1) were evaluated by immunocytochemistry using specific antibodies. Representative images of each condition are shown. Results are mean values ± SEM from at least 4 independent experiments. **p<0.01 and *p<0.05 *vs*. WT MNs; ##p<0.05 *vs*. mSOD1 MNs, two-tailed unpaired Student's *t* test with Welch's correction when required. Scale bar: 40 µm.

2. Effects of MN secretome from non-modulated and modulated WT and mSOD1 cells on microglia activation

ALS is nowadays considered a non-cell autonomous disease where glial cells play a crucial role (Peters et al. 2015). It is thought that disease onset is probably on neurons that communicate with glia in an attempted to survive (Yamanaka et al. 2008), leading to an astrocytic and microglial activation which exacerbates even more neuronal injury and neurodegeneration. The involvement of microglia in ALS has been widely characterized and activated microglia usually increase their number during disease progression, representing a continuum between neuroprotective (M2) and neurotoxic (M1) phenotypes (Geloso et al. 2017). Our previous results showed that NSC-34 mSOD1 MNs release exosomes with increased content of miR-124 that, when incubated in N9 microglia cells, promote a decrease in phagocytic ability and alterations in microglial phenotype (Pinto et al. 2017). Thus, we incubated N9 cells with the secretome from WT or mSOD1, non-modulated or modulated with either pre-miR-124 (WT cells) or anti-miR-124 (mSOD1 cells), for 4 h in order to evaluate alterations in microglia and their reversal by miR-124 modulation.

2.1. Secretome from WT and mSOD1 MNs non-modulated and modulated for miR-124 does not affect N9 microglia viability

To make sure that incubation of MNs secretome does not significantly affect microglia viability we assessed cell death by flow cytometry analysis using Guava Nexin® Reagent. As shown in **Table III.1** no differences were observed for microglia incubated with the MN culture medium and with the secretome from miR-124 modulated or non-modulated MNs.

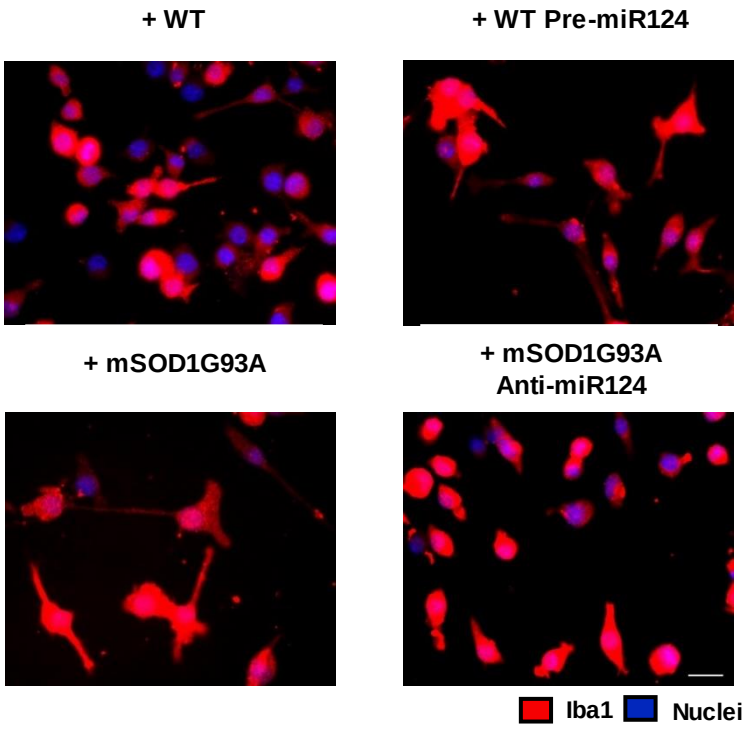
Table III.1. Number of viable and apoptotic microglia after for 4 h incubation with the motor neuron (MN) culture medium and secretome from miR-124 modulated and non-modulated MNs.

	Viable Cells (%)	Early Apoptosis (%)	Late Apoptosis (%)
N9 + MN ctl medium	90.8 ± 2.1	8.5 ± 1.6	0.3 ± 0.1
N9 + WT NCM	93.3 ± 0.6	6.0 ± 1.0	0.2 ± 0.1
N9 + WT pre-miR-124 NCM	96.3 ± 1.7	4.4 ± 0.3	0.3 ± 0.2
N9 + mSOD1 NCM	94.3 ± 3.7	7.7 ± 1.3	0.4 ± 0.3
N9 + mSOD1 anti-miR-124 NCM	93.2 ± 5.0	9.2 ± 3.1	0.5 ± 0.3

MN, motor neuron; mSOD1, mutated superoxide dismutase; WT, wildtype, NCM, neuronal conditioned medium;

2.2. Upregulation of miR-124 leads to microglia morphological alterations suggestive of cell activation, which are prevented by anti-miR-124 modulation in mSOD1 MNs

Here, we stained cells with microglia specific marker Iba1 and estimate cell area, perimeter, Ferret's diameter, and circularity, since morphological changes are useful to illustrate microglial activation (Cunha et al. 2016). As represented in **Figure.III.7**, our results showed that area ($p<0.01$), perimeter ($p<0.05$) and Ferret's diameter ($p<0.01$) of N9 microglia incubated with neuronal conditioned medium (NCM) from mSOD1 MNs was increased, suggesting increased reactivity. Cells also showed a less round shape with more and longer ramifications ($p<0.05$). The same pattern was observed in microglial exposed to NCM from WT pre-miR-124. Interestingly, microglia morphological alterations were reverted to control levels when these cells were incubated cells with NCM of mSOD1 anti-miR-124 MNs.



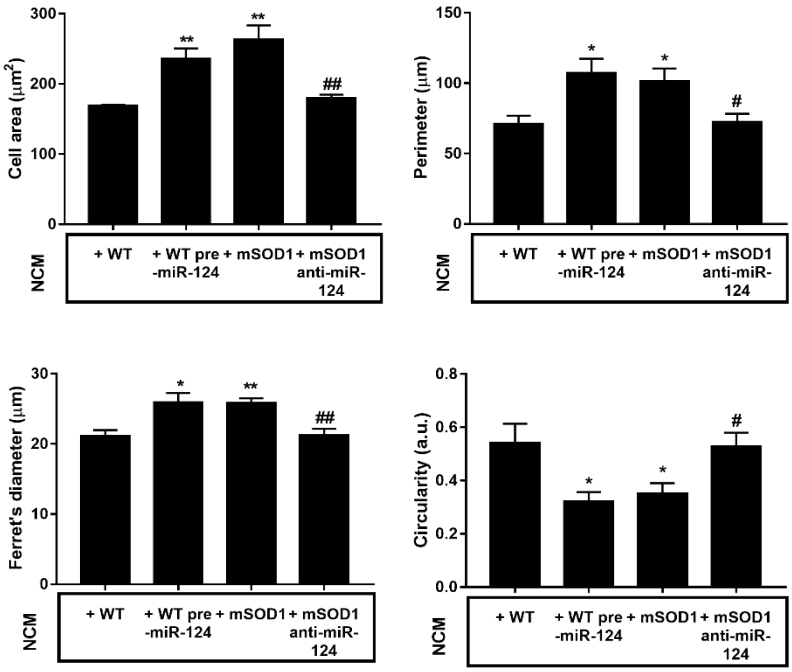


Figure III.7. Secretome from mSOD1 MNs and WT pre-miR-124 MNs activates N9 microglia that undergo morphological changes, which are prevented when secretome derives from mSOD1 MNs treated with anti-miR-124. N9 microglia cells are differentiated for 24 h and incubated with the secretome from modulated (WT with pre-miR-124 and mSOD1 with anti-miR-124) and non-modulated MNs for 4 h. For morphological analysis, cells were stained with microglia specific Iba1 antibody and the evaluation of cell area, perimeter, and Ferret's diameter performed by using the computer program ImageJ. Representative images of each condition are shown. Results are mean values ± SEM from at least 5 independent experiments **p<0.01 and *p<0.05 *vs*. N9 + WT neuronal conditioned medium (NCM); $^{\#\#}$p<0.01 and $^{\#}$p<0.05 *vs*. N9 + mSOD1 NCM, two-tailed unpaired Student's *t* test with Welch's correction when required. Scale bar: 20 μm.

2.3. Microglia lose their phagocytic ability upon incubation with secretome from mSOD1 MNs, as well as from pre-miR-124 treated WT or anti-miR-124 treated mSOD1 MNs

Microglial phagocytic ability is one of the most relevant neuroprotective functions and our previously published results revealed an accentuated decrease of this capacity after incubation with mSOD1 MNs exosomes (Pinto et al. 2017). The percentage of phagocytic and non-phagocytic cells after 4 h incubation with neuronal secretome is represented in **Figure III.8**. It is showed that the phagocytic loss observed before with mSOD1 MNs exosomes, was again verified with the incubation of MNs whole secretome. The same effect was observed with WT pre-miR-124 NCM. However, the phagocytic ability was not recovered by NCM from mSOD1 MNs with inhibition of miR-124. This means that although enough to lead to a phagocytic loss by microglia, downregulation of miR-124 in MNs is not enough to completely alter mSOD1 MN secretome in terms of molecules that can be recognized by microglia in order to promote their phagocytic ability. Another hypothesis is that secretome from mSOD1 anti-miR-124 MNs by improving MN homeostasis does not induce phagocytosis since these levels are similar to the levels found in microglia non-incubated with NCM (80% non-phagocytic cells, 20% phagocytic cells, data not shown).

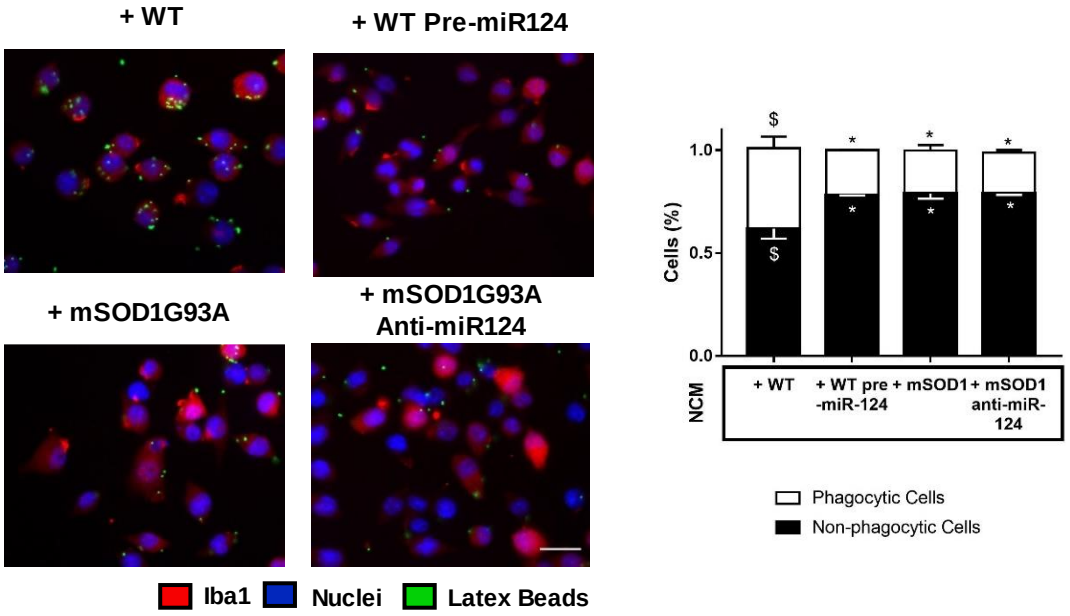


Figure III.8.Secretome from mSOD1 MNs and WT pre-miR-124 MNs determines a sustained and marked decrease in the N9 microglia phagocytic ability, which is not prevented by anti-miR-124. N9 microglia cells are differentiated for 24 h and incubated with the secretome from modulated (WT with pre-miR-124 and mSOD1 with anti-miR-124) and non-modulated MNs for 4 h. Representative results of one experiment, showing engulfed latex beads (in green) by the Iba1 stained microglia. The results are expressed as percentage of phagocytic and non-phagocytic cells relatively to the total number of cells. Results are mean ± SEM from 3 independent

experiments. $p<0.001 *vs.* non-incubated N9, *p<0.05 *vs.* + WT neuronal conditioned medium (NCM), two-tailed unpaired Student's *t* test with Welch's correction when required. Scale bar represents 20 µm.

2.4. Secretome from MNs with upregulated miR-124 induces M1/M2 microglia polarization that disappears if mSOD1 MNs are modulated with anti-miR-124

In order to understand the effect of NCM in N9-microglia phenotype, we assessed both protein expression and mRNA levels of iNOS, usually associated with M1 microglial phenotype, and Arg1, usually associated with M2 subtype.

RT-PCR data depicted in **Figure III.9.A, B** show that NCM from mSOD1 MNs and WT pre-miR-124 MNs lead to upregulation of both M1 (iNOS, $p<0.01$ and $p<0.01$, respectively) and M2 (Arg1, $p<0.001$ and $p<0.05$, respectively) markers, which suggest a shift to a mixed M1/M2 population.

As indicated in **Figure III.9.C**, when we evaluated the expression of these two markers by immunocytochemistry, microglia incubated with NCM from mSOD1 and WT pre-miR-124 MNs simultaneously express increased levels of iNOS and Arg1 ($iNOS^{hi}$/ $Arg1^{hi}$, orange), thus contradicting the existence of a mixed population in favor of cells with concomitant elevated expression of both markers. We also observed a decrease of cells in steady state condition (iNOSlo/Arg1lo, black), namely after treatment with secretome from mSOD1 and WT cells overexpressing miR-124 reinforcing that the elevated expression of miR-124 determines the activation of microglia. Upon incubation with secretome from mSOD1 MNs, expression levels of both markers decrease towards control levels, suggesting again that inhibition of miR-124 in MNs prevent microglia activation.

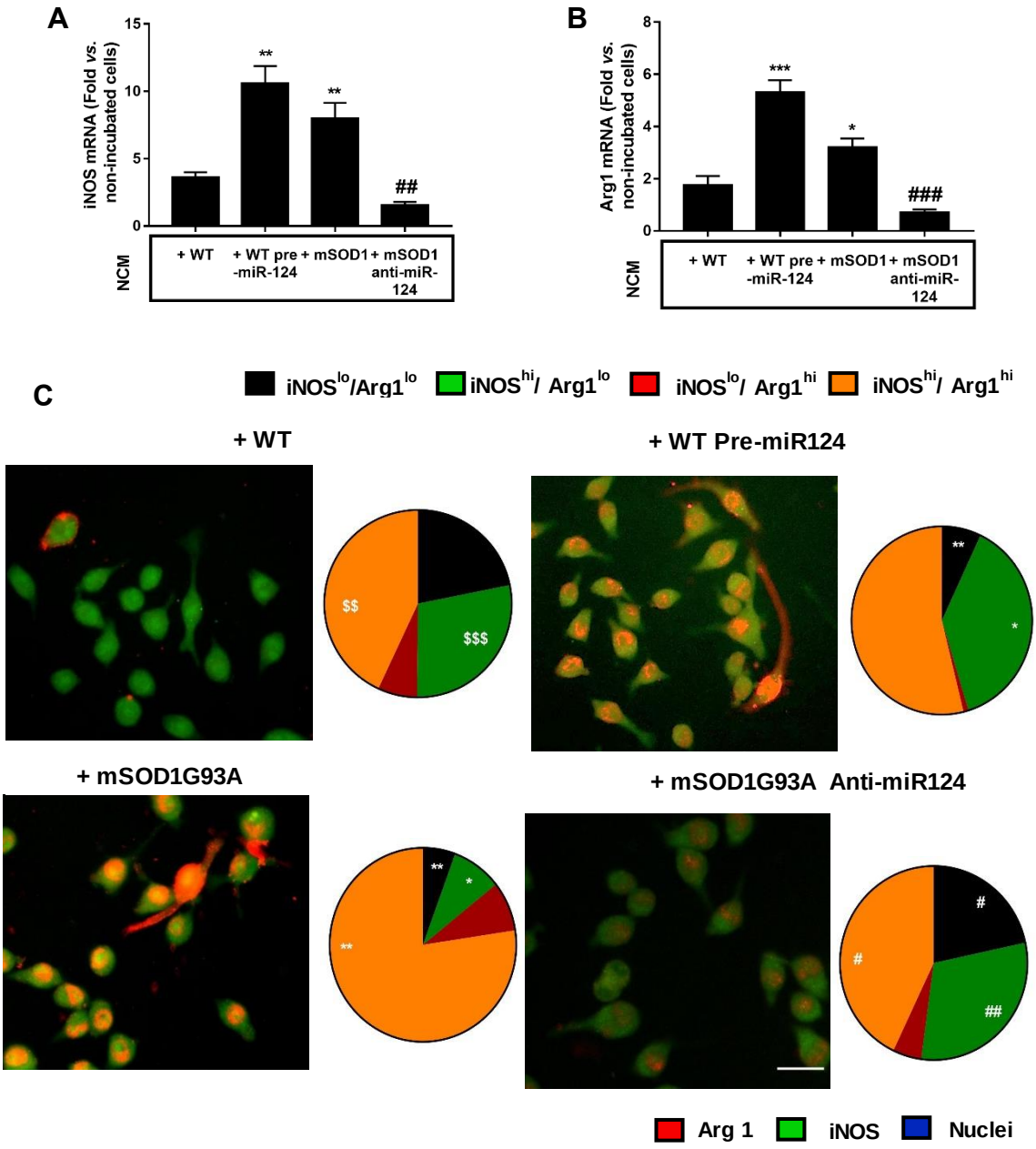


Figure III.9. Secretome from mSOD1 MNs and WT pre-miR-124 MNs induces the increase of M1 (iNOS) and M2 (Arginase1) markers, which are unnoticed when in the presence of secretome form anti-miR124 mSOD1 MNs. N9 microglia cells are differentiated for 24 h and incubated with the secretome from modulated (WT with pre-miR-124 and mSOD1 with anti-miR-124) and non-modulated MNs for 4 h. mRNA levels of **(A)** inducible nitric oxide synthase (iNOS) and **(B)** Arginase 1 (Arg1) were evaluated by quantitative Real-Time PCR (qRT-PCR). Results are expressed as fold change *vs.* non-incubated N9. **(C)** Protein levels of iNOS and Arg1 were evaluated by immunocytochemistry using specific antibodies. Percentage of iNOS and Arg1 high and low cells were obtained with cell profiler software. A representative image and the percentage of high and low Arg1 and iNOS cells from each condition are shown. Results are shown as mean values ± SEM from at least 5 independent experiments. $^{\$\$\$}p<0.001 and $^{\$\$}p<0.01 *vs.* non-incubated N9. ***p<0.001, **p<0.01 and *p<0.05 *vs.* WT neuronal conditioned medium (NCM); $^{\#\#\#}p<0.001 and $^{\#\#}p<0.01 *vs.* + mSOD1 NCM, two-tailed unpaired Student's *t* test with Welch's correction when required. Scale bar: 20 µm.

2.5. Microglia treated with mSOD1 MN-derived secretome show increased M1 and decreased M2 markers, which are counteracted by neuronal anti-miR-124 modulation

To better assess the inflammatory state of microglia after incubation with NCM from non-treated mSOD1 MNs. we also used secretome from WT MNs with or without pre-miR-124, as well as mSOD1 MNs with anti-miR-124. Expression of pro- and anti-inflammatory markers were assed. Our results revealed that microglia incubated with mSOD1 MNs and WT pre-miR-124 NCM have increased mRNA levels of pro-inflammatory cytokines TNF-α and IL-1β and decreased levels of the anti-inflammatory CD206, a mannose receptor known as a typical M2 marker. The increase of gene expression of the three markers was rebalanced toward normal values when secretome was derived from mSOD1 anti-miR-124 MNs. Interestingly, the levels of HMGB1 were very similar to the previous analyzed HMGB1 mRNA levels in modulated MNs (**Figure III.10** and **Figure III.3**, respectively). This suggests that HMGB1 is probably being released by mSOD1 MNs to the extracellular space, and as an alarmin is subsequently inducing the production of HMGB1 by the surrounding cells, as is the case of microglia.

Overall, our results show that NCM with upregulated levels of miR-124 triggers a pro-inflammatory phenotype in microglia accompanied by a loss in their phagocytic ability, more associated with the M2 state. Additionality led to an increase of microglia area, associated also with a reactive state. This reactive pro-inflammatory state was prevented with NCM from mSOD1 anti-miR-124 MNs, though it was unable to prevent microglia phagocytic reduced ability.

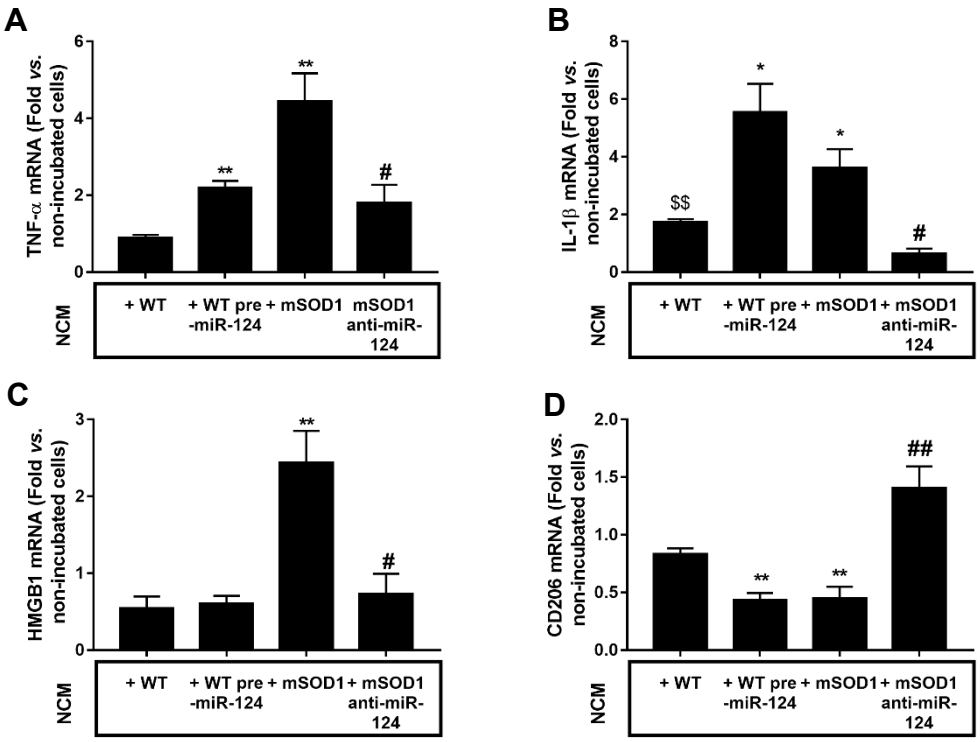


Figure III.10. Addition of secretome from mSOD1 MNs to N9 microglia leads to an increase of pro-inflammatory cytokines IL-1β and TNF-α, as well as the alarmin HMGB1, while decrease the M2 marker CD206, all prevented when secretome results from anti-miR-124 modulated mSOD1 MNs. N9 microglia cells are differentiated for 24 h and incubated with the secretome from modulated (WT with pre-miR-124 and mSOD1 with anti-miR-124) and non-modulated MNs for 4 h. mRNA levels of **(A)** TNF-α, **(B)** IL-1β, **(C)** high-mobility group box 1 (HMGB1) and **(D)** CD206 were evaluated by quantitative Real-Time PCR (qRT-PCR). Results are mean values ± SEM from at least 5 independent experiments and are expressed as fold change *vs*. non-incubated N9. $^{\$\$}$p<0.01 *vs*. non-incubated N9; **p<0.01 and *p<0.05 *vs.* + WT neuronal conditioned medium (NCM); $^{\#\#}$p<0.01 and $^{\#}$p<0.05 *vs.* + mSOD1 NCM, two-tailed unpaired Student's *t* test with Welch's correction when required.

3. Effects of MN secretome from non-modulated and miR-124 modulated WT and mSOD1 cells on WT and Tg spine astrocyte GLT-1 and GFAP reactive markers

Astrocytes have been pointed as major contributors of ALS neurodegeneration and disease progression by the release of toxic factors (Trias et al. 2017). Recently, our group demonstrated that mSOD1 mice cortical astrocytes have an aberrant phenotype with decreased levels of both GFAP and GLT-1. When co-cultured with MNs these astrocytes showed an early and evident harmful effect (Gomes et al. 2018).

However, how injured MNs have a direct effect on astrocytes aberrant phenotype is still poorly investigated. To assess if secretome from mSOD1 MNs contributes to astrocytic aberrancy in ALS and whether miR-124 is involved, we used astrocytes isolated from the SC of 8-days mice pups with or without SOD1 mutation, which were incubated for 24 h with the secretome from non-treated mSOD1 MNs, as well as secretome from WT MNs without or with pre-miR-124, in addition to mSOD1 MNs with anti-miR-124.

3.1. Secretome from mSOD1 MNs and pre-miR-124 treated WT MNs reduces astrocytic GLT-1 and GFAP levels, an effect not observed if mSOD1 MNs are downregulated for miR-124

Excitotoxicity is one of the most widely described mechanism for MN death in ALS (Menon et al. 2017). Glutamate receptor GLT-1 is usually found downregulated in ALS patients and in mSOD1 mouse models (Zarei et al. 2015), which results in increased glutamate concentration and in an over-stimulation of postsynaptic receptors, ultimately leading to MN death. Additionally, downregulation of GLT-1 in astrocytes is associated with their aberrant phenotype (Diaz-Amarilla et al. 2011; Gomes et al. 2018). Moreover, influence of exosomal miR-124 on the astrocytic expression of GLT-1 was previously noticed and its content in exosomes demonstrated (Morel et al. 2013; Pinto et al. 2017).

Therefore, we decided to evaluate whether elevated miR-124 in mutated MNs and overexpressed in WT MNs would be associated with GLT-1 impairment SC astrocytes, as compared with the results for the secretome from WT, and if anti-miR-124 in mutated MNs was able to counteract such effect.

We first confirmed that MN culture medium, used for having the whole secretome, does not interfere with astrocyte cell viability, when assessed by flow cytometry analysis using Guava Nexin® Reagent. No differences were found, though a small increase in viability with decreased levels of early and late apoptosis was found after astrocyte treatment with the MN culture medium, probably due to the presence of soluble growth factors (WT astrocytes:

90.2±3.3% viable cells, 6.5±1.9% cells in early apoptosis and 2.7±1.3% cells in late apoptosis; WT astrocytes + MN culture medium: 93.8±1.8% viable cells, 4.7±1.6% cells in early apoptosis and 0.9±0.3% in late apoptosis).

Expression of GLT-1 was determined by immunocytochemistry and as depicted in **Figure III.11**, secretome from mSOD1 MNs led to a decrease in the expression of GLT-1 both in WT astrocytes ($p<0.05$) and mSOD1 ones ($p<0.01$), which revealed the lowest levels.

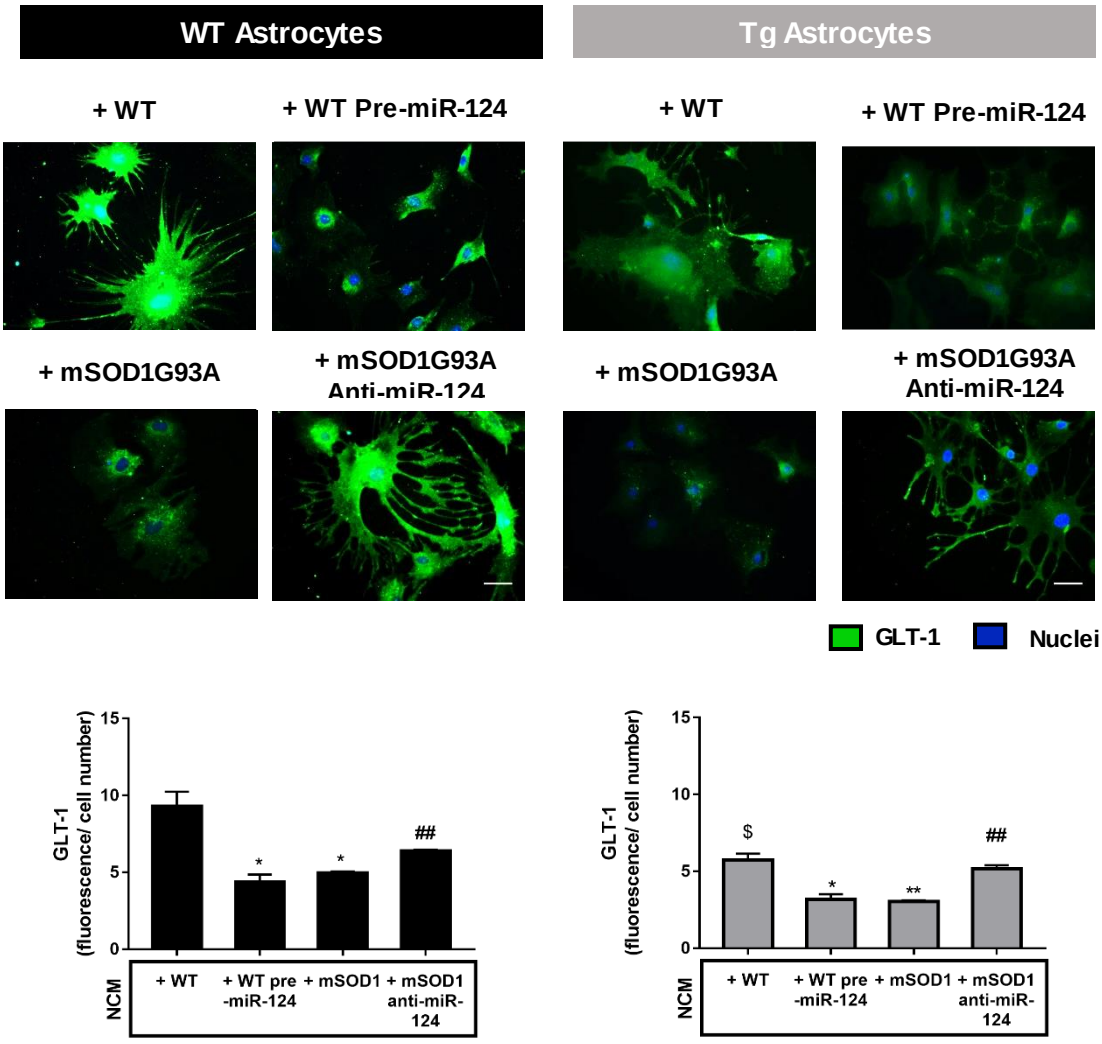


Figure III.11. Secretome from mSOD1 MNs and WT pre-miR-124 MNs determines a reduction of GLT-1 in WT astrocytes and aggravates its low levels in mSOD1 astrocytes, which is prevented when secretome results from anti-miR-124 modulated mSOD1 MNs. Astrocytes were isolated from the spinal cord (SC) of 8-day-old mice, either wild type (WT) or transgenic (Tg) animals expressing human SOD1 with G93A mutation (mSOD1). After 11 days in vitro, cells were incubated with the MN secretome from WT MNs untreated or treated with pre-miR-124, as well as from mSOD1 MNs untreated or treated with anti-miR-124, for 24 h. Protein levels of GLT-1 were evaluated by immunocytochemistry using specific antibodies. Representative images for each condition are shown. All results are mean values ± SEM from at least 3 independent experiments. $^{\$}p<0.05$ *vs.* non-incubated WT astrocytes or non-incubated Tg astrocytes, $^{**}p<0.01$ and $^{*}p<0.05$ *vs.* respective + WT neuronal conditioned medium (NCM),

respectively, ##p<0.01 *vs.* respective + mSOD1 NCM two-tailed unpaired Student's *t* test with Welch's correction when required. Scale bar: 40 µm.

Interestingly, we observed that the secretome from mutated MNs was even able to further reduce the diminished values exhibited by the Tg astrocytes treated with the secretome from WT MNs ($p<0.05$). Similar reduced levels ($p<0.05$) were obtained in both WT and Tg astrocytes after treatment with the secretome derived from WT MNs modulated with pre-miR-124, indicating an inverse correlation between miR-124 expression and that of GLT-1. When mSOD1 MNs were modulated with anti-miR-124, GLT-1 levels despite not achieving the values of WT astrocytes, were found more elevated than when mSOD1 MN secretome was used ($p<0.01$) (left panel in **Figure III.11**). Similar effects were noticed in Tg astrocytes, in which the use of anti-miR-124 in MNs revealed to sustain the GLT-1 levels of mSOD1 astrocytes treated with secretome from WT MNs, and to counteract the additional decrease caused by the secretome from mutated MNs ($p<0.01$) (right panel in **Figure III.11**). It may then be concluded that apart from the low levels of GLT-1 in mSOD1 astrocytes, the secretome from mSOD1 MNs with upregulated miR-124 additionally enhance such impairment, whereas the anti-miR-124 modulation annul such effect.

Since GFAP, a classical astrocytic reactive marker (Bachoo et al. 2004), was found downregulated in our previous studies and n those from others in SC and cortical astrocytes from mSOD1 rodents (Diaz-Amarilla et al. 2011; Gomes et al. 2018), we started by confirming this issue in the Tg astrocytes used in the present work, what was validated. Actually, we confirmed that GFAP expression by immunocytochemistry was reduced in Tg spine astrocytes, when compared to WT ones (data not shown, $p<0.05$).

Secretome from mSOD1 MNs and from WT MNs overexpressing miR-124 led to a significant reduction of GFAP expression in WT astrocytes ($p<0.001$ and $p<0.05$, respectively), with values close to that found in Tg mice astrocytes (**Figure III.12**). The same effect was not observed when secretome from such cells with upregulated miR-124 were added to Tg astrocytes, due to their already reduced levels of GFAP expression. In sum, the harmful effects of the secretome coming from the mSOD1 MNs in decreasing the expression of GFAP is only sensed by WT astrocytes, thus contrasting with GLT-1 that was further decreased in Tg astrocytes by such condition (**Figure III.11**). Nevertheless, the benefits of using anti-miR-124 modulation in mSOD1 MNs in inducing the expression of GFAP close to normal WT astrocyte levels, were noticed on both WT and Tg astrocytes (**Figure III.12**), reinforcing the idea that neuronal miR-124 may play a role in regulating the astrocyte phenotypic polarization and aberrant signatures.

Globally, the modulation of mSOD1 MNs with anti-miR-124 has extended effects in ameliorating MN function (decrease of SOD1 expression, alarmin levels, redistribution of inflamma-miRs, reestablishment of axonal anterograde transport, and recovery of synaptic function and mitochondria dynamics). Moreover, their secretome has added valuable effects in preventing secretome-mediated activated microglia (decrease of pro-inflammatory cytokines) and astrocyte aberrancy (rescue of GFAP and GLT-1 levels). In conclusion, this work highlights targeting of miR-124 in mSOD1 MNs as a potential extended therapeutic strategy in ALS, acting in preventing neurodegeneration and secretome-mediated activation of glial cells, thus impacting directly and indirectly in several cells and multiple targets, what may be a real key in the treatment of ALS disease.

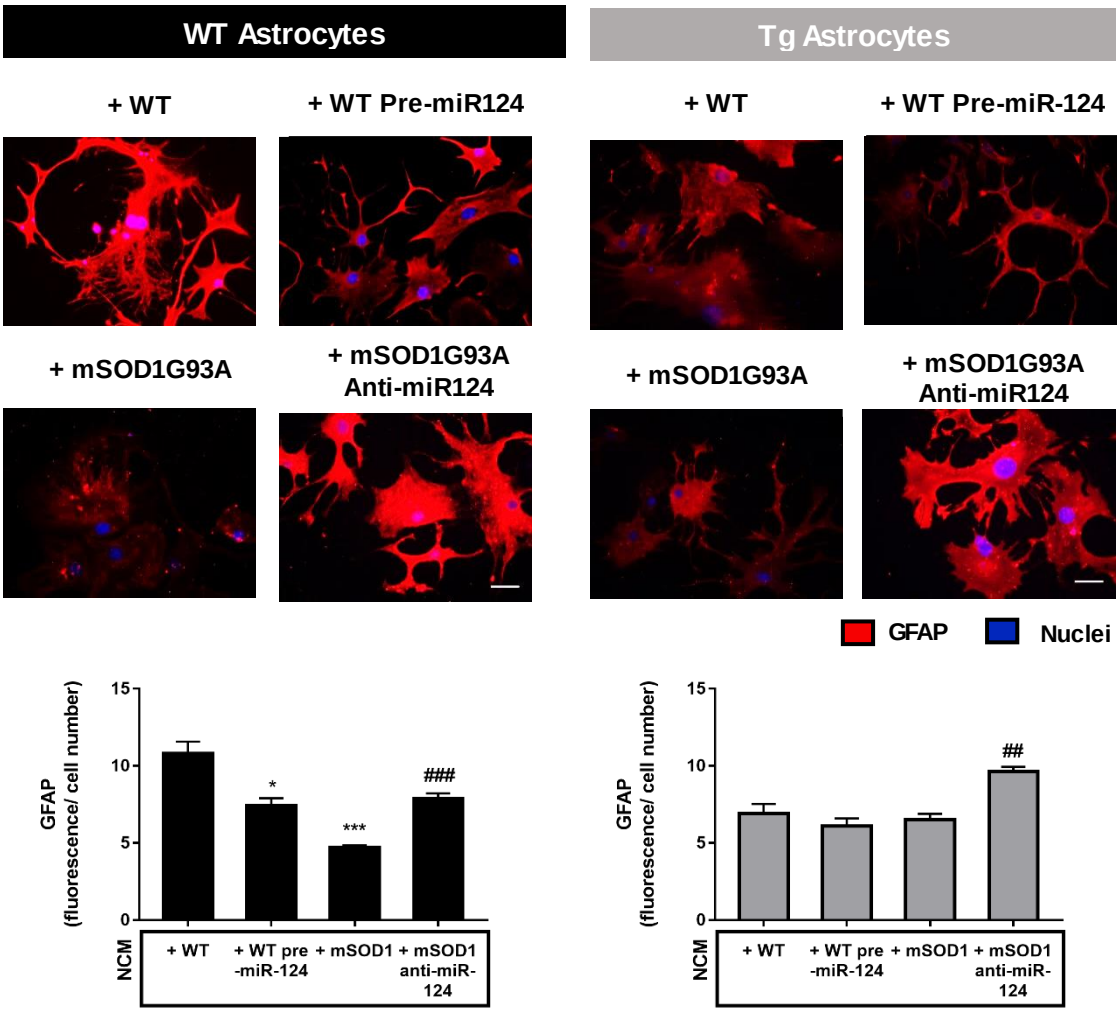


Figure III.12. Secretome from MNs with upregulated miR-124 reduces GFAP expression in WT astrocytes, without further aggravating its low levels in mSOD1 astrocytes, while secretome from anti-miR-124-treated mSOD1 MNs virtually sustains its expression in WT astrocytes or restores normal values in Tg astrocytes. Astrocytes were isolated from the spinal cord (SC) of 8-day-old mice, either wild type (WT) or transgenic (Tg)

animals expressing human SOD1 with G93A mutation (mSOD1). After 11 days in vitro, cells were incubated with the MN secretome from WT MNs untreated or treated with pre-miR-124, as well as from mSOD1 MNs untreated or treated with anti-miR-124, for 24 h. Protein levels of GFAP were evaluated by immunocytochemistry using specific antibodies. Representative images for each condition are shown. All results are mean values ± SEM from at least 3 independent experiments. ***p<0.001 and *p<0.05 *vs*. respective + WT neuronal conditioned medium (NCM), respectively, $^{###}$p<0.001 and $^{##}$p<0.01 *vs.* respective + mSOD1 NCM two-tailed unpaired Student's *t* test with Welch's correction when required. Scale bar: 40 µm.

IV. Discussion

Alterations of miRNAs in ALS patients and disease models have been identified and related with cell survival and neuroinflammation (Rajgor 2018). For example, the inflammatory-associated miR-155 was found elevated in microglia and anti-miR-155 modulation found to be able to significantly increase the survival of ALS mice model (Butovsky et al. 2015; Koval et al. 2013). Another important inflammatory-associated miRNA is the miR-124. It is one of the most abundant in the CNS and changes on its levels are indicative of CNS altered status and disease progression (Sun et al. 2015). In ALS, miR-124 upregulation was associated with neurodegenerative sites, both in SC and brainstem of transgenic mice (Zhou et al. 2018). In addition, we recently demonstrated to be upregulated in NSC-34 mSOD1 MNs and in their derived membrane-bound vesicles (exosomes) (Pinto et al. 2017), which are then collected by microglial cells. After the uptake microglia become activated and exhibit early M1 and late mixed M1/M2 phenotypes. Therefore, the MN elevation in miR-124 seems to have an important role in modulating neighboring microglia reactivity. However, it is not yet clarified if the overexpression of miR-124 is directly related with MN dysfunction in ALS, or instead is a cell defensive mechanism, as well as if the secretome from mSOD1 MNs overexpressing miR-124 directly affects glial cell immunoregulation. Therefore, if differential miRNA expression pattern in patients suffering from ALS can represent a biosignature and be valuable for detecting the early onset of the disease, it is also important for developing new miRNA-based therapeutics. Actually, miRNAs are released as circulating molecules or as exosome cargo into bodily fluids and miRNA-based new drugs are currently being developed for several diseases, including neurodegenerative ones (Chakraborty et al. 2017; Zhao et al. 2016). For instance, MRG-107 that targets miR-155 is being tested in preclinical and enabling phase 1 studies for ALS (http://www.miragen.com/pipeline/).

The present thesis was designed to assess how upregulation and downregulation of miR-124 levels differently affect MN function, and whether its downregulation in mSOD1 MNs overexpressing miR-124 may represent a therapeutic potential in ALS. For that, we modulated its expression in WT NSC-34 MNs with pre-miR-124, and in mSOD1 NSC-34 MNs with anti-miR-124 for 12h. New medium was added and have analyzed the cells after 48 h incubation (4 DIV). Since SOD1 post-transcriptional regulation mediated by miRNAs is almost unexplored (Milani et al. 2011), we firstly evaluated if miR-124 modulation affected SOD1 accumulation in our ALS MN model, by assessing SOD1 levels. As expected and previously described by our group in this cellular model, mSOD1 accumulation was increased in mSOD1 MNs at 4 DIV (Vaz et al. 2015). WT cells treated with pre-miR-124 did not produce SOD1 variations in terms of protein expression, although after treatment with anti-miR-124 in mSOD1 MNs, the SOD1

accumulation decreased, suggesting that upregulation of miR-124 is a consequence of the overall dysfunctional mechanisms associated to SOD1 accumulation in ALS MNs but not a direct cause of such accumulation. Interestingly, SOD1 was previously predicted as a target of miR-355, i.e. miR-377 reduced SOD1 protein quantifies in mesangial cells (Wang et al. 2008). The decrease we found for SOD1 accumulation with anti-miR-124 may associate with enhanced autophagy and reduced misfolded protein accumulation since miR-124 was identified as a negative regulator of the autophagic pathway by targeting beclin1 and p62 (Mehta et al. 2017). To also note that a predicted target of miR-124 is IRE1α, which is an ER transmembrane sensor that activates the clearance of protein aggregates by UPR activation (Bartoszewska et al. 2013). Therefore, downregulation of miR-124 by activating UPR may turn in a decrease of SOD1 accumulation.

MiR-124 was shown to promote neuronal differentiation (Makeyev et al. 2007) and to control the choice between neuronal and astrocyte differentiation (Neo et al. 2014). Lately, it was shown to target vimentin and to control axonal morphology, as well as mitochondria morphology and function in mouse primary MNs (Yardeni et al. 2018). Thus, next, we evaluated how miR-124 dependent neuronal function and structure, by using anti-miR-124 and pre-miR-124, and which impaired mechanisms usually found in ALS MNs may be associated with the expression of upregulated or downregulated miR-124 levels.

Since one of the earliest events of MN degeneration in ALS is axonal degeneration (Gatto et al. 2018; Iwai et al. 2016), we evaluated how miR-124 expression was associated with neurite growth and branching. Similarly, to mSOD1 MNs with upregulated miR-124, WT pre-miR-124 MNs also presented a reduced neurite number, suggesting axonal degeneration. Interestingly, mSOD1 MNs showed an extension of neurite length, which was recently proposed to occur before neurodegeneration in a study using mSOD1 transgenic mice MNs (Osking et al. 2018). The increase of neurite length was also observed in WT pre-miR-124 MNs. This observation is not without precedent since it is described as being involved in neuronal cytoskeleton organization and neurite outgrowth during neuronal development (Yu et al. 2008). It is worthwhile to note that the inhibition of neurites in an inflammatory environment was shown to be counteracted by miR-124 in PC12 neurons (Hartmann et al. 2015). Then we can associate an increase of neurite extension in mSOD1 MNs to a miR-124 elevation in these cells. At first sight, this was not necessarily a negative effect of miR-124 elevation in mSOD1 MNs, since the increase of presynaptic markers is associated with the outgrowth of processes leading to an attempt of communication with the surrounding cells (Antonova et al. 2009). However, when we evaluated the mRNA expression of synaptic vesicle markers, we observed an increase of the pre-synaptic marker synaptophysin and a decrease of the post-synaptic PSD-95 in mSOD1 and WT pre-miR-124 MNs, showing trans-synaptic signaling unbalance,

that might be, dependent of miR-124 expression. Indeed, normal levels of miR-124 expression are required to sustain homeostatic synaptic plasticity, and both elevation and downregulation are related to abnormal synaptic function (Hou et al. 2015). Connectivity failure has been associated with defects either in pre-synaptic or post-synaptic terminals, and the number of postsynaptic densities were found reduced in ALS motor cortex from sporadic (Genç et al. 2017), compromising proper function.

Axonal transport is essential for neuronal function, in which anterograde transport supplies distal axons with newly synthesized proteins and lipids, while retrograde transport leads to the removal of aged proteins and organelles from the distal axon to sustain homeostasis (Maday et al. 2014). Pathological axonal transport is commonly observed neurodegenerative diseases, including ALS (De Vos and Hafezparast 2017). In fact, impaired axonal transport is considered a very early event ALS MNs (Hardiman et al. 2017). In this study, we have observed decreased anterograde transport and increased retrograde transport in mSOD1 MNs, as evaluated by kinesin decreased expression increased dynein levels. Similar findings were observed in transgenic mice mSOD1 MNs (De Vos et al. 2007; Warita et al. 1999). We observed the same pattern of expression of dynein and kinesin by overexpressing miR-124 in WT MNs, that has been very recently associated with impairment of axonal transport by affecting anterograde transport (Yardeni et al. 2018). We may hypothesize that the inhibition of anterograde transport in mSOD1 MNs is also a consequence of miR-124 upregulation. Tightly associated with axonal transport impairment is mitochondrial dysfunction, also a remarkable feature in ALS (Delic et al. 2018; Magrane et al. 2014; Onesto et al. 2016; Vaz et al. 2015). Our results demonstrated that mitochondrial dysfunction usually observed in mSOD1 MNs may be associated with miR-124 upregulation. Recent studies report that miR-124 regulates mitochondrial activity and localization in MNs and in ALS mSOD1 mice decreased levels of mitochondria in MNs were associated with increased retrograde transport of both miR-124 and mitochondria (Gershoni-Emek et al. 2018; Yardeni et al. 2018). Taken together, such evidence points to an important association between miR-124 and regulation on mitochondrial function, Moreover, our results also show an impaired in mitochondrial dynamics in MNs with elevated levels of miR-124, leading to high expression of the fission protein DRP-1, inducing mitochondrial fragmentation. Decreased expression of fusion proteins with elevated levels of fission proteins, as DRP-1, was found to occur in the SC at the onset of the disease in SOD1G93A mice (Liu et al. 2013). Moreover, it was recently demonstrated that peptide inhibitor P110 that blocks the association of DRP-1 with mitochondria was able to restore mitochondrial dynamics and to enhance motor activity and life span in the same animal model (Joshi et al. 2018), thus reinforcing the therapeutic potential of the anti-miR-124 strategy in ALS therapeutics.

Increased levels of neuronal levels of neuronal inflammatory markers were found to be expressed by SH-SY5Y cells transfected with the Swedish mutant of APP_{695}, as an *in vitro* model of Alzheimer's disease, and its secretome to have immunoregulatory properties in human CHME3 microglia (Fernandes et al. 2018). Neuronal activity has been suggested to be associated to neuroinflammation and neurons were lately considered to be the inflammatory problem, and the release of inflammatory cytokines and signaling molecules can be released from all cell types, including neurons (Tracey 2018; Xanthos and Sandkuhler 2014). We found that HMGB1 and iNOS levels were increased in mSOD1 MNs in a non-dependent manner of miR-124 elevation since it was not increased in WT pre-miR-124 MNs. In the case of S100B, only WT pre-miR-124 MNs showed elevated levels of this alarmin. However, both alarmins decreased in mSOD1 MNs treated with anti-miR-124, again, pointing out such mechanisms to be affected by miR-124 levels but not directly dependent on this miRNA. These findings are important if we consider that these markers are associated with neuroinflammation, communication with surrounding glial cells and activation of the inflammatory response (Kato and Svensson 2015). To go deeper in neuronal inflammatory status associated to mSOD1 MNs with upregulated miR-124, we assed associated inflamma-miRNAs profile, which we found to be deregulated in such miR-125b was overexpressed and miR-21/-146a were downregulated. Similarly to miR-124, miR-125b is associated with neuronal differentiation, neurite outgrowth and synaptic function and structure (Edbauer et al. 2010). However, in ALS miR-125b upregulation is usually associated with microglia reactivity and consequent NF-kβ activation, neuroinflammation exacerbation and ultimately neuronal death (Parisi et al. 2016). Thus, miR-125b increase in our cells may have two different roles: (i) in neurons, it can also be involved in neurite outgrowth and synaptic function alterations observed with the elevation of miR-124; (ii) it may be additionally exported to exosomes or by soluble factors, mediating neuronal communication with glia, regulating microglia activation and inflammatory response. Expression of miR-21 in neurons is often associated with axonal regeneration (Strickland et al. 2011). So, the decrease in MNs observed by us may be associated with the decrease of neurite number also observed in mSOD1 and WT pre-miR-124 MNs. In the case of miR-146a, one of its targets in neurons is ROCK1, a protein kinase usually associated with apoptotic response, though it is a key regulator of the actin cytoskeleton, as well (Wang et al. 2016a). Thus, miR-146a downregulation may also be associated with the morphological alterations observed in neurons. However, is important to keep in mind the importance of neuronal communication with glial cells and that in comparison with miR-124 these miRNAs have much less expression in MNs. Their alterations may not have a huge impact comparing with miR-124 and, importantly, these miRNAs are also immunomodulators with important functions in glial cells. Therefore, miR-21 and miR-146a decrease in the cell may not mean they are directly affecting MNs but probably that they are signaling surrounding cells via exosomes and soluble factors.

Importantly, either mSOD1 MNs with upregulated miR-124 or WT MNs transfected with pre-miR-124 revealed the same 125b^{high}/miR-21^{low}/miR-146a^{low} signature.

Considering that miR-124 is released from neurons, either in soluble form or inside exosomes, and may impact on microglia activation and astrocyte reactivity, we explored the effects of the neuronal secretome on the stimulation of these glial cells, even more considering that ALS is nowadays considered a non-cell autonomous disease where glial cells play a crucial role and are associated with neurodegeneration areas, suggesting a potential feed-forward loop. Thus, is important to develop therapies that target not only neurons but also have an effect in glial cells. To evaluate how MNs secretome differently affects microglia activation depending on miR-124 levels, we incubated secretome derived from modulated and non-modulated MNs in N9 microglia during 4 h, in order to evaluate short time response of microglia, usually associated with a more evident change of its inflammatory state.

We looked for microglia basic functions as phagocytic ability, that is usually increased upon activation and important for adequate microglia response to damage (Arcuri et al. 2017). Neuronal secretome globally induced a decrease in the phagocytic ability, when derived from mutated MNs (modulated with anti-miR-124 or not) or WT MNs expressing upregulated miR-124, suggesting that the correction of miR-124 is not enough to rescue microglia phagocytic ability. Interestingly, mSOD1 modulated with anti-miR-124 led to microglia morphology resemblance with WT secretome and annulled the enhanced are and perimeter by overexpressed miR-124. Since neuroinflammation is indicated to be a key player in ALS pathology and to be associated with microglia activation (Liu and Wang 2017), we evaluated the inflammatory profile of microglia after incubation with the secretome from WT and mSOD1 MNs with/without miR-124 modulation. First, we looked for M1/M2 polarization classical markers: iNOS and Arginase1, respectively. Besides the expected upregulation of iNOS (Pinto et al. 2017) by mSOD1 secretome, we also found upregulated Arg1, both identified in the same cells when miR-124 is upregulated, pointing to mixed phenotype. When transfected into microglia, miR-124 was shown to increase M2 microglia polarization in intracerebral hemorrhage (Yu et al. 2017) and to promote microglial quiescence in experimental encephalomyelitis after its peripheral administration (Ponomarev et al. 2011). Here we found that in the absence of neuroinflammation, the secretome from mSOD1 MNs induces increased gene expression of pro-inflammatory TNF-α and HMGB1, together with anti-inflammatory IL-10, supporting M1/M2 phenotype. This comes in defense of what has been though about microglia polarization in the past years: it is a continuous process with changes between M1 and M2 pro- and anti-inflammatory mediators and overlapping between typical phenotypes (Cunha et al. 2017). Recent studies showed indeed that microglia phenotype is remarkably different from that of macrophages meaning that it is not possible to do a straightforward

comparison between their phenotypic markers (Lisi et al. 2017; Zarruk et al. 2018). Anyway, microglia revealed to not only los the phagocytic ability, but to also decrease the expression of CD206, whenever secretome derived from MNs with upregulated miR-124, further indicating decreased endocytic activity and validating reduced phagocytosis. Again, anti-124 in mSOD1 MNs revealed to have benefits in counteracting all these effects associated with microglia activation and loss of function by their released secretome. Altogether, our study highlights that anti-miR-124 modulation in mSOD1 MNs is a promising strategy to recover both neuronal and microglia function while sustaining their steady state and avoiding the activation of signaling pathways leading to neuroinflammation.

Astrocytes have an important role in CNS homeostasis and by detecting the level of neuronal activity may release transmitters that influence neuronal function (Fellin 2009). In ALS, astrogliosis has been associated with MN degeneration in both patients and mice models (Philips and Rothstein 2014). ALS astrocytes were described as having an aberrant reactive phenotype, with low expression of both GFAP and GLT-1 (Cunha et al. 2017; Diaz-Amarilla et al. 2011). Lately, our group has demonstrated that astrocytes derived from the SC of mSOD1 mice also have decreased levels of the reactive marker GFAP (unpublished data). In the present work we confirmed the low expression of GLT-1 and GFAP in astrocytes isolated from mSOD1 mice pups as compared with WT animals. Intriguingly, secretome from WT MNs with upregulated miR-124 also determined a reduced expression of GLT-1 in both WT and mutated astrocytes and of GFAP in WT ones. GLT-1 glutamate receptor decrease in astrocytes is considered a major ALS hallmark (Pardo et al. 2006), and here we report for the first time that mSOD1 MN secretome causes a decrease of this transporter in healthy astrocytes in a manner that appears to be miR-124 dependent. Recently, it has been observed a similar effect with another miRNA (miR-218) that is released from dying motor neurons in ALS (Hoye et al. 2018). Now, we verified that mSOD1 and WT pre-miR-124 neuronal secretome cause GLT-1 decrease as in mSOD1 astrocytes. Though the secretome from WT MNs treated with pre-miR-124 also decreased GFAP immunostaining in WT astrocytes, as observed with that from mutated cells, it was not able to modify such expression in mutated astrocytes. As observed for neurons and microglia the anti-miR-124 treatment in mSOD1 MNs led to a secretome with properties of sustaining GLT-1 and GFAP close to WT astrocyte normal values, reinforcing the neuroprotective potential of the secretome derived from MNs modulated for the miR-124 expression. Data highlight the relevance of cell secretome and cell-to-cell signaling in CNS homeostasis and pathology.

Looking at the results obtained in microglia and astrocytes, our work gives some cues for the understanding on how glial response to neuronal injury can be directly mediated by the secretome of affected neurons. Moreover, anti-miR-124 sorts from this study as having

therapeutic relevance in targeting different cells, signaling pathways and drivers of ALS pathophysiology.

CONCLUDING REMARKS

Our results showed that miR-124 is involved in mSOD1 MNs synaptic dysfunctionality (increase pre-synaptic marker synaptophysin and decrease post-synaptic PSD-95), cell cytoskeleton alterations, axonal loss, axonal transport deficits (with favored retrograde transport and reduced mitochondrial function) and mitochondria alterations in its dynamics balance (increased fission and decreased fusion). Moreover, miR-124 is also involved in inflamma-miRNA profile in mSOD1 cells (elevated miR-125b and decreased miR-21/miR-146a). The inhibition of miR-124 in mSOD1 MNs was shown to lead to the recovery of all these impaired mechanisms, besides indirectly counteracting the increase of alarmins. Additional to the benefit effects on neuronal functions, this study is innovative in demonstrating that secretome from MNs with upregulated levels of miR-124 is associated with microglia activation and astrocyte aberrant phenotype. In N9 microglia it leads to morphological changes, reduced phagocytosis, increase of both iNOS and Arg1, together with gene expression of pro-inflammatory mediators (IL-1β, TNF-α, and HMGB1). In astrocytes, secretome from mSOD1 MNs and WT MNs with upregulated miR-124 determined the reduction of GLT-1 and GFAP in both WT and mSOD1 astrocytes isolated from mice spinal cord. Secretome from anti-miR-124-treated mSOD1 MNs protected astrocyte and microglia dysfunctional properties and exerted immunomodulatory effects. Therefore, anti-miR-124 therapy in ALS MNs acts like a combined strategy directed to multiple targets and may be uses as a miRNA-based therapeutics for ALS.

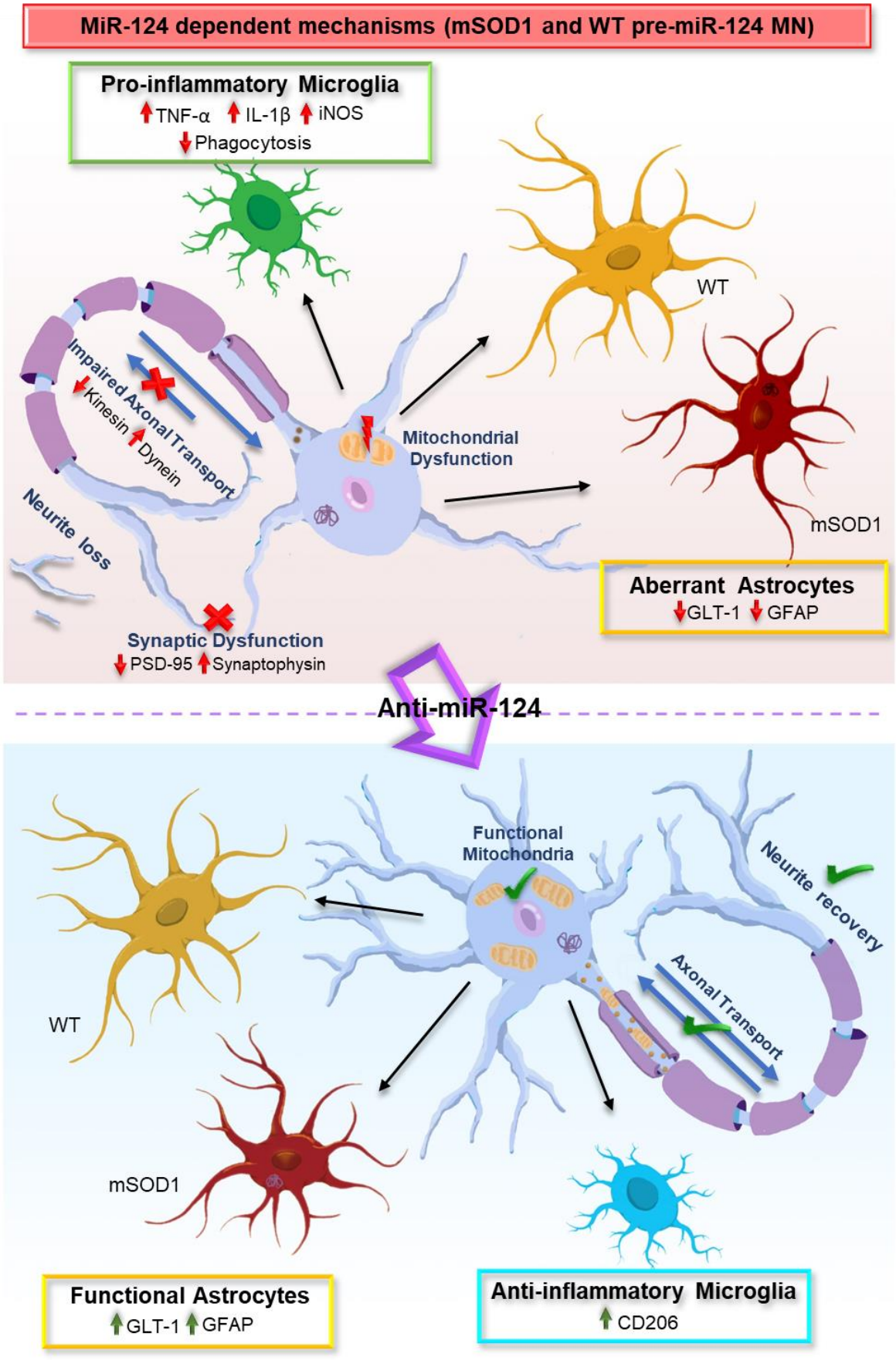

MiR-124 dependent mechanisms (mSOD1 and WT pre-miR-124 MN)
Pro-inflammatory Microglia
TNF-α IL-1β iNOS
Phagocytosis
WT
Impaired Axonal Transport
Kinesin Dynein
Mitochondrial Dysfunction
Neurite loss
mSOD1
Aberrant Astrocytes
GLT-1 GFAP
Synaptic Dysfunction
PSD-95 Synaptophysin
Anti-miR-124
Functional Mitochondria
Neurite recovery
Axonal Transport
WT
mSOD1
Functional Astrocytes
GLT-1 GFAP
Anti-inflammatory Microglia
CD206
MiR-124 downregulation in mSOD1 MN

Figure IV.1. Schematic representation of the major findings ensuing from this thesis. NSC-34 motor neurons (MNs) with Amyotrophic Lateral Sclerosis (ALS) mSOD1 mutation (mSOD1) showing miR-124 upregulation and dysregulated inflammatory-associated microRNAs (miRNAs), as well as NSC-34 MNs (wild type, WT) transfected with pre-miR-124, evidence similar functional and structural alterations when compared with WT MNs. Cells overexpressing miR-124 present synaptic dysfunction (increase of pre-synaptic synaptophysin and decrease of post-synaptic postsynaptic density protein 95 (PSD-95)), cell cytoskeleton defects, axonal loss, axonal transport deficits with favoured retrograde transport (high dynein) and depressed anterograde transport (low kinesin levels), together with changes in mitochondria dynamics (increased fission processes by elevated dynamin-related protein 1 (DRP-1)). Secretome from MNs overexpressing miR-124 disturbs the steady state of N9 microglia by changing their morphology, while decrease phagocytic ability and increase gene expression of pro-inflammatory mediators (Tumour necrosis factor α (TNFα), Interleukin-1β (IL-1β) and inducible nitric oxide synthase (iNOS)), including mixed M1/M2 polarized phenotypes. Interestingly, when added to astrocytes isolated from the spinal cord of WT and mSOD1 mice it also induces the decrease of glutamate transporter 1 (GLT-1) (in both) and Glial fibrillary acid protein (GFAP) (in WT), thus promoting the astrocytic aberrant phenotype characteristic of ALS. Upon treatment with anti-miR-124, mSOD1 MNs recover all these impaired mechanisms that were assessed towards a phenotype closer to the one observed in WT MNs. Treatment of mSOD1 MNs with anti-miR-124 recovered normal neuronal function and structure. Additionally, when incubated in glial cells, secretome from these modulated anti-miR-124 cells sustains the N9 microglia morphological shape and steady state functionality, while also preserving GLT-1 and GFAP expression in both WT and mSOD1 spine astrocytes.

FUTURE PERSPECTIVES

In the present study, we describe how miR-124 upregulation in NSC-34 mSOD1 and WT MNs contributes to alterations in their structure and function, thus promoting neurodegenerative processes, and how anti-miR-124 is able to counteract such dysfunctionalities. Since NSC-34 is a mice cell line, it will be important to validate if miR-124 also impact on MN function in models closer to the ALS human disease, namely by using MNs generated from iPSCs obtained from ALS patients' fibroblasts. This will give us important information not only about the role of miR-124 in the familiar SOD1-linked mutations, but also in other forms of familial ALS and, in particular, in the sporadic ones that represent a challenge due to the absence of models that mimic the disease.

Additionally, it would be interesting to assess the alterations on the synaptic vesicle glycoprotein 2A (SV2A) that regulates action potential-dependent neurotransmitters release and is a specific binding site for certain antiepileptic drugs and radioactive chemicals, what allows PET image in Alzheimer's patients (Chen et al. 2018). Once this protein is expressed in mitochondria (Chen et al. 2018; Stockburger et al. 2016) and anti-miR-124 showed to improve mitochondria dynamics, it would be interesting to evaluate whether this anti-miRNA therapy will have benefits at this levels in the iPSCs-MN ALS model, as well. Therefore, besides SV2A immunostaining it would be also important to check mitochondrial location within the axon before and after anti-miR-124 transfection into MNs.

Moreover, since we show that secretome from MNs with elevated levels of miR-124 has a negative impact on glial function and leads to cell activation, mostly reverted when we treat mSOD1 MNs with anti-miR-124, we believe that it would be relevant to investigate whether such effects are mediated by soluble factors or by exosomes. Based on the knowledge that miR-218 also negatively affects glial function in ALS (Hoye et al. 2018), it may be important to study the dynamics of miR-124 and miR-218 inside neuronal cells to check if they are dependent on each other, and if they act in synergy or by independent mechanisms. Recently, our ongoing data revealed that miR-124 is also elevated in mSOD1 astrocytes (unpublished data), what deserves future studies to assess how miR-124 modulation in astrocytes may impact on their function, aberrancy, and if targeting miR-124 simultaneously in astrocytes and MNs will also be advantageous for neuroprotection in ALS.

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ANEX 1

Mind-Brain College of ULisboa

LISBOA UNIVERSIDADE DE LISBOA

Mind-Brain College

14th - 15th November

REITORIA DA UNIVERSIDADE DE LISBOA

4th Students Meeting of Mind-Brain College

INVITED SPEAKERS

14th Nov 10h00

Manuel Carreiras
Basque Center on Cognition, Brain and Language

15th Nov 12h00

Luciano Fadiga
University of Ferrara

ROUND-TABLES

14th Nov 14h00

Diálogos mente-cérebro

15th Nov 14h30

Ciência & Sociedade – o futuro da ciência

Academia · Sociedade Portuguesa de Neurociências · PAPA-LETRAS® · LISBOA · justNews · Sea4Us

Elevated expression of miR-124 is associated with motor neuron impairment in ALS and contributes to glia reactivity

Vizinha D. (1), Sequeira C. (1), Vaz A.R. (1,2), Brites D. (1,2)
1-Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal; 2- Department of Biochemistry and Human Biology, Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal.

Amyotrophic lateral sclerosis (ALS) is characterized by motor neuron (MN) loss and glial dysfunction. Expression of microRNA (miR)-124 was found elevated in the MN-like NSC-34 cell line overexpressing SOD1 with G93A mutation (mSOD1) and in their exosomes that caused diverse microglia activated phenotypes (Pinto *et al.* 2017). Here, we investigated how changes in miR-124 expression differently modulate MN function and impact on secretome-mediated glial reactivity. Wild type (wt) MN-like NSC-34 cells were transfected with pre-miR-124, while anti-miR-124 was used in mSOD1 MN, and cells evaluated for structure and function. Glial reactivity of N9-microglia and spine astrocytes from wt and mSOD1 mice was assessed after treatment with neuronal secretome (NS).

mSOD1-MN revealed increased miR-125b and decreased miR-146a/miR-21 levels, synaptic dysfunctionalities (increased synaptophysin/decreased PDS-95), neurite loss, reduced mitochondria viability and axonal transport deficits (increased dynein/decreased kinesin). While anti-miR-124 prevented such disturbing effects in mSOD1 cells, pre-miR-124 induced the pathological phenotype in wt ones. N9-microglia incubated with NS-mSOD1-MN showed increased expression of IL-1β/TNF-α and morphological alterations (increased ramifications/enlarged cell shape), which were reproduced with NS-wt-MN/pre-miR-124, but counteracted by NS-mSOD1-MN/anti-miR-124. Decreased GLT-1/GFAP in mSOD1 spine astrocytes was reproduced in wt cells with either NS-mSOD1-MN or NS-wt-MN/pre-miR-124, but prevented with NS-mSOD1-MN/anti-miR-124. Most important, NS-wt-MN/pre-miR-124 was able to increase GLT-1/GFAP expression in mSOD1 spine astrocytes.

Overall, by recovering mSOD1 MN function and preventing secretome-inducing glial reactivity, data highlight miR-124 targeting as a promising therapeutic strategy in ALS.

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Keywords: Pre-/anti-miR-124 modulation; mSOD1 spine astrocytes; mSOD1 MN-like NSC-34 cells; N9-microglia activation; GFAP/GLT-1 astrocyte aberrancies;

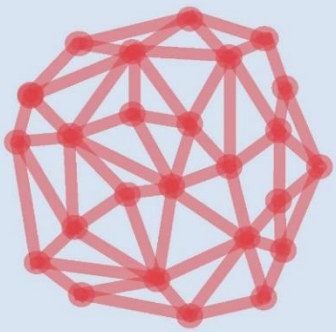
ANEX 2

10th iMed.ULisboa Postgraduate

STUDENTS MEETING

& 3rd i3DU Meeting

July 24-25, 2018



BOOK OF ABSTRACTS

MiR-124 Targeting in ALS Motor Neurons Reduces Genes Associated with Alarmin Production and Synaptic Loss, while Favours Anterograde Axonal Transport

Neuron-Glia Biology in Health and Disease

Vizinha D. * [1], Sequeira C. [1], Vaz A.R. [1,2], Brites D. [1,2]

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

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease causing motor neuron (MN) loss. MN overexpressing human superoxide dismutase 1 with G93A mutation (mSOD1) is the most widely used model to study MN degeneration. Our previous work using the mouse mSOD1 MN-like NSC-34 cell line revealed SOD1 intracellular accumulation, activation of MMP-9, mitochondrial dysfunction and apoptosis [1]. Lately, we identified an increased expression of microRNA(miR)-124 in those MNs and transfer into cell-derived exosomes, which induced early M1 and late M1/M2 microglia phenotypes [2]. However, it is not known whether such miR-124 upregulation has beneficial or harmful effects on MN function.

To better assess the role of miR-124 upregulation in SOD1 MNs we transfected pre-miR-124 in wild type (wt) MNs and anti-miR-124 in mSOD1 MNs.

We observed that pre-miR-124 selectively induced the gene expression of the alarmin HMGB1 in the wt MNs, while anti-miR-124 significantly reduced its expression in the mSOD1 MNs. Such effect may prevent neurite degeneration and inflammatory stimulation, known to be associated to the release of HMGB1 to the extracellular medium. Interestingly, anti-miR-124 in mSOD1 MNs recovered the control levels of the post-synaptic protein PSD-95 that showed to be reduced in ALS MNs. Since pre-miR-124 decreased its expression, though not significantly, we assume that miR-124 has a role in disturbing synaptic functionality. Additionally, anti-miR-124 reduced the retrograde axonal transport, while induced the anterograde one, suggesting that miR-124 upregulation causes deficient transport of new synthesized regenerative genes and cytoskeleton proteins to the axon tip, as well as impairs synaptic vesicle and mitochondria movement, thus contributing to neurodegeneration. Data highlight anti-miR-124 targeting as having therapeutic potential in mSOD1 MNs, but further studies are required to assess the effects in cell-to-cell communication in ALS disease.

Funding: Research Grant of the Santa Casa Scientific Research Program on ALS (SCML-Project ELA-2015-002 (to DB) and in part by iMed.ULisboa (UID/DTP/04138/2013) from Fundação para a Ciência e a Tecnologia (FCT). ARV is recipient of a Post-doc fellowship from FCT (SFRH/BPD/76590/2011).

Keywords: MicroRNA-124 Downregulation; Mutated SOD1 Motor Neurons; Synaptic Proteins; HMGB1; Axoplasmic Transport

References:

1 - Vaz, A.R., et al. Mol Neurobiol 2015, 51, 864-777.

2 - Pinto, S., et al. Front Neurosci 2017, 11, 273.

MiR-124 Targeting in ALS Motor Neurons Reduces Genes Associated with Alarmin Production and Synaptic Loss, while Favours Anterograde Axonal Transport

Vizinha D. (1), Sequeira C. (1), Vaz A.R. (1,2), Brites D. (1,2)

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2- Department of Biochemistry and Human Biology, Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease causing motor neuron (MN) loss. MN overexpressing human superoxide dismutase 1 with G93A mutation (mSOD1) is the most widely used model to study MN degeneration. Previous work from our group using the mouse mSOD1 MN-like NSC-34 cell line revealed SOD1 intracellular accumulation in mutant cells, activation of MMP-9, mitochondrial dysfunction and apoptosis [1]. Lately, we identified an increased expression of microRNA(miR)-124 in those mSOD1 MNs but not of other inflammatory-associated miR [2]. Interestingly, this miR-124 was transferred into their derived exosomes, which induced an early M1 and late M1/M2 microglia phenotypes in N9 cell line [2]. However, it is not known whether such miR-124 upregulation has beneficial or harmful effects on MN function.

AIMS

To assess the beneficial/harmful effects of miR-124 upregulation in MNs, either by overexpressing its levels in naïve human SOD1 (WT) MNs or by using mSOD1 MNs.

EXPERIMENTAL DESIGN

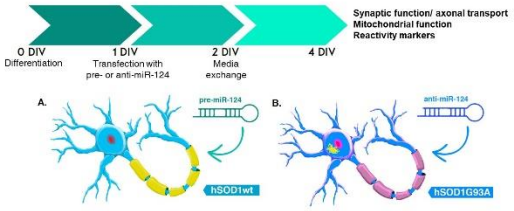


Figure 1: Experimental design schematic representation. (a) NSC-34 cells, transiently transfected with human superoxide dismutase 1 (SOD1), wild type or with G93A mutation [NSC-34 hSOD1wt (wt MNs) or NSC-34 hSOD1G93A (mSOD1 MNs)], were differentiated for 4 days in vitro (DIV) in order to induce SOD1 accumulation, as described by our group1. At 2 DIV, wt MNs (A) were transfected with pre-miR-124 and mSOD1 MNs (B) were transfected with anti-miR-124. Culture media was exchanged 12 hours after transfection and cells were maintained in this media during 48 hours and then were collected for different assays

RESULTS

1 Transfection with anti- and pre-miR-124 is efficient in NSC-34 without affecting cell viability

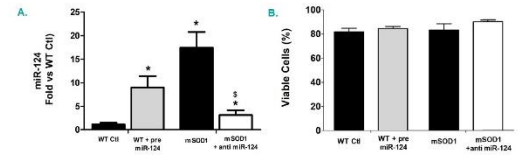


Figure 2: Expression of miR-124 (A) was measured by qRT-PCR. Cell viability was evaluated by flow cytometry using Nexin Reagent®. Results are mean (+SEM) from at least three independent experiments; *p<0.05 vs. MN over-expressing human wild type SOD1 (WT Ctl). $p<0.05 vs. non-transfected NSC-34 overexpressing human SOD1 with G93A mutation (mSOD1).

2 Elevated levels of miR-124 induce HMGB1 increased levels in NSC-34-MNs, which are prevented by miR-124 downregulation

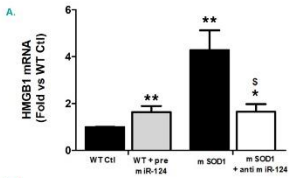


Figure 3: Expression of HMGB1 (A), was measured by qRT-PCR. Results are mean (+SEM) from at least three independent experiments; *p<0.05 and **p<0.01 vs. MN over-expressing human wild type SOD1 (WT Ctl). $p<0.05 vs. non-transfected NSC-34 overexpressing human SOD1 with G93A mutation (mSOD1).

3 Elevated levels of miR-124 slightly increase neurite outgrowth while reducing neurite branching in NSC-34-MNs

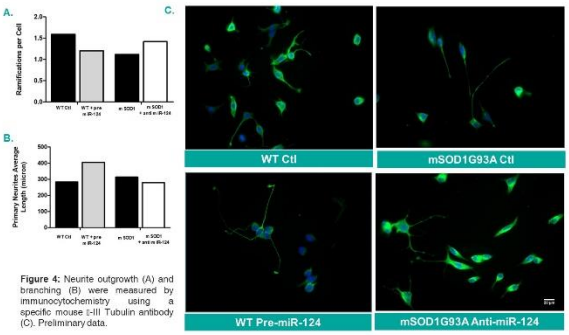


Figure 4: Neurite outgrowth (A) and branching (B) were measured by immunocytochemistry using a specific mouse (-III Tubulin antibody (C). Preliminary data.

4 Both mSOD1-MN and WT-MN overexpressing miR-124 have PSD-95 downregulation

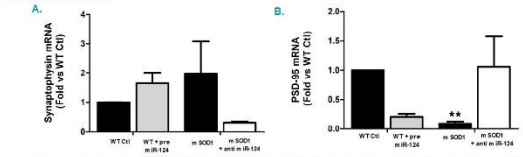


Figure 5: Levels of pre-synaptic vesicles marker synaptophysin (A) and post-synaptic vesicles marker PSD-95 (B) were measured by qRT-PCR. Results are mean (+SEM) from at least three independent experiments; **p<0.01 vs. MN over-expressing human wild type SOD1 (WT Ctl).

5 Downregulation of miR-124 reduces mitochondrial dysfunction and the retrograde axonal transport, while inducing the anterograde one, in mSOD1-MNs

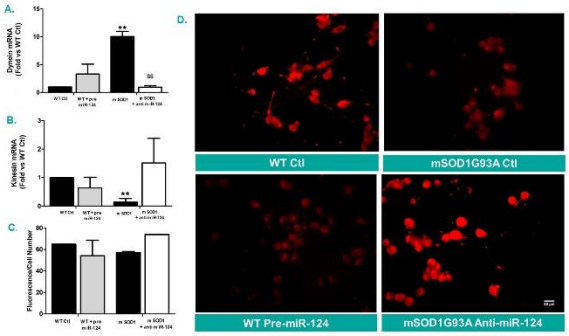
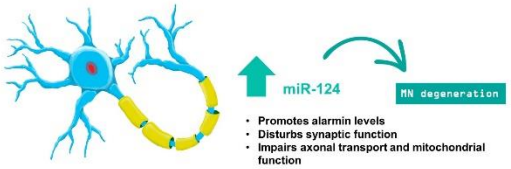


Figure 6: Levels of retrograde axonal transport marker dynein (A) and anterograde axonal transport maker kinesin (B) were measured by qRT-PCR. Results are mean (+SEM) from at least three independent experiments; *p<0.05 and **p<0.01 vs. MN over-expressing human wild type SOD1 (WT Ctl). $p<0.05 and $$p<0.01 vs. non-transfected NSC-34 overexpressing human SOD1 with G93A mutation (mSOD1). Mitochondrial viability was determined by Mitotracker Staining (C, D). Preliminary data.

CONCLUSIONS



MiR-124 upregulation impairs several neuronal mechanisms, contributing to motor neurodegeneration. Our data highlight that miR-124 downregulation by anti-miR-124 in mutated cells generally restores synaptic function, axonal transport and mitochondrial function, suggesting that therapeutic miR-124 targeting may have potential in mSOD1 MNs.

References:
[1] Vaz, A.R., et al. Mol Neurobiol 2015, 51, 864-777.; [2] Pinto, S., et al. Front Neurosci 2017, 11, 273

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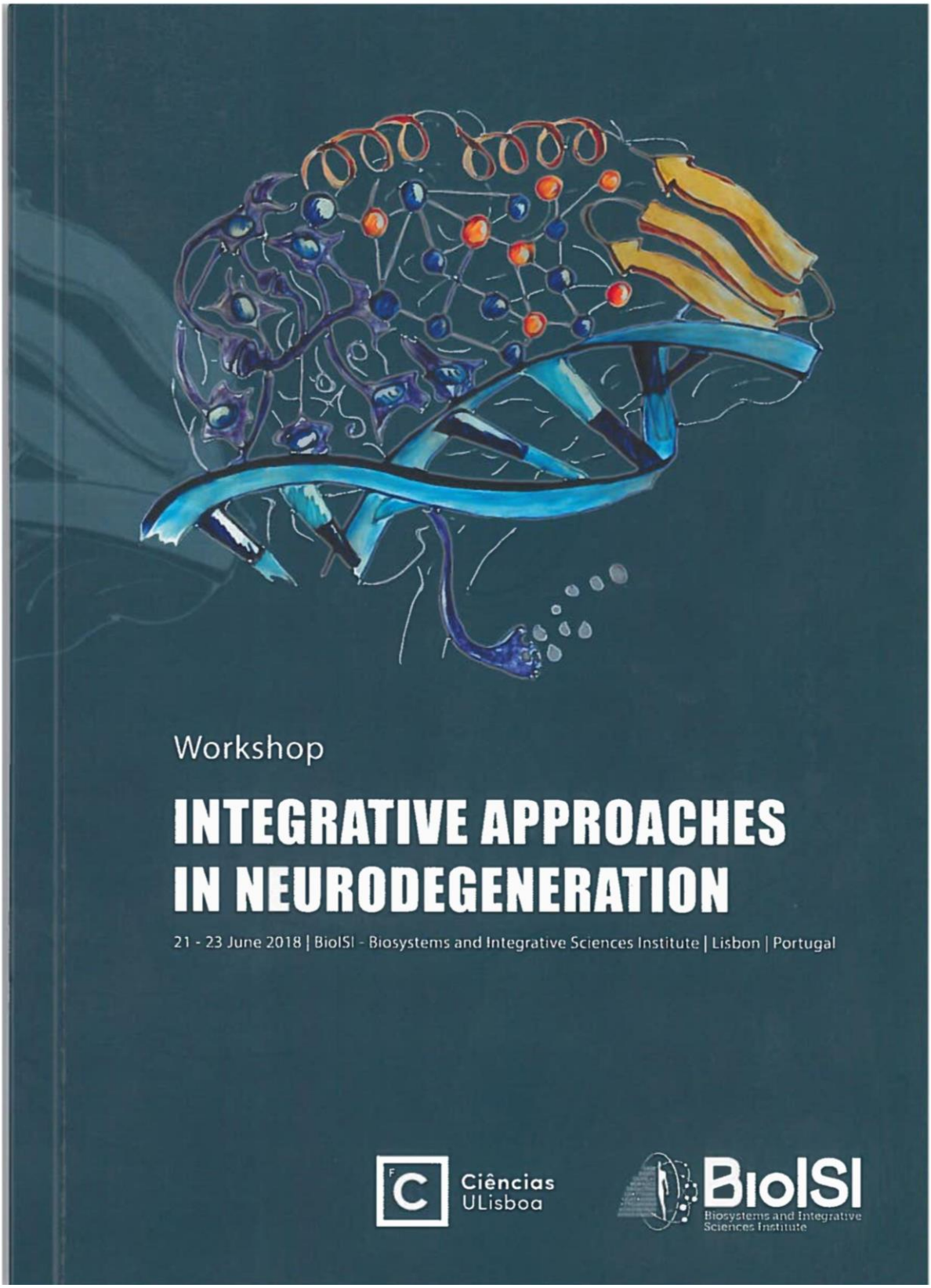
ANEX 4


Workshop
INTEGRATIVE APPROACHES IN NEURODEGENERATION
21 - 23 June 2018 | BioISI - Biosystems and Integrative Sciences Institute | Lisbon | Portugal
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Workshop on Integrative Approaches in Neurodegeneration

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Targeting astrocyte and motor neuron specific miRNAs to recover cell function in ALS

Amyotrophic lateral sclerosis (ALS) is a fatal disease characterized by motor neurons (MNs) loss and dysfunctional astrocytes. G93A mutation in SOD1 (mSOD1) is the most used model to study ALS pathological mechanisms. Cell-to-cell trafficking of inflammatory miRNAs are associated with glia activation and neurodegeneration. We demonstrated that NSC-34-mSOD1-MN and their exosomes have increased miRNA(miR)-124 levels[1]. We additionally observed upregulated miR-155 in the spinal cord (SC) of mSOD1 mice[2]. In contrast, miR-146a was found downregulated either in the cortical brain (CB) tissue of symptomatic mSOD1 mice, or in isolated astrocytes from their pups[3].

Here, we evaluated the effects of miR-155/146a/124 modulation in astrocytic/MN function. For that, we used primary astrocytes from CB or SC of mSOD1 mice pups, and NSC-34-mSOD1-MN. Cells were transfected with anti-miR-155 (spinal astrocytes), premiR-146a (CB astrocytes) or anti-miR-124 (mSOD1-MN). Astrocytes from non-transgenic mice and NSC-34-wild-type-SOD1-MN were used as controls.

MiR-155 downregulation in mSOD1 spinal astrocytes reduced inflammatory profile, by decreasing IL-1β/HMGB1 and increasing IL-6, although not directly affecting astrocyte phenotype (GFAPlow/S100Blow/Cx43high). However, upregulation of miR-146a led to a decrease in inflammatory mediators (HMGB1/inflamma-miRs) and neutralized the aberrant astrocyte phenotype (GFAPlow/S100Blow/Cx43high). Finally, by downregulating miR-124 in MNs, we restored HMGB1 and S100B normal levels, as well as the retrograde and anterograde axonal transport, all deregulated in mSOD1-MNs.

Overall, our results highlight that combined modulation of specific miRNAs in different target cells may represent a promising therapeutic potential in ALS disease.

Supported by: SCML-ELA-2015-002(DB); FCT-UID/DTP/04138/2013(iMed.ULisboa); FCT-SFRH/BPD/76590/2011(ARV).

[1]Pinto et al Front Neurosci. 2017
[2]Cunha et al Mol Neurobiol. 2018
[3]Gomes et al Mol Neurobiol. (in revision)

Targeting astrocyte and motor neuron specific miRNAs to recover cell function in ALS

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Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal disease characterized by motor neurons (MNs) loss and dysfunctional astrocytes. G93A mutation in SOD1 (mSOD1) is the most used model to study ALS pathological mechanisms. Cell-to-cell trafficking of inflammatory miRNAs have been suggested to be associated to glia activation and neurodegeneration.

We have shown that NSC-34-mSOD1-MN and their exosomes have increased miRNA(miR)-124 levels. Transfer of such exosomes into microglia promoted cell activation[1]. We additionally observed upregulated miR-155 in the spinal cord mSOD1 mice[2] as well as in isolated astrocytes from their pups (unpublished). In contrast, miR-146a was found downregulated either in the cortical brain tissue of symptomatic mSOD1 mice, or in isolated astrocytes from their pups[3].

Aims

To investigate the effects of specific miR-155/146a/124 modulation in different ALS cellular models overexpressing human SOD1 with G93A mutation (mSOD1), namely in: (i) MN-like cell line; (ii) primary astrocytes either from cortical brain or spinal cord of mSOD1 mice pups.

Experimental design

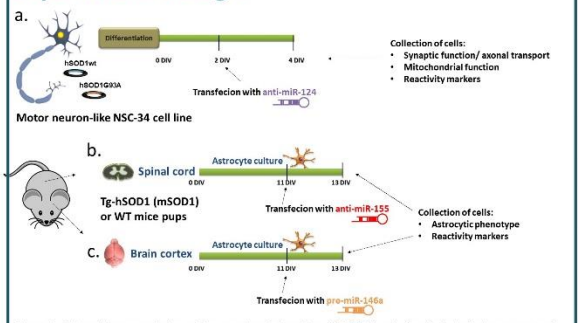


Figure 1: Schematic representation of the experimental models. (a) NSC-34 cells, transfected with human superoxide dismutase 1 (SOD1), wild type or with G93A mutation [NSC-34 hSOD1wt (wt MNs) or NSC-34 hSOD1G93A (mSOD1 MNs)], were differentiated for 4 days in vitro (DIV)) to induce SOD1 accumulation, as described by us[4]. At 2 DIV, cells were transfected with anti-miR124. Primary astrocyte cultures were obtained from (b) spinal cord or (c) brain cortex of 7-day-old B6SJL-TgN (SOD1G93A) 1Gur/J (Tg. mSOD1) or non tg (WT) mice and maintained for 11 DIV and then transfected with (b) anti-miR155 or (c) pre-miR146a. In all cell models, media was replaced 12 hours after transfection, and the cells were maintained in their proper media for 48 h. After this period, cells were collected for the different assays.

Results

1 Downregulation of miR-124 in mSOD1 MNs prevents alarmin induction and restores mitochondrial function and axonal transport

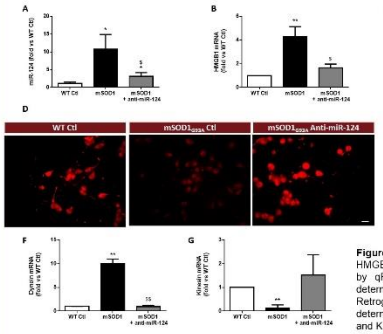


Figure 2: Expression of miR-124 (A), HMGB1 (B) and S100B (C), was measured by qRT-PCR. Mitochondrial viability was determined by Mitotracker Staining (D,E). Retrograde and anterograde transport was determined by quantification of Dynein (F) and Kinesin (G) expression, also by qRT-PCR. Results are mean (+SEM) from at least three independent experiments; $^{*}p<0.05$ and $^{**}p<0.01$ *vs.* MN over-expressing human wild type SOD1 (WT Ctl). $^{\S}p<0.05$ *vs.* non-transfected NSC-34 overexpressing human SOD1 with G93A mutation (mSOD1).

2 MiR-155 downregulation in mSOD1 spinal astrocytes does not change astrocytic aberrancies

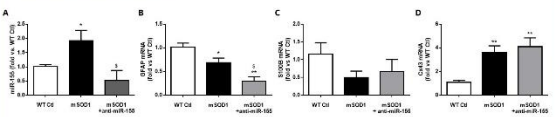


Figure 3: Expression of miR-155 (A), GFAP (B), S100B (C) and Connexin43 (Cx43) (D) was measured by qRT-PCR. Results are mean (+SEM) from at least three independent experiments; $p<0.05$ and $^{**}p<0.01$ *vs.* naïve astrocytes (WT Ctl) $^{\S}p<0.05$ *vs.* non-transfected astrocytes overexpressing human SOD1 with G93A mutation (mSOD1).

3 MiR-155 downregulation in mSOD1 spinal astrocytes restrains inflammatory gene expression

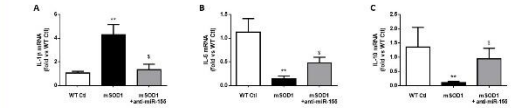


Figure 4: Expression of IL-1β (A), IL-6 (B) and IL-10 (C) was measured by qRT-PCR. Results are mean (+SEM) from at least three independent experiments; $p<0.05$ and $^{**}p<0.01$ *vs.* naïve astrocytes (WT Ctl) $^{\S}p<0.05$ *vs.* non-transfected astrocytes overexpressing human SOD1 with G93A mutation (mSOD1).

4 Upregulation of miR-146a in mSOD1 cortical astrocytes recovers miR-21 and HMGB1 control levels

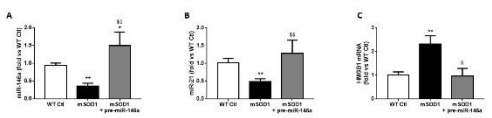


Figure 5: Expression of miR-146a (A), miR-21 (B) and HMGB1 (C) was measured by qRT-PCR. Results are mean (+SEM) from at least six independent experiments: $p<0.05$ and $^{**}p<0.01$ *vs.* naïve astrocytes (WT Ctl); $^{\S\S}p<0.01$ $^{\S}p<0.05$ *vs.* non-transfected astrocytes overexpressing human SOD1 with G93A mutation (mSOD1).

5 Upregulation of miR-146a in mSOD1 cortical astrocytes counteracts astrocytic aberrancies

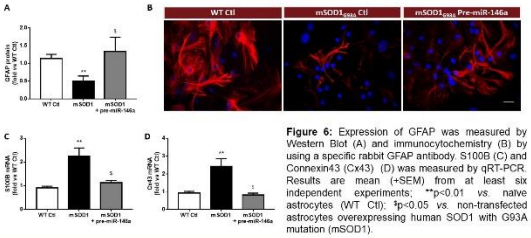
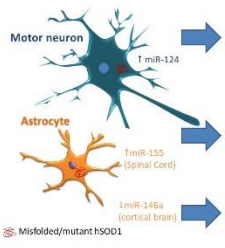


Figure 6: Expression of GFAP was measured by Western Blot (A) and immunocytochemistry (B) by using a specific rabbit GFAP antibody. S100B (C) and Connexin43 (Cx43) (D) was measured by qRT-PCR. Results are mean (+SEM) from at least six independent experiments; $^{**}p<0.01$ *vs.* naïve astrocytes (WT Ctl); $^{\S}p<0.05$ *vs.* non-transfected astrocytes overexpressing human SOD1 with G93A mutation (mSOD1).

Conclusions



anti-miR-124
- Prevention of miR-124 upregulation in mSOD1 MNs reestablishes cell function;

anti-miR-155
- Prevention of miR-155 upregulation in mSOD1 spinal astrocytes reduces inflammation but not cell aberrancy;

pre-miR-146a
- Reestablishment of miR-146a normal levels in mSOD1 cortical astrocyte reduces both inflammation and cell aberrancy.

Overall, our results by highlighting the different pathological role of **specific inflammatory miRNAs** in **target cells** at **distinct ALS affected regions** suggest that a combined therapeutic modulation may represent a promising strategy in the disease.

References:
[1]Pinto *et al.* Front Neurosci. 2017; 17;11:273; [2]Cunha *et al.* Mol Neurobiol. 2018; 55:4207-4224; [3]Gomes *et al.* Mol Neurobiol. (under revision); Vaz *et al.* Mol Neurobiol. 2015;51:864-77.

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