ALS pathogenesis: role of motor neuron-derived exosomes in microglia activation and dysfunction

Sara Filipa Castro da Costa Pinto
Dissertação de Mestrado

Orientador: Dora Maria Tuna de Oliveira Brites
Investigadora Coordenadora e Professora Catedrática Convidada
Faculdade de Farmácia da Universidade de Lisboa

Co-orientador: Ana Rita Mendonça Vaz Botelho, PhD
Bolseiro de Pós-Doutoramento da FCT
Faculdade de Farmácia da Universidade de Lisboa

MESTRADO EM CIÊNCIAS BIOFARMACÊUTICAS
2016
ALS pathogenesis: role of motor neuron-derived exosomes in microglia activation and dysfunction

Sara Filipa Castro da Costa Pinto
Dissertação de Mestrado

Orientador: Dora Maria Tuna de Oliveira Brites
Investigadora Coordenadora e Professora Catedrática Convidada
Faculdade de Farmácia da Universidade de Lisboa

Co-orientador: Ana Rita Mendonça Vaz Botelho, PhD
Bolseiro de Pós-Doutoramento da FCT
Faculdade de Farmácia da Universidade de Lisboa

MESTRADO EM CIÊNCIAS BIOFARMACÊUTICAS
2016
The studies presented in this thesis were performed in the Neuron Glia Biology in Health and Disease group, at the Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, under the supervision of Dora Brites, Ph.D. and Ana Rita Vaz, Ph.D.
Part of the results discussed in this thesis was presented in the following occasions:

*Paper to be submitted in a special issue by invitation*


*Communications in National Scientific Meetings*

Brites D., Pinto S., Cunha C., Gomes C., Barbosa M., Cunha C., Vaz A.R. Exosomes as mediators of neuroinflammation and pathogenicity in ALS. 30ª Reunião do Grupo de Estudos de Envelhecimento Cerebral e Demência, June 3-4th, 2016, Hotel Açores, Lisboa (oral presentation) (Annex VI.2)

Pinto S., Barbosa M., Cunha C., Vaz A.R., Brites D. Extracellular vesicles from ALS motor-neurons trigger microglia activation. 8th Postgraduate iMed.ULisboa Students Meeting, July 14th, 2016 (poster presentation) (Annex VI.3)

The work presented in this master thesis was supported by Santa Casa da Misericórdia de Lisboa (ELA Project 2016) and by Fundação para a Ciência e Tecnologia – FCT, Portugal (UID/DTP/04138/2013 to iMed.ULisboa, SFRH/BPD/76590/2011 to Vaz A.R., SFRH/BD/91316/2012 to Cunha C. and SFRH/BD/102718/2014 to Gomes C.).
Abstract

Exosomes are nanosized (30-100 nm) extracellular vesicles that are formed by nearly all types of cells and derive from the endocytic pathway and intracellular multivesicular bodies. They are released when the multivesicular bodies fuse with the plasma membrane. Exosomes mediate intercellular communication and have an important role in the spreading of neurodegenerative diseases, probably also of Amyotrophic Lateral Sclerosis (ALS). Recently, release of exosomes derived from motor neuron (MN)-like NSC-34 cells overexpressing human superoxide dismutase 1 mutated in G93A (mSOD1), was suggested to be implicated in cell-to-cell transfer of mSOD1 toxicity. However, how the uptake of such exosomes by receptor cells, such as microglia, contributes to their activation or loss of function was never investigated. Here we evaluated a selected set of promising markers and mediators of inflammatory response to establish: (i) the pro- and anti-inflammatory microRNA profile in NSC-34 MN-like cell line and in their derived exosomes; (ii) alterations in microglia function and generated polarized microglia subtypes triggered by the mSOD1 MN-derived exosomes; and (iii) the cellular distribution of labelled exosomes in a MN-microglia co-culture system. For that, we used mouse NSC-34 cells expressing either wild-type SOD1 (wt) or the G93A mutation (mSOD1) and the mouse N9 microglial cell line. Exosomes were isolated from the cell culture medium by differential ultracentrifugation and incubated with microglia for 2, 4 and 24 hours, or with the MN-microglia co-cultures for 24 hours. We assessed microglia phagocytic ability and senescence, nitric oxide (NO) production, matrix metalloproteinase (MMP)-2 and MMP-9 activity in the extracellular media, nuclear factor-kappa B (NF-κB) activation, and gene and microRNA expression by quantitative Real-Time PCR. We observed that the overexpression of microRNA (miR)-124 in mSOD1 MNs was reproduced in their derived exosomes. Such exosomes led to a loss of microglia phagocytic ability, acute release of NO, MMP-2 and MMP-9, and interleukin (IL)-1β and tumor necrosis factor (TNF)-α expression, together with lasting NF-κB activation and delayed increase of senescent-like cells. Interestingly, the early decrease in miR-124 and miR-146a expression induced by both types of exosomes was followed by their increase after 24 hours of incubation with the mSOD1 MN-derived exosomes, where enhanced miR-155 expression was similarly observed. Finally, we observed that the distribution of exosomes was preferentially towards microglia than to MNs, in the co-culture system. Preliminary data also suggest that mSOD1-associated exosomes increase the microglial expression of IL-1β and TNF-α, together with that of alarmin HMGB1. However, further studies are needed to confirm and assess the relevance of these pilot results. Overall, data highlight exosomes from mSOD1 MNs as inducers of microglia activation and dysfunction, different microglia subsets and inflammatory mediators’ production.

Keywords: exosomes; amyotrophic lateral sclerosis; microglia activation/dysfunction; neuroinflammation-associated mediators; microRNA profiling.
Resumo

Exossomas são nano-vesículas extracelulares (30-100 nm) formadas por praticamente todos os tipos de células e que derivam da via endocítica e corpos multivesiculares intracelulares. Estas vesículas são libertadas aquando da fusão do corpo multivesicular com a membrana plasmática das células. Os exossomas mediam a comunicação intercelular e têm um papel importante na disseminação de doenças neurodegenerativas, como possivelmente a Esclerose Lateral Amiotrófica (ELA). Recentemente foi proposto o envolvimento de exossomas provenientes das células motor neuron-like NSC-34, transfetadas com a superóxido dismutase 1 humana com mutação em G93A (mSOD1), na propagação da toxicidade desta proteína. Contudo, o efeito da incorporação destes exossomas pelas células receptoras, tal como as células de microglia, na sua ativação ou perda de função nunca foi investigado. No presente estudo, pretendeu-se avaliar um conjunto específico de marcadores e mediadores da resposta inflamatória, de forma a estabelecer: (i) o perfil de microRNAs pro- e anti-inflamatórios na linha celular NSC-34 e nos seus respetivos exossomas; (ii) alterações na funcionalidade das células de microglia, assim como os diferentes subtipos de polarização desencadeados pela exposição aos exossomas provenientes dos neurônios motores mSOD1; e (iii) a distribuição celular de exossomas marcados num sistema de co-cultura neurônios-microglia. Para isso, foram usadas células NSC-34 que expressam tanto a proteína SOD1 normal (wt) como mutada em G93A (mSOD1) e a linha celular de microglia N9. Os exossomas foram isolados do meio de cultura celular por ultracentrifugação diferencial e incubados com a microglia durante 2, 4 e 24 horas, ou nas co-culturas durante 24 horas. Os parâmetros avaliados foram a capacidade fagocítica da microglia e a sua senescência, produção de óxido nítrico (NO) e atividade das metaloproteinases (MMP)-2 e MMP-9 no meio extracelular, ativação do factor nuclear-kappa B (NF-κB), e expressão génica e de microRNAs por PCR quantitativo em tempo real. Nos neurônios motores mSOD1 foi observada a sobre-expressão do microRNA (miR)-124, que se reproduziu nos exossomas provenientes destas células. Estes exossomas induziram a perda da capacidade fagocítica da microglia e a sua senescência, produção de óxido nítrico (NO) e atividade das metaloproteinases (MMP)-2 e MMP-9 no meio extracelular, ativação do factor nuclear-kappa B (NF-κB), e expressão génica e de microRNAs por PCR quantitativo em tempo real. Nos neurônios motores mSOD1 foi observada a sobre-expressão do microRNA (miR)-124, que se reproduziu nos exossomas provenientes destas células. Estes exossomas induziram a perda da capacidade fagocítica da microglia, libertação aguda de NO, MMP-2 e MMP-9, e expressão da interleucina (IL)-1β e factor de necrose tumoral (TNF)-α, juntamente com a ativação prolongada do NF-κB e aumento tardio do número de células do tipo senescente. Curiosamente, a diminuição inicial na expressão do miR-124 e miR-146a induzida pelos dois tipos de exossomas foi seguida pelo seu aumento após 24 horas de incubação com os exossomas dos neurônios motores mSOD1, sendo igualmente observado o aumento da expressão do miR-155. Por fim, verificou-se que, quando em sistema de co-cultura, os exossomas são preferencialmente internalizados pelas células de microglia do que pelos neurônios motores. Dados preliminares sugerem ainda que os exossomas associados aos neurônios mSOD1 aumentam a expressão de IL-1β e TNF-α na microglia, juntamente com a alarmina HMGB1. No entanto, são necessários mais estudos que confirmem e atestem a
relevância destes resultados. Os resultados obtidos evidenciam os exossomas derivados dos neurónios motores mSOD1 como induutores de ativação e disfunção da microglia, assim como diferentes subconjuntos de microglia e produção de mediadores inflamatórios.

**Palavras-chave:** exossomas; esclerose lateral amiotrófica; ativação/disfunção da microglia; mediadores associados à neuroinflamação; perfil de microRNAs.
Agradecimentos

Como não poderia deixar de ser, o meu primeiro agradecimento é dirigido à Professora Doutora Dora Brites, por me ter recebido tão bem no seu grupo. Durante este ano tive a oportunidade de conhecer melhor, e de perto, o mundo da investigação e foi sem dúvida uma experiência muito enriquecedora, a todos os níveis. Muito obrigada por toda a ajuda, pelo apoio e incentivo ao longo deste trabalho!

De seguida, um muito obrigado a ti, Rita! Agradeço toda a orientação ao longo deste percurso e toda a disponibilidade que sempre demonstraste para me ajudar, mesmo quando eu não te queria incomodar com os meus “problemas”. Obrigada por tudo o que me ensinaste ao longo deste ano, por todas as correções e discussões de resultados, e ainda pelas palavras de incentivo para concluir esta etapa! Por tudo isto, e talvez por mais, obrigada.

Um agradecimento também aos Professores Rui, Adelaide, Sofia e Alexandra, e ainda à Cláudia, pela simpatia e boa disposição com que me receberam.

Carolina, isto já não deve ser novidade para ti, mas como é óbvio não podia deixar de te agradecer! Desde o início que fui acompanhando o teu trabalho e foi contigo que aprendi grande parte daquilo que sei hoje. Estiveste sempre lá para me ajudar quando algo corria menos bem, para oferecer soluções ou para esclarecer as minhas dúvidas existenciais. “Só problemas”! Sem dúvida que és a alma do grupo, com o teu empenho e boa disposição! Obrigada por me chamares tantas vezes a atenção para não falar tanto... Espero poder continuar a acompanhar o teu trabalho, que com certeza irá ser brilhante.

Ao resto das meninas que me acompanharam ao longo deste ano: Cátia, nunca me irei esquecer do dia em que me aplaudiste depois da minha cantoria, foste o meu melhor público até agora! Obrigada pela simpatia e por também tu estares sempre disponível para me ajudar. Martinha, vieste sem dúvida dar mais alegria à cave com a tua boa disposição! Obrigada pela tua ajuda ao longo deste trabalho, foste essencial para que conseguisse cumprir alguns dos objectivos. Foi um prazer trabalhar e discutir resultados contigo. Muito boa sorte para esta nova fase da tua vida! Gisela, vou ter saudades de te ver “implicar” com a Marta… Foi um prazer enorme conhecer-te, oxalá houvesse mais tempo! Ainda, um beijinho para todas as outras meninas (e menino!) com que me fui cruzando na cave, nem que por pouco tempo: Maria, Carla, Mafalda e Filipe.

Agora, um agradecimento hiper mega especial à Margarida e à Tânia! Vocês partilharam comigo esta nova fase desde o início e foram quem mais ouviu os meus desabafos, frustrações, piadas secas e conversas sem sentido. Não dá para transmitir por palavras tudo aquilo que tenho para vos agradecer, mas fica só a ideia de que sem vocês isto não teria metade da piada!
Por fim, obrigada à minha família e amigos! Ao Luís, mesmo não percebendo nada desta tese, sempre demonstraste interesse em saber aquilo que andava a fazer e a descobrir. Que me continues a aturar, a mim e às minhas células, por muito mais tempo! Às minhas “bestis”, um obrigado não chega, já são muitos anos a virar frangos! Aos meus “putxis”, Laura e Tiago, os melhores manos que podia ter! E por fim, o mais importante, aos meus pais, sem os quais nada disto seria possível. Obrigada por todos os dias me incentivarem a ser uma pessoa melhor, tanto a nível pessoal como profissional. Aprendo muito com vocês todos os dias e espero que também tenham aprendido alguma coisa aqui com a jovem! Muito obrigada, do fundo do coração!
1.3. Antibodies.......................................................................................................................35
1.4. Equipment..........................................................................................................................36
1.5. Cell lines ..........................................................................................................................36
2. Methods ..............................................................................................................................37
2.1. Cell lines ..........................................................................................................................37
2.1.1. NSC-34 cell line.........................................................................................................37
2.1.2. N9 cell line ................................................................................................................37
2.2. Cell treatments................................................................................................................37
2.2.1. NSC-34 cell line.........................................................................................................37
2.2.2. N9 cell line and incubation with exosomes from NSC-34 cells.....................................37
2.2.3. NSC-34-N9 co-cultures ..............................................................................................38
2.3. Differential ultracentrifugation..........................................................................................39
2.4. Labelling with PKH67 fluorescent probe .........................................................................40
2.5. Determinations ...............................................................................................................41
2.5.1. Microglial phagocytosis .............................................................................................41
2.5.2. Microglial senescence ...............................................................................................41
2.5.3. NF-κB activation ........................................................................................................42
2.5.4. Extracellular nitric oxide ............................................................................................43
2.5.5. Gelatin zymography ..................................................................................................43
2.5.6. Quantitative Real-Time PCR .....................................................................................43
2.5.7. Exosome distribution ................................................................................................45
2.6. Statistical analysis ..........................................................................................................45

III. RESULTS ...............................................................................................................................47
1. NSC-34 inflamma-miR profiling indicates that only miR-124 is upregulated in mSOD1 MNs, which is recapitulated in cell-derived exosomes .................................................................................47
2. Exosomes from mSOD1 MNs induce loss of microglia phagocytic ability and increase the number of senescent cells ..............................................................................................................48
3. Evaluation of microglial inflammatory response to exosomes released by wt MNs and mSOD1 MNs .............................................................................................................................................51
3.1. Exosomes from mSOD1 MNs lead to an early microglial production of inflammatory mediators ..........................................................................................................................51
3.2. Microglia increasingly release pro-inflammatory cytokines upon interaction with exosomes from mSOD1 MNs .................................................................................................................52
3.3. Exosomes from mSOD1 MNs trigger a delayed upregulation of inflammatory miRNAs ..................................................................................................................................................55
4. Exosomes from MN-microglia donors preferentially distribute in microglia when both recipient cells are considered ..................................................................................................................56
Figure Index

I. INTRODUCTION
   Figure I. 1 – Biological characteristics of exosomes: biogenesis, general composition and cargo ................................................................. 6
   Figure I. 2 – Exosomal uptake by recipient cells occurs by different mechanisms .......... 9
   Figure I. 3 – Involvement of exosomes in interneuronal communication .......................12
   Figure I. 4 – ALS is a neurodegenerative disease, resulting from the degeneration of UMN and/or LMNs ......................................................... 16
   Figure I. 5 – Role of glial cells and exosomes in the progression and dissemination of ALS ............................................................................... 22
   Figure I. 6 – Inflammatory response of microglia cells in the presence of mutant SOD1 ....25
   Figure I. 7 – Microglia assume different phenotypes during ALS disease progression .......27
   Figure I. 8 – Therapeutic uses of exosomes in neurological diseases ..............................32
   Figure I. 9 – Schematic representation of the specific aims of the present thesis ............34

II. MATERIALS AND METHODS
   Figure II. 1 – Schematic representation of the experimental model for the isolation of exosomes and incubation in the recipient cells ................................................. 39
   Figure II. 2 – Schematic procedure for the isolation of exosomes from the extracellular media .................................................................................. 40

III. RESULTS
   Figure III. 1 – Exosomes from mSOD1 MNs contain an increased expression of microRNA (miR)-124, reflecting their cells of origin ...................................................... 47
   Figure III. 2 – Exosomes from mSOD1 MNs produce a lasting decrease in the microglia phagocytic ability .................................................................................. 49
   Figure III. 3 – Microglia exposed to exosomes from mSOD1 MNs display signs of dysfunction, with increased SA-β-gal activity and dystrophic features ....................... 50
   Figure III. 4 – Microglia exposure to exosomes from mSOD1 MNs lead to an immediate microglial release of NO, MMP-9 and MMP-2 .................................................. 52
   Figure III. 5 – NF-κB is activated in the microglia exposed to exosomes from mSOD1 MNs, leading to increased expression of TNF-α and IL-1β ........................................... 54
   Figure III. 6 – Microglial HMGB1 expression is maintained unchanged upon incubation with exosomes from wt and mSOD1 MNs ..................................................... 54
   Figure III. 7 – Expression of microRNA (miR)-124, miR-146a and miR-155 in microglia cells after 24 hours of incubation indicate the acquisition of several microglia phenotypes .............................................................................. 56
   Figure III. 8 – Exosomes released from motor neurons (MN) and microglia are preferentially sorted in microglia that in MNs ................................................................. 57

IV. DISCUSSION
   Figure IV. 1 – Schematic representation of the major findings in this thesis .................. 66
Table Index

II. MATERIALS AND METHODS
   Table II. 1 – List of primer sequences used in qRT-PCR..................................................44

III. RESULT
   Table III. 1 – Expression of TNF-α, IL-1β and HMGB1 in microglia co-cultured with wt and mSOD1 motor neurons (MNs), in the presence and absence of added exosomes isolated from microglia-MN co-cultures (either wt or mSOD1)........................................58
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CD14</td>
<td>Cluster of differentiation 14</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CX₃CL1</td>
<td>Fractalkine</td>
</tr>
<tr>
<td>DIV</td>
<td>Days in vitro</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>EAAT</td>
<td>Excitatory amino acid transporter</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ESCRT</td>
<td>Endosomal Sorting Complexes Required for Transport</td>
</tr>
<tr>
<td>EVs</td>
<td>Extracellular vesicles</td>
</tr>
<tr>
<td>fALS</td>
<td>Familial Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>G418</td>
<td>Geneticin 418 sulfate</td>
</tr>
<tr>
<td>GluR2</td>
<td>Glutamate receptor 2</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High-mobility group box 1</td>
</tr>
<tr>
<td>Hsp70</td>
<td>Heat shock protein 70</td>
</tr>
<tr>
<td>Iba1</td>
<td>Ionized calcium-binding adaptor molecule 1</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ILV</td>
<td>Intraluminal vesicle</td>
</tr>
<tr>
<td>Inflamma-miRs</td>
<td>Inflammatory microRNAs</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MFG-E8</td>
<td>Milk-fat globule EGF factor-8</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>miRNAs</td>
<td>MicroRNAs</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MN</td>
<td>Motor neuron</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNAs</td>
</tr>
<tr>
<td>mSOD1 MNs</td>
<td>Mutant SOD1 motor neurons (NSC-34/hSOD1&lt;sup&gt;G93A&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MVB</td>
<td>Multivesicular body</td>
</tr>
<tr>
<td>NEAA</td>
<td>Nonessential amino acids</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffer saline</td>
</tr>
<tr>
<td>PDL</td>
<td>Poly-D-Lysine</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>Penicillin/Streptomycin</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Real-Time PCR</td>
</tr>
<tr>
<td>Rab</td>
<td>Ras-related in brain</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>sALS</td>
<td>Sporadic Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>SA-β-gal</td>
<td>Senescence-associated β-galactosidase</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SOD1</td>
<td>Superoxide dismutase 1</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>wt MNs</td>
<td>Wild-type motor neurons (NSC-34/hSOD1&lt;sup&gt;wt&lt;/sup&gt;)</td>
</tr>
</tbody>
</table>
1. Extracellular Vesicles and Exosomes – Role in Homeostasis and Cellular Dysfunction

Almost all living cells release extracellular vesicles (EVs) that facilitate and modulate intercellular communication by carrying proteins, lipids and nucleic acids. Increasing evidences suggest that soluble factors and EVs within the secretome provide a major contribution to paracrine activity, generating a tissue microenvironment that may be either beneficial or neurotoxic.

The release of membrane-enclosed vesicles from cells was described more than 40 years ago. Originally, these EVs were thought to bud directly from the plasma membrane (Crawford, 1971). However, in 1985, a research using transmission electron microscopy (TEM) showed that, during maturation of reticulocytes to erythrocytes, small vesicles were formed by inward budding inside an intracellular endosome, leading to the formation of a multivesicular body and to vesicle sorting (Pan et al., 1985). In 1987, the word “exosome” was proposed for these EVs and they were first isolated from immature sheep reticulocytes (Johnstone et al., 1987). Later, in 1989, it was established that such vesicles had biological value, helping maturing erythrocytes get rid of transferrin receptors and other unnecessary proteins (Johnstone et al., 1989).

Nowadays, EVs are categorized into exosomes and ectosomes (also called microvesicles or shed vesicles), based on their mechanisms of biogenesis and biophysical properties, such as size and surface protein markers. Exosomes are homogenous small particles, with 30-100 nm in size and a cup-shaped appearance by TEM, and are derived from the endocytic recycling pathway. Consequently, exosomes contain specific endosomal proteins that are often used as exosomal markers. Ectosomes constitute a more heterogeneous population of EVs, with 100-1000 nm in size. They are produced directly through the outward budding and fission of membrane vesicles from the plasma membrane, so their surface markers are largely dependent on the composition of the membrane of origin (Lee et al., 2012b; Kastelowitz and Yin, 2014). Despite their differences, the functions of ectosomes are largely analogous to those of exosomes (Cocucci and Meldolesi, 2015). Along the years, accumulating evidence shows that several cell types have the capacity to secrete EVs, including neuronal and glial cells. From now on, we will be focused on exosomes in this Introduction Section, since isolation method and experimental work indicated in the present work only considered exosomes.
1.1. Biogenesis of Exosomes

Through exocytosis, cells deliver newly synthetized proteins and/or lipids to either the plasma membrane or the extracellular space. By the opposite process of endocytosis, cells remove plasma membrane components and deliver them to internal compartments called endosomes, from where they can be recycled to the plasma membrane or delivered to lysosomes for degradation (Alberts et al., 2008). These processes complement each other, maintaining homeostasis in the cell membrane.

The endosomal system consists of primary endocytic vesicles, early endosomes and late endosomes, also called multivesicular bodies (MVBs). Early endosomes are located near the plasma membrane where they contact with the primary endocytosed vesicles, resulting in the transfer of cargo and addition of membrane. Early endosomes then mature into late endosomes (or MVBs) and during this process they accumulate intraluminal vesicles (ILVs) in their lumen. This is possible by invagination of the MVB membrane, which creates a membrane enclosed compartment in which the lumen is equivalent to the cytoplasm of the cell. The ILVs that are formed sequester proteins, lipids and nucleic acids that are specifically sorted. Interestingly, the fact that two membrane inversions occur during the formation of an exosome, one during the endocytic internalization and other during the formation of the ILVs, allows exosomes to contain transmembrane cargo in the same orientation relative to the plasma membrane (Klumperman and Raposo, 2014). When the MVB is formed, it can have several fates: (i) degradation; (ii) recycling; or (iii) exocytosis. Degradation occurs via fusion of the MVB with lysosomes, allowing the cell to remove excessive membrane and unnecessary proteins. MVBs can also traffic to the Golgi for redistribution and recycling of their cargo, or fuse with the plasma membrane, leading to release of ILVs into the extracellular environment as exosomes (Figure I.1). Consequently, when released, exosomes can communicate with the surrounding cells, having a role in several physiological processes. However, in pathological conditions, exosomes can carry toxic forms of aggregated proteins that would be targeted for degradation, under normal conditions, contributing to the spreading and progression of neurodegenerative diseases, such as Amyotrophic Lateral Sclerosis (ALS).

1.1.1. Mechanisms of Biogenesis

At the limiting membrane of MVBs, some mechanisms act jointly to allow vesicular formation and cargo sorting. The main mechanism of biogenesis requires the Endosomal Sorting Complexes Required for Transport (ESCRT) machinery (Kowal et al., 2014). These protein complexes are transiently recruited from the cytoplasm to the endosomal membrane, where they function sequentially in the sorting of transmembrane proteins and in the
formation of ILVs. Many studies use ESCRT inhibition as a tool to inhibit secretion of exosomes. However, MVBs and ILVs can also form in the absence of ESCRT machinery. This was demonstrated by the inactivation of four proteins of the four different ESCRT complexes, in mammalian cells, which still allowed the MVB formation (Stuffers et al., 2009). Ceramide and phosphatidic acid, two lipids present in the limiting membrane of MVBs, induce inward curvature of MVBs and thus formation of ILVs (Trajkovic et al., 2008; Ghossoub et al., 2014).

After sorting and budding of ILVs, MVBs are transported to the plasma membrane, followed by docking and fusion of the lipid bilayers, so that exosomes can be released into the extracellular space. Several components of the endocytic machinery are involved in secretion of exosomes, such as Rab GTPases (Ras-related in brain), cytoskeleton regulatory proteins and SNAREs (soluble NSF-attachment protein receptor) (Théry et al., 2001; Fader et al., 2009; Colombo et al., 2014).

1.2. Characteristics of Exosomes

The composition and content of exosomes depends on the cell of origin, but proteins, lipids and nucleic acids are the main components, with some of them considered as hallmarks of the ILVs due to their endosomal origin. In the past few years, numerous studies have reported changes in protein and lipid composition, as well as in RNA content, of secreted vesicles induced by different extracellular environments or different physiological states of the secreting cells. Hypoxia, inflammation and cancer were some of the conditions studied (de Jong et al., 2012; Tauro et al., 2013). These variations between normal and diseased cells, depending on cell context, have garnered much interest in exosomes as disease biomarkers and may prove vital to the understanding of the involvement of exosomes in pathological disorders.

1.2.1. Membrane Composition

The exosomal membrane is mainly composed by transmembrane proteins and lipids (Figure I.1).

Proteomic studies revealed that exosomes contain a specific subset of proteins, some of which depend on the donor cell type, whereas others are from endosomes, the plasma membrane and the cytosol. Proteins from other intracellular organelles, like nucleus, mitochondria, endoplasmic reticulum (ER) and Golgi complex, are minimal (Théry et al., 2001). Exosomes expose at its surface the extracellular domain of transmembrane proteins. Surface transmembrane proteins serve as exosomal markers and include major histocompatibility complex (MHC) class I and II molecules, adhesion molecules (such as
integrins), several members of tetraspanins (CD9, CD63, CD81 and CD82) and lipid raft-associated proteins (including flotillin-1) (Théry et al., 2001; Wubbolts et al., 2003; Subra et al., 2010; Baietti et al., 2012). Exosomes also contain proteins that are involved in specific cell functions, probably addressing exosomes to target cells, through receptor-ligand interactions (Segura et al., 2005; Purushothaman et al., 2016). Moreover, the presence of proteins involved in all major neuropathological hallmarks of neurodegenerative diseases, such as amyloid-β for Alzheimer’s disease, α-synuclein for Parkinson’s disease, and Cu/Zn superoxide dismutase 1 (SOD1) for ALS, suggests the involvement of exosomes in dissemination of these diseases, as discussed in Section 1.3.2. Most interesting, if exposed at the surface, these proteins may be recognized by therapeutic agents.

Besides proteins, exosomes contain abundant phospholipids composing their limiting membrane. Although the published works do not provide a completely unified view of this composition, in a general way exosomes contain lipid rafts enriched in cholesterol, glycosphingolipids, sphingomyelin and ceramide, together with phosphatidylserine, a lipid usually present at the cytosolic side of the plasma membrane (Wubbolts et al., 2003; Laulagnier et al., 2004; Trajkovic et al., 2008; Llorente et al., 2013). It has been reported that different lipid compositions are directly related to rigidity and delivery efficiency of exosomes to other cells, hence contributing to the diffusion of certain diseases (Parolini et al., 2009). Exosomes can also have saccharide groups on their outer surface. It was observed that exosomes are enriched in mannose, poly lactosamine, α-2,6 sialic acid and complex N-linked glycans, which indicates that glycosylation has a role in protein sorting (Batista et al., 2011).

1.2.2. Cargo of Exosomes

Another interesting feature of exosomes is their cargo and how it can modulate the recipient cells. Exosomes are known to contain within the cytosolic lumen various types of cytosolic proteins and nucleic acids (Figure I.1).

It is believed that proteins are target for entry into ILVs by ubiquitination of their cytosolic domains and are recognized by the ESCRT machinery (Katzmann et al., 2001). Cytosolic proteins include cytoskeletal proteins, like tubulin and actin, annexins and Rab proteins, all participating in intracellular transport and membrane fusion, as well as molecules involved in signal transduction, such as syntenin, and heat shock proteins, such as Hsc70. Exosomes may likewise contain specific proteins involved in the MVB biogenesis, including Alix and Tsg101 (Théry et al., 2001; Wubbolts et al., 2003; Subra et al., 2010).

Regarding nucleic acids, exosomes have been reported to contain significant amounts of messenger RNAs (mRNA) and small RNAs, including microRNAs (miRNAs). The RNA species were termed “exosomal shuttle RNA” for their properties of being delivered to
another cells and altering their gene expression and protein profiles (Valadi et al., 2007; Iguchi et al., 2010; Montecalvo et al., 2012). Interestingly, many of the mRNA found was not present in the cytoplasm of the donor cell, suggesting a preferential sorting of certain mRNAs to exosomes (Valadi et al., 2007). Concerning small RNA species, the nature of miRNAs present in exosomes is not entirely clear. However, since exosomes fuse and release their miRNA content in recipient cells and repress target mRNAs, inhibiting their expression, it is believed that these miRNAs play a functional role in intercellular communication. For instance, in normal conditions, exosomal miR-124a is transferred from neurons to astrocytes and regulates the levels of the excitatory amino acid transporter 2 (EAAT2), involved in synaptic modulation. In the spinal cord of end-stage SOD1\textsuperscript{G93A} mice, the mouse model of ALS, miR-124a is selectively reduced and, probably because it will be reduced in the released exosomes, it may determine the pathological loss of EAAT2 and neuronal excitotoxicity demonstrated to be associated (Morel et al., 2013). The mechanism of miRNA sorting is not yet clear, but it also seems to be preferential for some of them. This sorting may be controlled by specific RNA-binding proteins that deliver miRNAs into the MVB. Recently, it was described the involvement of one particular protein, the protein heterogeneous nuclear riboprotein A2B1 (hnRNPA2B1) (Villarroya-Beltri et al., 2013). This protein recognizes and binds specific RNA motifs in miRNAs, called EXOmotifs, and controls their sorting into exosomes when sumoylated, pointing to sumoylation as controlling the binding of hnRNPA2B1 to miRNAs.

Other non-coding RNAs were also found enriched in exosomes, relative to its cellular content, including vault-RNA, $\gamma$-RNA and specific transfer RNAs (Nolte'T Hoen et al., 2012). Moreover, it was also reported that exosomes released from astrocytes and glioblastoma cells contained mitochondrial DNA (Guescini et al., 2010).
Figure I. 1 – Biological characteristics of exosomes: biogenesis, general composition and cargo. Extracellular vesicles are composed by ectosomes and exosomes. Ectosomes are produced directly through the outward budding and fission of the plasma membrane. Exosomes are derived from the endocytic recycling pathway and are formed by the inward budding of the multivesicular body (MVB) membrane, originating the intraluminal vesicles (ILVs) in their luminal space. After being formed, MVBs can be (i) degraded via lysosomes, (ii) recycled to the Golgi network or (iii) released into the extracellular space as exosomes. Exosomal membrane is mainly composed by transmembrane proteins and lipid rafts, in the same orientation as the cell membrane, and the exosomal lumen is equivalent to cytoplasm. Surface transmembrane proteins serve as exosomal markers and include major histocompatibility complex (MHC) class I and II molecules, adhesion molecules, lipid raft-associated proteins and several members of tetraspanins. In a general way, lipid rafts are enriched in cholesterol, glycosphingolipids, sphingomyelin and ceramide. Furthermore, exosomes contain inside various types of cytosolic proteins and nucleic acids. Cytosolic proteins include cytoskeletal proteins, proteins involved in intracellular transport and membrane fusion, molecules that participate in signal transduction, heat shock proteins, as well as proteins implicated in MVB biogenesis. Nucleic acids already identified in exosomes are messenger RNAs (mRNA) and microRNAs (miRNAs). Other small non-coding RNAs found in exosomes are vault-RNA, γ-RNA and specific transfer RNAs.
1.2.3. Isolation Methods

One major ongoing challenge in the isolation of exosomes is the lack of methods that allow its isolation with high purity and the precise characterization of exosome populations. Accurate discrimination between exosomes and microvesicles is also a challenge.

Exosomes have been successfully purified from cell culture conditioned medium or body fluids. The original and most commonly used protocol for exosome purification involves differential ultracentrifugation (Raposo et al., 1996), which sequentially pellets cells, microvesicles and exosomes. Several variants of this method are used nowadays, in which the first centrifugation steps are replaced by a single filtration step (Théry et al., 2006). Other slightly modified versions of this protocol were designed for purifying exosomes from body fluids. Because of the viscosity of some fluids, it is necessary to dilute them and to increase the speed and length of centrifugation (Caby et al., 2005; Théry et al., 2006). A different purification procedure has also been described, using ultrafiltration instead of ultracentrifugation that is especially useful for purifying exosomes from large volumes of conditioned medium (Lamparski et al., 2002). When the goal is to isolate only a subpopulation of marker-positive exosomes, beads coated with antibodies specific for exosomal surface molecules can be used (Clayton et al., 2001; Caby et al., 2005). Furthermore, an extra purification step, after obtaining the pellet of exosomes, may be through a sucrose cushion. This step eliminates most of contaminants, such as proteins non-specifically associated with exosomes or large protein aggregates, which are sedimented by centrifugation but do not float on a sucrose gradient (Théry et al., 2006). In addition to these traditional isolation techniques, in the last few years several kits have been commercialized, such as ExoQuick™ and Total Exosome Isolation™.

Recently, Van Deun and colleagues (2014) have compared different types of exosome isolation protocols and showed that both differential ultracentrifugation and density gradient centrifugation originates the purest exosome preparations, which are enriched in exosomal marker proteins, when compared with commercial kits. Most importantly, it was shown that differential ultracentrifugation results in a high yield of protein and RNA, thus making it a suitable method for exosome isolation. However, it must be taken into account that, after obtaining the isolated EVs, they have to be characterized to confirm the specific presence of exosomes (Arroyo et al., 2011; Van Deun et al., 2014). Further characterization requires at least two different techniques, including complementary biochemical, mass spectrometry and imaging techniques. Exosomes can be examined using dynamic light scattering analysis (DLS) or TEM (Raposo et al., 1996; Jella et al., 2014). To visualize specific markers, exosomes can be previously immunolabelled with antibodies against proteins known to be exposed on exosomal membranes (van Niel et al., 2001). Also,
quantification of exosomes can be made by nanoparticle tracking analysis (NTA), which allows determining the mean size of the population of vesicles analyzed and the particle concentration estimation in the sample (Soo et al., 2012). Furthermore, to characterize the purified vesicles as exosomes, it is important to show that most of the common exosomal proteins (as well as more specific proteins) are present, which can be accomplish by immunoblotting.

1.3. Biological Functions of Exosomes

Nowadays, it is known that exosomes are involved in normal homeostasis, as well as in pathological conditions, serving as mediators of cell-to-cell communication. Cells utilize exosomes to dispose of unwanted proteins or to exchange signals with neighboring cells. Once released from a cell, exosomes are internalized into recipient cells, transferring exosomal molecules from one cell to another. Exosomes transfer both proteins and functional RNA species, resulting in alterations in the recipient cell (Valadi et al., 2007; Skog et al., 2008). For instance, exosome-mediated small interfering RNA (siRNA) delivery has been shown to knockdown target gene expression (Alvarex-Erviti et al., 2011a), and administration of exosomes loaded with luciferin substrate to luciferase-expressing cells results in production of bioluminescence (Montecalvo et al., 2012). Furthermore, exosomal uptake has been visualized directly, by using fluorescent lipid membrane dyes to stain EV membranes, such as PKH67 (Fitzner et al., 2011).

Various mechanisms for exosomal uptake have been proposed, many involving exosomal protein interactions with the plasma membrane of target cells that facilitate subsequent endocytosis (Christianson et al., 2013). Exosomes that bind to cell surface receptors lead to the endocytic uptake of the vesicle, but direct fusion of exosomes with the plasma membrane is also possible (Parolini et al., 2009) (Figure I.2). Several studies propose endocytosis as the primary method of exosome uptake and visualization of exosomes inside cells occur within 15 minutes after initial introduction (Feng et al., 2010). Endocytosis can occur in many ways, including phagocytosis (Feng et al., 2010), macropinocytosis (Fitzner et al., 2011; Tian et al., 2014), clathrin-, caveolin- and lipid raft-mediated endocytosis (Escrevente et al., 2011; Nanbo et al., 2013; Svensson et al., 2013; Tian et al., 2014), however there seems to be little agreement in the literature as to which mechanisms are most important. It is possible that, due to their heterogeneity, a population of exosomes can simultaneously trigger different gateways into a cell, depending on the cell type and its constituents. Recently, it has also been proposed that exosomes do not need to be internalized to lead to a phenotypic response. Receptor–ligand interactions between
Chapter I. Introduction

Exosomes and the cell surface may be sufficient to permit signal transduction and subsequent downstream signaling effects in the recipient cell (Raimondo et al., 2015).

Figure I.2 – Exosomal uptake by recipient cells occurs by different mechanisms. When released from cells, exosomes can bind to neighboring cells, transferring exosomal molecules from one cell to another. They transfer both proteins and functional RNA species, producing changes in the recipient cell. The adhesion of exosomes to the recipient cell utilizes the interaction of various exosomal surface proteins and cellular receptors. Once bound, the exosome may fuse with the cellular membrane and transfer its content into the cytoplasm of the recipient cell (1), be endocytosed by phagocytosis, macropinocytosis, and by clathrin-, caveolin- and lipid raft-facilitation (2), or may elicit signal transduction via intracellular signaling cascades through ligand-receptor interaction (3). After being endocytosed, exosomes can fuse with the membrane of endosomes and release their content in the recipient cell, or remain in the endosome to be recycled/degraded.

1.3.1. Exosomes in Interneuronal Communication

Initially, exosomes were thought to be a mechanism for cells to remove unwanted proteins however, they also appear to have a function in intercellular communication. Many studies have reported the release of EVs (ectosomes and exosomes) by all cells that comprise the central nervous system (CNS): neurons (Fauré et al., 2006), oligodendrocytes (Krämer-Albers et al., 2007), astrocytes (Fauré et al., 2006; Taylor et al., 2007) and microglia (Potolicchio et al., 2005). It is proposed that exosomes are linked to a number of different biological processes in the CNS, such as synaptic plasticity, neuroprotection, metabolic support and regulation of myelin membrane biogenesis (Figure I.3).

Exosomes secreted by neurons have been implicated in synaptic plasticity. These can be incorporated into neurons or into surrounding glial cells however, the machinery for exosome targeting remains unclear. In neurons, MVBs are more abundant in soma and dendrites, being noted an association of MVBs with postsynaptic sites (Cooney et al., 2002),
and the secretion of exosomes is regulated by glutamatergic synaptic activity. In the cortex and hippocampus, calcium (Ca\textsuperscript{2+}) entry through ionotropic glutamate receptors, such as N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, activates the MVB fusion to the plasma membrane and, thereby, exosome secretion. Recently, Chivet and colleagues (2014) demonstrated that exosomes released upon synaptic activation bind specifically to other neurons, and not to glial cells. The secreted exosomes can carry AMPA glutamate receptor 2 (GluR2) subunits as a way of regulating the amount of these postsynaptic receptors and, consequently, adjusting the strength of neuron's excitatory synapses, maintaining homeostasis (Lachenal et al., 2011). Synaptic plasticity can also be achieved by the delivery of specific proteins and miRNAs via exosomes. This was observed for synaptotagmin 4, a membrane-trafficking protein, that when released by presynaptic terminals and transmitted to postsynaptic cells, activates retrograde signaling and synaptic growth (Korkut et al., 2010), and also for miR-124a that, as previously mentioned, is released by neurons and can modulate the expression of EAAT2 in neighboring astrocytes, consequently controlling the glutamate uptake levels (Morel et al., 2013). Furthermore, neuron-derived exosomes can regulate neuritic elimination through microglial activation. It was found that neurite removal is accelerated when microglial cells are pre-incubated with exosomes, by upregulating complement factors in microglia (Bahrini et al., 2015).

Besides the transfer of exosomes from neuron to neuron (or to other cells), glial cells can also release exosomes that are internalized by neurons. It is proposed a bidirectional communication between neurons and oligodendrocytes. Release of the neurotransmitter glutamate by neurons triggers oligodendroglial exosome secretion into the periaxonal space, through NMDA and AMPA receptors on the surface of oligodendrocytes (Frühbeis et al., 2013), whereas these exosomes, containing myelin and stress-protective proteins, help maintaining axonal integrity and improve cellular viability under conditions of cell stress, such as oxygen/nutrient deprivation (Krämer-Albers et al., 2007; Fröhlich et al., 2014). Neurons internalize oligodendroglial exosomes by endocytosis and utilize their cargo. Furthermore, oligodendrocyte-derived exosomes can communicate with other cells in the brain. It has been shown that oligodendroglial exosomes inhibit differentiation of oligodendrocytes and control myelin formation, in an autocrine way (Bakhti et al., 2011), and are taken-up by microglia, by macropinocytosis, which confirms the role of these cells in removing debris (Fitzner et al., 2011). Interestingly, such exosomes do not provoke an inflammatory response by microglia, since they are preferentially taken-up by microglia that do not have antigen-presenting capacity. In the peripheral nervous system (PNS), Schwann cells also communicate with neurons, contributing to axonal regeneration. After nerve damage,
Schwann cells dedifferentiate and proliferate, remove myelin and axonal debris, and support axonal regeneration (Chen et al., 2005). On the other hand, regenerating axons express adhesion molecules that promote Schwann cells alignment and migration (Yamauchi et al., 2008). A recent study demonstrates that Schwann cells secrete exosomes that can be selectively internalized by axons, increasing axonal regeneration. These exosomes carry on their surface the p75-neurotrophin receptor, a protein expressed by dedifferentiated Schwann cells (Lopez-Verrilli et al., 2013).

Astrocytes can also mediate neuroprotection through exosomes. In response to oxidative and heat stress, astrocytes secrete elevated levels of heat shock protein 70 (Hsp70) and synapsin I, a synaptic vesicle-associated protein implicated in neural development, in association with exosomes. This is associated with an increase in the survivability of neighboring neurons during injury (Taylor et al., 2007; Wang et al., 2011). Interestingly, astrocyte-derived exosomes have been reported to contain mitochondrial DNA, which can have some relevance in diseases involving mitochondrial alterations (Guascini et al., 2010). Furthermore, an important role of astrocytes in the CNS is the scavenging of extracellular glutamate through EAATs. These transporters have been reported in EVs, such as exosomes, suggesting a possible role in reducing excitotoxicity at an extracellular level (Gosselin et al., 2013).

Concerning microglia-derived exosomes, they have been proposed to provide metabolic support to neurons. Early studies demonstrated that microglia secrete exosomes containing the expected exosomal proteins, as well as a set of proteins previously reported for B cell- and dendritic cell-derived exosomes. They also carry the surface-bound aminopeptidase N (CD13) and the monocarboxylate transporter 1 (MCT1), as well as metabolically active enzymes and chaperones. Exosomal CD13 degrades enkephalins, enabling the bind of the ligands to the opioid receptor and thus influencing neuronal cyclic adenosine monophosphate (cAMP) levels. The presence of the lactate transporter MCT1, together with glycolytic enzymes, may attest their role in delivering energy substrates to neurons (Potolicchio et al., 2005). Recently, it has been suggested that release of serotonin from neurons induces secretion of microglia-derived exosomes. This suggests a neurotransmitter-dependent signaling pathway in microglial cells that regulates exosome release (Glebov et al., 2015).

Moreover, recent evidences suggest an exosome-mediated inflammasome signaling. When there is a trauma in the CNS, inflammasome is activated with secretion of exosomes containing inflammasome protein cargo into the cerebrospinal fluid (CSF). These exosomes then fuse with target cells to activate the innate immune response in peripheral tissues (De Rivero Vaccari et al., 2016). It is believed that exosomes trigger toll-like receptors (TLRs) in
monocytic cells, but possibly also in other immune cells, and induce the nuclear factor-kappa B (NF-κB) activation and release of cytokines (Bretz et al., 2013; Chow et al., 2014). Furthermore, dendritic cell-derived exosomes increase inflammation through the NF-κB pathway in a similar way to that of lipopolysaccharide (LPS) (Gao et al., 2016). Exosomes containing Hsp70, for instance astrocyte-derived exosomes, can also lead to NF-κB activation and tumor necrosis factor (TNF)-α release in macrophages (Anand et al., 2010).

Figure 1.3 – Involvement of exosomes in interneuronal communication. Many studies have reported the release of exosomes by neurons, oligodendrocytes, astrocytes and microglia. It is proposed that these exosomes are linked to a number of different biological processes in the central nervous system, such as synaptic plasticity, neuroprotection and metabolic support. Neuron-derived exosomes can carry AMPA glutamate receptor 2 (GluR2) subunits as a way of adjusting the strength of neuron’s excitatory synapses, synaptotagmin 4 (Syt4), a membrane-trafficking protein that, when released by presynaptic terminals and transmitted to postsynaptic cells, activates retrograde signaling and synaptic growth, and the microRNA(miR)-124a that modulates the expression of excitatory amino acid transporters (EAATs) in neighboring astrocytes. Glial cells can also release exosomes that are internalized by neurons. Oligodendrocyte-derived exosomes contain myelin and stress-protective proteins that help maintaining axonal integrity and improve cellular viability under conditions of cell stress. These exosomes can also be taken-up by microglia, which indicates the role of these cells in removing debris. Astrocytes mediate neuroprotection through exosomes containing elevated levels of the heat shock protein 70 (Hsp70) and synapsin I, a synaptic vesicle-associated protein implicated in neural development. Interestingly, astrocyte-derived exosomes have been also reported to contain mitochondrial DNA (mtDNA) and EAATs. Concerning microglia-derived exosomes, they have been proposed to provide metabolic support to neurons. Microglia secrete exosomes carrying the surface-bound aminopeptidase N (CD13) and the monocarboxylate transporter 1 (MCT1), as well as metabolically active enzymes and chaperones. In the peripheral nervous system, Schwann cells also communicate with neurons through exosomes, contributing to axonal regeneration. Schwann cells secrete exosomes that carry on their surface the p75-neurotrophin receptor (p75NTR), a protein expressed by dedifferentiated Schwann cells.
1.3.2. Exosomes in Disease Spread

It has been shown that part of the physiological role of exosomes is their ability to influence other cells through their protein and RNA-based cargo, though this also happens in pathological conditions. In fact, many studies have noted an increased production of EVs in diseased states when compared with non-diseased ones, such as in cancer and senescence (Parolini et al., 2009; Lehmann et al., 2012), as well as alterations in their composition and cargo (de Jong et al., 2012; Tauro et al., 2013). These findings suggest that these vesicles can contribute to the spreading of the disease in pathological conditions.

One mechanism associated to disease spreading involves the transfer of miRNAs and pathogenic proteins by exosomes, an issue highly explored in oncology. For example, glioblastoma-derived exosomes contain elevated levels of miR-21 and oncogenic receptors, such as EGFRvIII, that are taken-up by normal cells in the tumor environment, leading to further tumor growth and metastasis (Skog et al., 2008). Furthermore, exosomes may modulate the tumor environment into a more favorable niche for tumor growth and metastasis (Park et al., 2010). Besides cancer, exosomes are also implicated in the spread of neurodegenerative diseases. Indeed, since exosomes can be found in almost all body fluids, there might be no distance restriction in its propagation. Thus, exosomes could play an important role in transmitting the pathogenic proteins from the CNS to the PNS.

Since the discovery of prion diseases, the concept has emerged that a protein could be a transmissible pathogen and exosomes have been suggested to contribute to their release from host cells to other cells. Alzheimer’s disease is characterized by neurofibrillary tangles composed of hyperphosphorylated tau, and by extracellular deposits of the amyloid-β peptide, called amyloid plaques (Masters et al., 1985; Grundke-Iqbal et al., 1986). In neurons, it is proposed that amyloid-β is sorted to the MVBs and released in association with exosomes, being a component of the exosomal membrane. Consequently, this peptide can spread to the surrounding cells, forming aggregates in such cells. This is supported by the finding of exosomal proteins in the plaques of diseased brains (Rajendran et al., 2006). Furthermore, exosome-associated tau was found in human CSF, suggesting that tau may also be secreted via this mechanism (Saman et al., 2012). Similarly, in Parkinson’s disease, α-synuclein, a presynaptic neuronal protein that forms toxic inclusions, has similarly been detected in exosomes. Once again, exosomes can transfer and propagate the toxic inclusions to other cells (Emmanouilidou et al., 2010; Alvarez-Erviti et al., 2011b) and, more recently, it was proposed that exosomes accelerate the conversion of monomeric to fibrillar aggregates, since they provide catalytic environments for α-synuclein aggregation (Grey et al., 2014). Exosome-mediated transfer has also been described in ALS. This neurodegenerative disease, characterized by the degeneration of motor neurons, has been
recently associated with exosomes through the propagation of mutant and misfolded SOD1 and its cell-to-cell transmission (Grad et al., 2014b). This topic will be further detailed in Section 2, as it is the main focus of the present thesis.

1.3.3. Exosomes as Disease Biomarkers

As previously mentioned, exosomes can be found in most circulating body fluids, such as plasma (Caby et al., 2005), saliva (Ogawa et al., 2008), urine (Pisitkun et al., 2004) and CSF (Street et al., 2012), suggesting their role in intercellular communication and disease dissemination. Thus, exosomes can be used as potential biomarkers for many pathological conditions, while providing an easy and noninvasive way to evaluate the “donor conditions”.

Biomarkers are defined as indicators of a biological or pathological condition and include DNA, RNA, proteins and metabolites. The most advantageous feature of exosomes as biomarkers is the use of their cell- and condition-specific cargo. Besides the regular exosomal proteins associated to their endosomal origin, exosomes contain disease-specific proteins or RNAs that can be used to determine the state or progress of the disease. For instance, misfolded SOD1, a protein involved in the motor neuron degeneration in ALS, is found exposed at the outer surface of the exosomal membrane, unlike native SOD1 that normally resides in the exosomal lumen (Kim et al., 2013). This may allow its recognition and deactivation by potential pharmacological and immunological therapeutics (Grad et al., 2014b). In the context of RNA-based biomarkers, they can be easily determined by standard methods, like quantitative Real-Time PCR (qRT-PCR) and microarrays. Exosomes have been shown to contain miRNAs implicated in many diseases that seem to be promising for diagnosis (Cheng et al., 2014). Skog and colleagues (2008) were the first to demonstrate that vesicle-secreted miRNAs can be used for diagnosis of glioblastoma and to determine the stage of disease. These exosomes do not simply replicate the miRNA profile of the cell of origin but also contain a specific set of these genes, suggesting a selective packaging of miRNAs into exosomes. More interestingly, miRNAs are known to be dysregulated in neurodegenerative diseases. For instance, miR-9, miR-125b, miR-146a and miR-155 are increased in the CSF and extracellular fluid of Alzheimer’s disease patients (Alexandrov et al., 2012). In Parkinson’s disease, miR-7 expression is found decreased (Junn et al., 2009). Moreover, significant upregulation of miR-146a and miR-155 is found in ALS patients (Koval et al., 2013). Lately, it was found that miR-146a and miR-155 are present in exosomes from dendritic cells, reason why we hypothesize that they may serve as biomarkers (Alexander et al., 2015).
An interesting discovery for brain diseases was the observation that exosomes are able to cross the blood-brain barrier (BBB). The BBB is a physiological barrier between the brain and circulating blood, formed by brain capillary endothelial cells. These cells are linked by tight junctions between them and are surrounded by the basement membrane, as well as by pericytes and astrocytes, forming the neurovascular unit. The BBB confers protection to the CNS, since it restricts the influx and efflux of molecules (Chow and Gu, 2015). Whether exosomes are internalized by endothelial cells and undergo transcytosis, or affect the permeability/integrity of the BBB is still unknown. However, not only exosomes in the circulating fluids can provide information regarding the cells in the CNS, but also can target a specific cell type and deliver protein or nucleic acid content into such cell. Exosomes have been successfully studied for therapy by taking advantage of their low immunogenicity and unique delivering capability (Sun et al., 2010; Alvarez-Erviti et al., 2011a; Zhuang et al., 2011), as will be discussed in Section 4.

2. Amyotrophic Lateral Sclerosis (ALS): Cellular and Vesicular Players that Contribute to Motor Neuron Injury

Amyotrophic Lateral Sclerosis (ALS), also known as Lou Gehrig’s disease, is a fatal adult-onset neurodegenerative disorder, involving the degeneration of the upper and lower motor neurons (MNs), from the motor cortex, brainstem and ventral horn of the spinal cord (Rowland and Shneider, 2001) (Figure I.4). It affects the muscles of mobility, speech, swallowing and respiration, but usually does not affect the cognitive function, since only 5% of patients develop frontotemporal dementia (Phukan et al., 2007). Typically, the age of onset is around 48 years and patients die 2 to 5 years after due to respiratory failure. The diagnosis is clinical, based on the physical examination, and is usually supported by electrophysiological or neuropathological studies and neuroimaging and laboratory tests to exclude other diseases (Hardiman et al., 2011).

Etiologically, ALS can be divided into two categories, sporadic (sALS) and familial (fALS), with 90-95% of cases with no known cause. Both genetic and non-genetic forms of ALS have been described with a remarkable similarity in terms of disease progression and clinical manifestation (Lilo et al., 2013). In fALS, some of the causative genes identified were in Cu/Zn superoxide dismutase 1 (SOD1), transactive response DNA-binding protein (TARDBP), fused in sarcoma (FUS) and chromosome 9 open reading frame 72 (C9ORF72) (Ajroud-Driss and Siddique, 2015). Recently, new gene variants were found to be responsible for 3% of all ALS patients. The new gene, called NEK1, was the result of a big data analysis in ALS research, in more than 1,000 fALS and 6,000 sALS individuals. The study was conducted in 11 countries and involved 80 researchers. NEK1 has multiple roles,
including maintenance of neuronal cytoskeleton, mitochondrial membrane regulation and DNA repair. Understanding the role of NEK1 may provide novel drug driven therapies (Kenna et al., 2016).

Since the first SOD1 missense mutation was discovered in 1993 (Rosen et al., 1993), more than 180 different mutations have been identified. Mutations in SOD1 account for approximately 20% of the fALS cases. It is believed that these mutations lead to a gain of function where the misfolding and accumulation/aggregation of this protein in neurons and glial cells causes toxicity (Hayashi et al., 2016). Although the main hallmark is MN injury, glial cells were shown to be actively implicated in this disorder and its progression, through the release of soluble factors (Lasiene and Yamanaka, 2011). More recently, it has also been described the involvement of exosomes in the cell-to-cell transmission of the disease (Basso et al., 2013; Grad et al., 2014b).

**Figure I. 4 – ALS is a neurodegenerative disease, resulting from the degeneration of UMN strand/or LMNs.** Upper motor neurons (UMNs) prolong their axons from the motor cortex through brainstem to spinal cord, connecting with the lower motor neurons (LMNs). In Amyotrophic Lateral Sclerosis (ALS), MN degeneration begins focally in the central nervous system and then spreads contiguously, ultimately leading to an impairment of signal transmission from MNs to muscle. This leads to weakness and muscle atrophy and, ultimately, to muscle paralysis. Nowadays it is know that, besides MN degeneration, neuroinflammation has an important role in the progression of the disease and, eventually, on the onset as well.
2.1. Motor Neuron Degeneration – Involvement of SOD1

SOD1, a small 153-amino acid protein, is a cytosolic and mitochondrial antioxidant enzyme, converting superoxide to molecular oxygen and hydrogen peroxide (Klug et al., 1972). Wild-type SOD1 is a highly stable dimer, retaining significant conformation and enzymatic activity in the presence of strong protein denaturants. However, it is prone to misfolding and subsequent aggregation in the MNs, when aberrantly oxidized or mutated (Bosco et al., 2010). Indeed, new evidences suggest that the transitory formation of highly toxic trimeric SOD1 species is involved in the neurotoxic phenomena in ALS (Proctor et al., 2016). Although to date the majority of cases are unrelated to mutations in the SOD1 gene, it is suggested the existence of a common pathogenic pathway involving this gene, since SOD1-containing inclusions are seen in cases of sALS and fALS. This observation indicates that aggregation of this protein is a common event in ALS disease (Gruzman et al., 2007; Grad et al., 2014b). However, other common mechanisms in sALS and fALS were identified, such as astrocyte-mediated toxicity (Haidet-Phillips et al., 2011; Meyer et al., 2014; Re et al., 2014). Recently, astrocytes in ALS have been shown to reduce the expression of MHC class I molecules on MNs, increasing their susceptibility to astrocyte-induced cell death (Song et al., 2016). However, exactly how these astrocytes become toxic remains unclear.

It is believed that SOD1 displays “prion-like” properties. In vitro assays showed that wild-type and mutant SOD1 can seed aggregation of themselves (self-seeding) and of each other (cross-seeding) and can be transferred to other cells (Chia et al., 2010). Furthermore, misfolding of SOD1 can persist even in the absence of the original misfolded source in cell culture and, when exogenously applied, mutant SOD1 can be efficiently taken-up into living cells and trigger the aggregation of endogenously expressed SOD1 (Grad et al., 2011; Münch et al., 2011; Furukawa et al., 2013). Recently, it was discovered that SOD1 can enter the vesicle-mediated secretory pathway, becoming selectively incorporated into exosomes and exit the cell, which can explain the cell-to-cell transfer of toxicity (Gomes et al., 2007; Basso et al., 2013; Grad et al., 2014a). However, it is still unknown how SOD1 is packaged on exosomes. One explanation can be that the presence of misfolded and aggregated proteins begins to overwhelm protein clearance pathways, leading the cell to release exosomes containing these proteins. Another possibility is that mutant and misfolded SOD1 is released from dying cells and becomes bound to vesicles in the extracellular space that were secreted by other cells.

The mechanism by which aggregation of SOD1 causes MN death and degeneration is not completely known, but several hypothesis have been proposed: disruption of axonal transport (Bilsland et al., 2010), glutamate excitotoxicity (Rothstein et al., 1995; Howland et al., 2002), mitochondrial dysfunction (Cozzolino et al., 2009), ER stress (Kikuchi et al., 2006),
inhibition of the ubiquitin-proteasome system (Cheroni et al., 2009), caspase-mediated apoptosis (Tokuda et al., 2007) and neuroinflammation (Alexianu et al., 2001). Although being hard to understand the origin of cellular dysfunction, some common inflammatory-associated markers have been proposed in the context of ALS. An important role is given to matrix metalloproteinase (MMP)-9. In the presence of mutant SOD1, MMP-9 is activated in MNs, enhancing ER stress and triggering degeneration (Kaplan et al., 2014; Vaz et al., 2015). Accordingly, reduction in MMP-9 delays muscle denervation and prolongs survival in ALS model mice (Lorenzl et al., 2006; Kaplan et al., 2014). MMP-9 has also been found in serum of ALS patients (Beuche et al., 2000). Other identified marker is S100A6, a calcium- and zinc-binding protein that is overexpressed in ALS human patients, mouse models and within astrocytes, and that has recently been demonstrated to enhance SOD1 aggregation (Hoyaux et al., 2000, 2002; Botelho et al., 2012). Moreover, one should also consider that miRNA biogenesis and function can be disrupted by cytoplasmic protein aggregates (Paez-Colasante et al., 2015). Altogether, the complex interaction between genes, environmental exposure and altered intracellular and cell-to-cell signaling mechanisms contribute to ALS pathology.

2.2. ALS as a Non Cell-Autonomous Disease

Although initially ALS was thought to be a cell autonomous disease, accumulating evidence shows the involvement of other non-neuronal cells in pathogenesis, as well as extracellular vesicles, particularly in disease progression (Ilieva et al., 2009; Lasiene and Yamanaka, 2011) (Figure I.5). Studies with chimeric mice have demonstrated that SOD1<sup>G93A</sup>-overexpressing neurons when surrounded by healthy glia remain relatively intact and survive longer. However, when surrounded by mutant SOD1<sup>G93A</sup>-overexpressing glia, healthy MNs acquire an ALS phenotype (Clement et al., 2003). The role of distinct glial cell types, as well as exosomes and their cargo in ALS will be further detailed in this chapter.

2.2.1. Oligodendrocytes and Schwann Cells

Oligodendrocytes are the myelinating cells of the CNS, being responsible for the formation of the myelin sheaths around axons. The role of oligodendrocytes in ALS pathogenesis is not fully elucidated. However, these glial cells can be somehow involved, as myelin abnormalities were observed in spinal cords of SOD1<sup>G93A</sup> models of ALS, which induced loss of compact myelin and a decrease in lipid content. These morphological changes were observed even before disease onset (Niebroj-Dobosz et al., 2007; Kang et al., 2013). Furthermore, it was also reported that, in ALS, oligodendrocytes downregulate transporters that transfer glycolytic substrates to neurons, failing to provide metabolic
support, and oligodendrocyte progenitors exhibit enhanced proliferation and differentiation. Nevertheless, these newly differentiated oligodendrocytes fail to mature and, therefore, are unable to mediate remyelination (Kang et al., 2013; Philips et al., 2013). These findings may suggest a contributive role to MN degeneration in ALS.

Schwann cells, in the PNS, are also responsible for the myelin sheaths surrounding peripheral axons and participate in the clearance of debris and in guiding the axon after neuron damage. So far, little is known about Schwann cells involvement in ALS pathology. An interesting study demonstrated that excision of mutant SOD1$^{G37R}$ in these cells not only failed to slow but it dramatically accelerated disease progression, suggesting a protective role for mutant SOD1 in Schwann cells (Lobsiger et al., 2009). And, although a second study did not find an involvement of Schwann cells in the ALS disease onset or progression (Turner et al., 2010), a new one demonstrated changes in death, oxidative stress, and mitochondrial related genes in Schwann cells of presymptomatic SOD1$^{G93A}$ mice, eventually correlated to MN death in ALS (Alves et al., 2015).

2.2.2. Astrocytes

Astrocytes are the most abundant and diverse glial cells, and tile the entire CNS. One of the main important functions of astrocytes is to maintain low extracellular concentrations of ions and glutamate. Astrocytes have EAAT2 glutamate transporters that clear the excess of glutamate from the synaptic clefts. In ALS, the astrocytic function of clearing glutamate is impaired due to the loss of these transporters (Rothstein et al., 1995; Howland et al., 2002). Consequently, overabundance of glutamate leads to neuronal excitotoxicity due to excessive neuronal firing and increased influx of Ca$^{2+}$. Furthermore, Ca$^{2+}$ enters the cells through Ca$^{2+}$-permeable AMPA receptors. The permeability of AMPA receptors is largely determined by the GluR2 subunit and the GluR2-lacking receptors are permeable to Ca$^{2+}$ ions. It was previously shown that deletion of the GluR2 gene accelerates MN degeneration in mutant SOD1$^{G93A}$ mice, and is also known that mutant SOD1 astrocytes secrete factors that lower the expression of the GluR2 subunit in MNs, consequently leading to AMPA receptor-mediated excitotoxicity (Van Damme et al., 2005, 2007).

Another function of astrocytes is to release neurotrophic factors that are important for the maintenance of neuronal health. In ALS, it was recently reported a population of astrocytes with an aberrant and neurotoxic phenotype, releasing insufficient neurotrophic factors and producing soluble toxic factors, such as pro-inflammatory cytokines (Nagai et al., 2007; Díaz-Amarilla et al., 2011; Fritz et al., 2013). Re and colleagues (2014) discovered that fALS- and sALS-derived human adult astrocytes selectively kill MNs by neurotoxic factors associated to necroptosis. Furthermore, connexin 43 expression in such cells also seems to
contribute to MN toxicity (Almad et al., 2016). This and other mechanisms are currently being explored in our lab to a better understanding of the role of astrocytes in the ALS disease and to develop new therapeutic strategies.

2.2.3. Microglia

Microglia are distributed throughout the CNS, continuously surveying the environment with long cell processes (Nimmerjahn et al., 2005). These cells are the first line of defense of the brain, sensing and reacting to many types of damage and stimuli. In such conditions, microglia become activated changing their morphology from ramified to amoeboid, migrate to the lesion sites, and clear the debris of dead cells and pathogens (Nakamura et al., 1999; Kloss et al., 2001). During this process, microglia release not only neurotrophic factors, but also reactive oxygen species (ROS), nitric oxide (NO), pro-inflammatory cytokines [such as TNF-α, interleukin (IL)-1β, IL-6, interferon (IFN)-γ and others], complement factors and neurotoxic molecules, leading to a state of neuroinflammation (Nakamura et al., 1999; Hensley et al., 2003).

Neuroinflammation has been known as a component of ALS pathology, with microgliosis observed at sites of MN injury, specifically in motor cortex, brainstem and ventral horn of the spinal cord (Hall et al., 1998; Alexianu et al., 2001; Turner et al., 2004). The intensity of microglial activation is correlated with the severity of upper MN damage, suggesting an active involvement of microglial activation in the disease. Some studies have demonstrated the role of mutant SOD1 in microglia. It was shown that extracellular mutant SOD1 can be endocytosed by microglia, increasing the release of pro-inflammatory cytokines, NO and ROS (Weydt et al., 2004; Liu et al., 2009; Zhao et al., 2010). Furthermore, when MNs are co-cultured with mutant SOD1<sup>G93A</sup> microglia they reveal reduced survival rates, when compared to the effects of wild-type microglia, which indicates that the expression of mutant SOD1 results in activated and neurotoxic microglia (Xiao et al., 2007). In accordance, replacement of microglia was shown to slow neuron loss and disease progression in mice expressing mutant SOD1<sup>G93A</sup> suggesting that microglial cells can indeed influence disease pathogenesis and progression (Beers et al., 2006; Lee et al., 2012a).

2.2.4. Exosomes

As already mentioned, the mechanisms responsible for the intercellular transmission of propagated SOD1 misfolding are not fully elucidated. Moreover, there seems to be little agreement regarding the effects of its secretion. Turner and colleagues (2005) considered the secretion of mutant SOD1 as beneficial, noting that the release of the mutant protein attenuates the formation of toxic intracellular inclusions and prolongs cell survival. However,
Urushitani and colleagues (2006) reported that mutant SOD1 triggers microgliosis and death of MNs in culture, suggesting a pathogenic mechanism based on toxicity of secreted SOD1 mutant proteins.

Some studies demonstrated the production and extracellular secretion of cytosolic SOD1, by an unknown mechanism. They proposed that, since SOD1 is a cytoplasmic protein that lacks a signal peptide, there must be an alternative mechanism, different from the classical ER-Golgi secretory pathway, for the export of this protein (Mondola et al., 1996, 1998). In 2003, Mondola and colleagues showed for the first time that the enzyme was exported by secretory vesicles. Some years later, Gomes and colleagues (2007) showed that the NSC-34 MN-like cells secrete wild-type and mutant SOD1 via exosomes. More recently, it has also been shown that misfolded SOD1 (both wild-type and mutant forms) is released to the extracellular environment on the surface of exosomes and that these exosomes are capable of delivering SOD1 to naive cells (Grad et al., 2014b). Therefore, these EVs may constitute a way of intercellular communication and dissemination of mutant SOD1 toxicity. It is also known that the interaction between MNs and other cell populations plays a role in MN degeneration and death. One of these cells are astrocytes that when carrying mutant SOD1 release metabolites and proteins toxic for MNs into the extracellular space (Nagai et al., 2007; Diaz-Amarilla et al., 2011; Fritz et al., 2013). In 2013, it was shown for the first time that astrocytes also secrete mutant SOD1-containing exosomes, which are able to transfer mutant SOD1 into spinal neuron cultures and are sufficient to induce selective MN death. Exosomes from wild-type SOD1 astrocytes do not have a toxic effect in cells (Basso et al., 2013). Although it is possible that astrocytes use exosome release as a way of clearance for misfolded and potentially toxic proteins, it can have adverse effects on neighboring cells and facilitate aggregation and disease spreading.

However, as has been referred, exosomes can have beneficial effects. A recent study demonstrated that exosomes derived from stem cells are able to protect NSC-34 MN-like cells from oxidative damage, which is one of the main mechanisms of damage in ALS. This observation proposes a role for exosomes derived from stem cells as potential therapeutic strategy in the disease (Bonafede et al., 2016).

So far, this is all that is known about exosomes and their involvement in ALS pathogenesis. In such context, it is still required further studies to elucidate the relevance of exosomes and their cell-to-cell transfer in the pathogenic mechanisms and in the initiation and progression of ALS, namely in what concerns microglia, the focus of the present thesis.
Figure I. 5 – Role of glial cells and exosomes in the progression and dissemination of ALS. Many studies showed the influence of mutated superoxide dismutase 1 (SOD1)-expressing non-neuronal cells in the pathogenesis of Amyotrophic Lateral Sclerosis (ALS). In ALS, it was observed myelin abnormalities and impaired metabolic support due to mutant SOD1-expressing oligodendrocytes. Furthermore, ALS astrocytes have a deleterious behavior, with loss of glutamate transporters, which can cause neuronal excitotoxicity, and insufficient release of neurotrophic factors and production of soluble toxic factors. In microglia, when mutant SOD1 accumulates, they become activated and increase their proliferation, with production of pro-inflammatory cytokines and release of nitric oxide (NO) and reactive oxygen species (ROS). Regarding Schwann cells, their involvement is not yet fully elucidated, with some authors proposing a protective role for these cells. Nowadays, it is known that exosomes can have a pivotal role in the interaction between motor neurons (MNs) and other cell populations. MNs are able to secrete wild-type and mutant SOD1 via exosomes, as well as misfolded SOD1 on the surface of these vesicles. Astrocytes also secrete mutant SOD1-containing exosomes that, once transferred into spinal neuron cultures, induce selective MN death. Therefore, extracellular vesicles are nowadays considered as vehicles of intercellular communication and dissemination of mutant SOD1 toxicity.

3. Microglia as Main Players in ALS

Microglia, as the resident macrophages of the CNS and the first line of defense in the brain, may adopt different activation phenotypes. The surveilling microglia show a ramified appearance and monitor the surrounding environment by extending and retracting their processes (Nimmerjahn et al., 2005). When activated, they may acquire either a neuroprotective or neurotoxic potential.
3.1. Microglia Reactivity

An important neuroprotective role of microglia is phagocytosis. Apoptotic neurons present in their surface specific receptors that microglia can recognize. The milk-fat globule EGF factor-8 protein (MFG-E8) that binds to phosphatidyl serine exposed on the plasma membrane of apoptotic cells is recognized by microglia favoring the engulfment and removal of the dying neurons. MFG-E8 is upregulated upon CX₃CL1 (or fractalkine) stimulation in microglia, through its specific receptor CX₃CR1. The CX₃C axis has important functions in microglial/neuronal cross-talk either in homeostatic or in pathological conditions (Wolf et al., 2013). CX₃CL1 is released by neurons and, upon that signal, microglia produce increased amounts of MFG-E8. Since MFG-E8 is found in the surface of exosomes, it is suggested that it may occur upon stimulation by microglia (Leonardi-Essmann et al., 2005; Véron et al., 2005). TLRs that are also expressed in microglia were shown to mediate the phagocytosis of microbial pathogens through recognition of pattern-associated molecular patterns (PAMPs) (Bsibsi et al., 2002).

It has been demonstrated that a transformation from a neuroprotective phenotype to a neurotoxic one occurs in ALS during disease progression (Liao et al., 2012). In ALS, the neurotoxic potential of microglia is a strong documented issue, with microgliosis observed in presymptomatic and symptomatic SOD1<sup>G93A</sup> models of ALS (Hall et al., 1998; Alexianu et al., 2001; Gerber et al., 2012). Accordingly, the replacement of mutant SOD1 microglia for wild-type microglia showed to delay disease and to prolong mice survival (Lee et al., 2012a). However, microglia does not need to express mutant SOD1 to produce a neurotoxic response. In the presence of mutant SOD1, released to the extracellular environment by living or dying cells, microglia becomes activated and causes a significant decrease in MN viability (Zhao et al., 2010; Roberts et al., 2013). Previous studies suggest that mutant SOD1 is recognized through the CD14/TLR pathway, which leads to an exacerbated inflammatory response (Olson and Miller, 2004; Zhao et al., 2010) (Figure I.6). Binding of pathogens to CD14 (cluster of differentiation 14) and TLRs (such as TLR2 and TLR4) activates a pro-inflammatory cascade involving the NF-κB pathway, with increased production of pro-inflammatory mediators, NO and ROS, together with a reduced release of protective neurotrophic factors. TNF-α and its receptors, TNFR1 and TNFR2, were shown to be elevated in SOD1<sup>G93A</sup> mice and in serum of ALS patients (Poloni et al., 2000; Yoshihara et al., 2002). Several others cytokines are similarly elevated in the spinal cord of SOD1<sup>G93A</sup> mice, such as IFN-γ, IL-1β and others (Hensley et al., 2003). Moreover, exposure of microglial cells to IFN-γ was shown to induce the expression of inducible nitric oxide synthase (iNOS) and subsequent NO production (Kawahara et al., 2001) that cause neuronal damage (Bonfoco et al., 1995). Also to consider that endocytosis of mutant SOD1 by microglia activates caspase-
1, involved in the maturation of IL-1β, therefore enhancing the inflammatory response (Meissner et al., 2010). MMP-9 and MMP-2 expression are also associated with ALS pathology (Beuche et al., 2000; Fang et al., 2010). MMPs are a family of zinc-dependent endopeptidases that are responsible for extracellular matrix degradation. Their excessive expression may result in tissue damage, as in MNs where MMP-9 activation enhances ER stress and triggers degeneration (Kaplan et al., 2014; Vaz et al., 2015). Enhanced release of MMPs by the activated microglia leads to MN injury and cell death (Nuttall et al., 2007).

Besides binding to CD14, aggregated SOD1 may interact with the surface of glial cells and can be internalized in a lipid raft and scavenger receptor dependent manner (Roberts et al., 2013). To note, as previously mentioned, that exosomes by triggering TLR-dependent signaling pathways may activate microglial cells as well (Bretz et al., 2013).

Being ALS a non-cell autonomous disease, neurodegeneration involves a cross-talk between MNs and glial cells. High-mobility group box 1 (HMGB1) protein, also known as amphoterin, is an inflammatory factor that can be released by dying cells, serving as a signal of cell injury. When neurons are injured, secretion of HMGB1 activates microglia through receptors for advanced glycation end products (RAGE), TLRs and Mac1 receptors (Park et al., 2005; Kim et al., 2006; Casula et al., 2011; Gao et al., 2011). However, activated microglia can release pro-inflammatory mediators, inducing further neuronal death. In ALS, it was observed a reduction of HMGB1 immunoreactivity in degenerating neurons during the progression of the disease, which may reflect the loss of MNs, reduced synthesis or enhanced release of this protein (Lo Coco et al., 2007). Concerning reactive glial cells, cytoplasmic translocation of HMGB1 in activated microglia and astrocytes was observed, supporting the role of glial cells as major source of extracellular HMGB1 in ALS patients (Casula et al., 2011). Similarly to other proteins, HMGB1 has been reported to be secreted, at least in part, associated to exosomes (Liu et al., 2006). HMGB1 release by microglia promotes the secretion of IL-1β and IL-18 and, then, extracellular HMGB1 can bind to microglial receptors, leading to the activation of NF-κB pathway and forming a vicious cycle that sustains progressive neurodegeneration (Gao et al., 2011). A recent study demonstrated that HMGB1 can also activate the TLR2/MyD88/miR-155 pathway (Wen et al., 2013).
Figure I. 6 – Inflammatory response of microglia cells in the presence of mutant SOD1. It has been demonstrated that a transformation from a neuroprotective phenotype to a neurotoxic one occurs in ALS during disease progression. Mutant SOD1 is released in the extracellular environment and recognized by microglia through the CD14/TLR pathway, which leads to an exacerbated inflammatory response. Binding to cluster of differentiation 14 (CD14) and toll-like receptors (TLRs) activates a pro-inflammatory cascade involving the nuclear factor-kappa B (NF-κB) pathway, with increased production of tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), inducible nitric oxide synthase (iNOS) and others. Consequently, iNOS expression leads to nitric oxide (NO) production. Endocytosis of mutant SOD1 by microglia also activates caspase-1, which is expressed by the NLRP3 inflammasome and is required for the maturation of interleukin-1beta (IL-1β), enhancing the inflammatory response. When activated, microglia also release matrix metalloproteinases (MMPs). Furthermore, cytoplasmic translocation of high-mobility group box 1 (HMGB1) was observed in activated microglia and, when released, extracellular HMGB1 can bind to microglial receptors, leading to the activation of NF-κB pathway and forming a vicious inflammatory cycle. Ultimately, microglial release of these pro-inflammatory factors leads to MN injury and cell death. Exosomes may also be activating microglia through TLR-dependent signaling pathways.

3.2. Microglial Phenotypes

Microglial cells may assume a large number of phenotypes and many subtypes have been suggested, although M1 (inflammatory cells) and M2 (pro-regenerative cells) polarized cells are the most accepted ones.

M1 phenotype, also known as “classical activation” is cytotoxic and is characterized by the production and release of pro-inflammatory cytokines, such as TNF-α and IL-1β, chemokines, proteases, superoxide, NO and ROS. Their activation and proliferation are triggered by granulocyte macrophage colony-stimulating factor (GM-CSF) and IFN-γ, released by T helper cell type 1 (Th1), as well as LPS through TLR signaling (Nayak et al., 2010; Kraft and Harry, 2011; Chhor et al., 2013).
On the other hand, M2 phenotype or “alternate activation” is considered to be neuroprotective, since it inhibits inflammation and works to restore homeostasis, mainly the subclasses M2a and M2c that release IL-4, IL-10 and transforming growth factor (TGF)-β, among other cytokines and growth factors. M2 is promoted by IL-4 released from Th2, and by regulatory T cells that inhibit microglial activation (Butovsky et al., 2005; Zhao et al., 2012; Zhou et al., 2012; Rózsér, 2015). Besides the M2a microglia (associated with the production of anti-inflammatory cytokines and trophic factors) and the M2c (associated with phagocytosis and suppression of the innate immune system) another M2b phenotype was named “acquired deactivation” and is characterized by markers of both M1 and M2 subtypes (Colton, 2009; Brothers and Wilcock, 2013; Chhor et al., 2013).

Today it is believed that microglia can switch between these phenotypes and may exist in many intermediate states. In ALS, microglia function change along disease progression, with different consequences in MNs (Figure I.7). Liao and colleagues (2012) suggested that microglia display the M2 phenotype at an early stage of the disease, switching to the M1 phenotype during the late phase and inducing MN injury. These authors also observed that, when mutant SOD1 microglia were co-cultured with MNs, the M2 microglia collected at an early phase of the disease do not increased MNs death, in contrast with M1 microglia that decreased the number of living MNs. Contributing to the diminished neuroprotective function of microglia in the late disease stage may be the increased accumulation of misfolded and aggregated SOD1 proteins, as well as the chronic activation of microglial cells and their degeneration (Nikodemova et al., 2014). It should also be considered that microglia may acquire a dysfunctional/senescent phenotype during the late phase (Brites and Vaz, 2014; Brites, 2015). Late evidences indicate that microglia in the preonset phase of SOD1-mediated disease show a distinct anti-inflammatory profile and attenuated response to LPS challenge, revealing an upregulation of the IL-10 anti-inflammatory cytokine. The authors also showed that IL-10 overexpression significantly delayed disease onset and increased survival of ALS mice, suggesting that it may have therapeutic potential (Gravel et al., 2016).
Chapter I. Introduction

**Figure I. 7 – Microglia assume different phenotypes during ALS disease progression.** In a surveillant state, microglia acquire a ramified appearance and continuously monitor the surrounding environment. However, these cells can acquire different activation states in response to specific stimuli. In Amyotrophic Lateral Sclerosis (ALS), it is believed that microglia function change along disease progression, with different consequences in motor neurons (MNs). Microglia display the M2 phenotype at an early stage of the disease, induced by interleukin (IL)-4 and with increased production of anti-inflammatory cytokines, such as IL-4, IL-10 and transforming growth factor-beta (TGF-β). Then, there’s a switch to the M1 phenotype during the late phase, inducing MN injury, as much as IL-10 production is decreased by aging. Lipopolysaccharide (LPS) induces the M1 phenotype, with increased production of pro-inflammatory cytokines, such as IL-1β, tumor necrosis factor-alpha (TNF-α) and inducible nitric oxide synthase (iNOS). It should also be considered that, when facing prolonged stimuli, microglia become overactivated and senescent/dystrophic, ultimately compromising their normal function.

### 3.3. MicroRNAs Involved in Microglia Response

MiRNAs are small non-coding RNA species (~20–25 nucleotides) that are transcriptionally processed in the host cell and released extracellularly. MiRNAs regulate gene expression post-transcriptionally and play an important role in physiologic and pathologic conditions. Although generally resulting in gene expression repression (mRNA degradation and protein translation inhibition) they may also activate gene expression, directly or indirectly (Valinezhad Orang et al., 2014). Some miRNAs modulate the inflammatory response by inhibiting or activating inflammatory pathways and have been designed as inflamma-miRs (Olivieri et al., 2013a). Among them, the most known ones are miR-155, miR-124 and miR-146a that modulate the M1 and M2 phenotypes of microglia and macrophages (Guedes et al., 2013; Essandoh et al., 2016).

MiR-155 is considered pro-inflammatory and is directly linked to the M1 phenotype, contributing to a microglia-mediated neurotoxic response. In response to pro-inflammatory stimuli, miR-155 is upregulated in microglia and leads to the upregulation of several
inflammatory mediators characteristic of the M1 phenotype, including iNOS, IL-6 and TNF-α (Cardoso et al., 2012). Furthermore, recent studies have shown that miR-155 is able to target M2-associated genes, such as SOCS1 (suppressor of cytokine signaling 1) that acts as a negative regulator of several inflammatory pathways, further supporting its association to M1-like phenotypes (Louafi et al., 2010; Cardoso et al., 2012). However, miR-155 upregulation was recently correlated with an increased expression of IFN-β, which regulates the expression of anti-inflammatory mediators (Kim et al., 2014). This is probably related with a feedback mechanism to control the immune response. On the other hand, although miR-124 has been found to be the most abundant miRNA expressed in neurons (Mishima et al., 2007), it has also been described in microglia (Ponomarev et al., 2011). It is thought to keep microglia in a quiescent state, reason why miR-124 expression is downregulated when microglia become activated. It has also been reported to contribute to the M2 phenotype, since its overexpression leads to the downregulation of M1-associated markers and to an increase of proteins associated with the M2 phenotype (Ponomarev et al., 2011; Willemen et al., 2012). However, miR-124 may also have dual and opposing roles, since it was suggested to activate microglia via neuronal-microglial signaling, enhancing microglia activation and inflammatory cytokines in epileptogenesis (Brennan et al., 2016). Regarding to miR-146a, it can directly downregulate the production of pro-inflammatory cytokines by acting as a negative feedback effector of the inflammatory signaling pathway initiated by NF-κB (Taganov et al., 2006). It seems to be related with the microglia phagocytic capacity and to be upregulated in aged microglia, reason why it is proposed as a biomarker of age-associated senescence (Saba et al., 2012; Caldeira et al., 2014).

The dysregulation of miRNAs has been shown to influence the pathogenesis of a number of diseases, such as ALS. Two of the candidates that were found to be altered in ALS patients were miR-146a and miR-155 (Campos-Melo et al., 2013; Koval et al., 2013; Parisi et al., 2013). Interestingly, it was discovered that inhibition of miR-155 prolongs survival in the mutant SOD1<sup>G93A</sup> mice (Koval et al., 2013), as well as restores dysfunctional microglia and ameliorates disease (Butovsky et al., 2015), pointing to this miRNA as a promising therapeutic target for ALS. The release of these miRNAs within exosomes has not been demonstrated in ALS. However, some studies have shown that miR-155, as well as miR-146a, can be released from dendritic cells within exosomes and be subsequently taken-up by recipient cells. Exosomal miR-146a was shown to inhibit endotoxin-induced inflammation, while miR-155 promoted inflammation. It was also shown that exosomes loaded with miR-155 mimic are able to significantly increase its content in cells and tissues (Momen-Heravi et al., 2014; Alexander et al., 2015). This may suggest a potential role for exosomes in the dysregulation of these miRNAs in ALS, as well as a role as therapeutic
vehicles. Regarding miR-124, it was observed to be transferred from neurons to astrocytes by exosomes and to mediate the regulation of EAAT2 expression (Morel et al., 2013). It is also believed that the increased expression of this miRNA is influenced by interactions between neuronal cells and microglia, through cell-to-cell contacts like CX₃CL1-CX₃CR1 or CD200-CD200R (another axis involved in microglia-mediated neuroprotection), or through exosomal vesicles, what needs to be confirmed. Overall, miRNAs are a new and very promising field to better understand diseases, to be used as biomarkers and to be modulated by innovative therapeutic strategies.

4. Exosomes as a Promising Therapeutic Strategy

One of the issues that arose with the discovery of exosomes was the advantage or usefulness that they could have as therapeutic strategy. By now, we already know that exosomes can serve as disease biomarkers, since they reflect the donor conditions and can be isolated from all body fluids. Regarding to ALS, early and proper biomarkers of the disease are still scarce. However, it was reported that misfolded SOD1 is localized to the outer surface of the exosomal membrane, which allows its recognition and deactivation by potential pharmacological and immunological therapeutics, in addition to all the other exosomal markers (Grad et al., 2014a).

Due to their properties, extensive research has been made to exploit exosomes as gene and drug delivery vehicles. The benefits of using exosomes as delivery vehicles are (i) specificity, as exosomes can interact and deliver their cargo to a specific target; (ii) safety, since exosomes present low immunogenicity; and (iii) stability, not only the exosome itself but also the content of exosomes is protected from degradation. Currently, exosomes are being explored to be used as drug delivery vehicles and in interference RNA therapy (Alvarez-Erviti et al., 2011a; Koval et al., 2013; Butovsky et al., 2015) (Figure I.8).

These EVs can be used to transport therapeutic molecules/drugs via insertion into the lipid bilayer or loading into their aqueous core. A method commonly used for incorporating “cargo” into exosomes is overexpressing that cargo in the exosome-producing cells (Mizrak et al., 2012; Ohno et al., 2013). This method is being mainly used with RNAs, being the overexpressed RNAs all functional: mRNA is translated into protein and miRNAs induce target gene knockdown. Interestingly, overexpression of mRNA in exosome-producing cells also results in high expression of the protein for which this mRNA codes, and this protein is packaged into exosomes as well. An alternative method for engineering exosomes is via electroporation of purified exosomes, but electroporation may not be effective for all types of cargo and/or cells (Alvarez-Erviti et al., 2011a). In order to target exosomes to specific cellular receptors, exosomes can also be genetically altered to display at their surface a
targeting protein fused with an exosomal protein (Alvarez-Erviti et al., 2011a; Hartman et al., 2011).

4.1. Passage Across the Blood-Brain Barrier

The major challenge for the treatment of neurological diseases is the BBB, which isolates the CNS and only allows spontaneous diffusion of lipid-soluble molecules. However, exosomes can cross this barrier via a non-invasive route and do not induce an immune response, unlike other delivery vectors, such as liposomes (Alvarez-Erviti et al., 2011a). Another issue in the clinical application of therapeutic drugs is their low solubility and stability that results in a low systemic bioavailability. Curcumin, a drug exhibiting anti-inflammatory activity, is one of those cases. Interestingly, Sun and colleagues (2010) showed that the incorporation of curcumin into exosomes increases the solubility, the stability, and the bioavailability of the drug, enhancing its anti-inflammatory effect. The same authors showed that these vesicles can transport curcumin to the CNS via intranasal injection, thus allowing the drug to cross the BBB and reach the brain, being taken-up by microglial cells (Zhuang et al., 2011). Other studies have been made in order to support the potential of exosomes for targeted brain drug delivery, across the BBB (Haney et al., 2015; Yang et al., 2015). Therefore, we can conclude that exosomes cross the BBB, diffuse throughout the brain, and deliver the drugs locally.

4.2. Small Interference RNA Delivery

Small interference RNAs (siRNAs) are short (~21–23 nucleotides) double-stranded RNA molecules that bind to mRNAs and lead to post-transcriptional gene silencing, either by degrading mRNA or blocking mRNA translation (Zamore et al., 2000; Elbashir et al., 2001). In recent years, the use of siRNAs has emerged as a promising strategy for diseases associated to gene-based pathophysiology. When using systemic delivery strategies, siRNAs can be immunogenic and are susceptible to degradation by endonucleases, what limits its therapeutic potential (Deng et al., 2014). Thus, they require an efficient delivery vehicle, like exosomes, which can preserve and deliver functional RNAs to the target cells (Valadi et al., 2007; Skog et al., 2008).

Alvarez-Erviti and colleagues (2011a) were one of the first to demonstrate the delivery of siRNA into the brain by systemic injection of targeted exosomes. As well as efficient and specific delivery of siRNA through the BBB, these exosomes produced little or no toxicity or immunogenicity even after repeated administration. Several other studies have been made in order to demonstrate the exosome-mediated delivery of siRNA to the brain and specific gene knockdown (Cooper et al., 2014; Liu et al., 2015; Didiot et al., 2016). The
advantages of this approach are the specific targeting of exosomes to the brain, the ability to load the desired RNA into the exosomes and the ability to escape an immune response.

4.3. Exosomal MicroRNA Modulation

Besides siRNA, exosomes can be loaded with miRNAs. Nowadays, two miRNA-based therapeutic strategies are being explored: miRNA mimics and anti-miRNAs.

miRNA mimics are modified double-stranded RNA molecules that resemble miRNA precursors. Once introduced into cells, this RNA fragment, mimicking an endogenous miRNA, can bind specifically to its target gene and produce translational inhibition of the gene. The therapeutic strategy of these mimics is to replace the function of natural miRNAs that can be lost or expressed at reduced levels and decrease the specific protein level itself (Bader et al., 2010). It was recently evidenced that exosomes loaded with miR-155 and miR-146a mimics were able to significantly increase its content in cells and tissues (Momen-Heravi et al., 2014; Alexander et al., 2015). In these cases, miRNA mimics can be a useful therapeutic strategy and using exosomes as delivery vehicles might increase their value.

Another miRNA-based therapy is to use anti-miRNAs, in order to create a loss-of-function in the miRNA of interest. In certain conditions in which miRNAs are overexpressed, the aim is to block these miRNAs by using a complementary RNA sequence that binds to and inactivates the target miRNA. Krutzfeldt and colleagues (2005) designed “antagomirs,” which are RNA fragments conjugated with cholesterol molecules to help the RNA enter cells. However, antagomirs cannot cross the BBB so, once again, exosomes can be a good alternative to deliver anti-miRNAs. Once overexpression of miR-155 was observed in both sALS and fALS and the use of anti-miR-155 was shown to extend the survival of ALS model mice, while restoring dysfunctional microglia (Koval et al., 2013; Butovsky et al., 2015), the use of exosomes with anti-miRNAs to modulate miR-155 upregulation, as well as that of others, may derive a promising strategy for the treatment of neurodegenerative diseases.

Nevertheless, cautious should be undertaken because of miRNA off-target effects, saturation of their machinery and interference with the normal functioning of the cell. In what concerns miRNA replacement, miRNA mimics should be viewed as a new class of therapeutics in which non-specific off-target effects are unlikely since they behave similarly to endogenous miRNAs (Bader et al., 2010). The main challenge will be its in vivo delivery and successful translation into clinics.
There are different methods for incorporating “cargo” into exosomes, one is overexpressing that cargo in the exosome-producing cells and then isolate exosomes (A); the other is engineering exosomes (B). In order to target exosomes to specific cellular receptors, exosomes can be genetically altered to display at their surface a targeting protein fused with an exosomal protein, and then loaded with the cargo of interest. Exosomes can transport (i) therapeutic drugs that can act in specific cells; (ii) small interference RNA (siRNA) that binds to messenger RNA (mRNA) and leads to gene silencing, either by degrading mRNA or blocking mRNA translation; (iii) microRNA (miRNA) mimics that bind specifically to its target gene and produce translational inhibition of the gene; or (iv) anti-miRNA that binds to complementary miRNA and inactivates the target miRNA.
5. Aims

In this thesis, the overall goal is to investigate the specific role of mutant SOD1 MN-derived exosomes in the activation and dysfunction of microglia, using an in vitro model of mutant SOD1 MNs. As general aims, we intend to identify the MN-derived exosomal cargo, and how they influence the activation and dysfunction of the recipient microglial cells, and inflammatory mediators produced and released to the extracellular media. We also aim to assess whether exosomes will be preferentially allocated by microglia or by MNs.

Therefore, the specific aims are to evaluate:

1. Inflamma-miR profile in NSC-34 MN-like cell line and in their derived exosomes. For this, NSC-34 cell line expressing either human wild-type SOD1 or mutant in G93A [NSC-34/hSOD1\textsuperscript{wt} (wt MNs) or NSC-34/hSOD1\textsuperscript{G93A} (mSOD1 MNs), respectively] will be maturated and exosomes isolated from the extracellular media.

2. Changes in microglia performance and proneness to cell senescence upon exosome interaction. For this, healthy N9 microglia cell line will be incubated with NSC-34-derived exosomes and microglia phagocytic properties and cell-associated senescence will be analyzed after 2, 4 and 24 hours of incubation.

3. Inflammatory mediators at intra- and extracellular levels and expression of inflamma-miRs subsequent to microglia challenge by MN-derived exosomes. We will use the protocol previously indicated to determine the release of NO, MMP-2 and MMP-9, the activation of NF-\(\kappa\)B, the expression of the pro-inflammatory cytokines IL-1\(\beta\) and TNF-\(\alpha\), as well as of the inflammatory miRNA (miR)-124, miR-146a and miR-155.

4. The distribution of exosomes collected from the extracellular media of microglia and MNs (used as donor cells) towards MNs and microglia (used as recipient cells), and which effects are produced. For this, we will use co-cultures of NSC-34 cell line expressing either human wild-type SOD1 (wt MNs) or mutant in G93A (mSOD1 MNs), and healthy N9 microglial cell line. Exosomes isolated from those cell supernatants will be re-incubated for 24 hours in a new similar co-culture, to assess whether they show preferential distribution for one cell type, and if so, the type of alterations they origin.
Chapter I. Introduction

Figure I. 9 – Schematic representation of the specific aims of the present thesis. Under the scope of clarifying the specific role of mutant SOD1 motor neuron (MN)-derived exosomes in the activation and dysfunction of microglia it will be assessed: (1) the inflammatory microRNA (inflamma-miR) profile in the NSC-34 MN-like cell line and their derived exosomes; (2) the changes in microglia performance related with the phagocytic ability and propensity to senescence-associated features upon exosome interaction; (3) the inflammatory mediators at intra- (activation of NF-κB and expression of pro-inflammatory cytokines such as TNF-α and IL-1β) and extracellular (NO, MMP-2 and MMP-9) levels, as well as the expression of inflamma-miRs, subsequent to microglia challenge by MN-derived exosomes; and (4) the distribution of exosomes collected from the extracellular medium of microglia and MNs (used as donor cells) towards MNs and microglia (used as recipient cells), and subsequent effects. NF-κB, nuclear factor-kappa B; TNF-α, tumor necrosis factor-alpha; IL-1β, interleukin-1beta; MMPs, matrix metalloproteinases.
II. MATERIALS AND METHODS

1. Materials

1.1. For cell culture

Dulbecco’s modified Eagle’s medium-Ham’s F12 medium (DMEM-Ham’s F12) and DMEM (high glucose with glutamine; w/o pyruvate), were purchased from Biochrom AG (Berlin, Germany). Roswell Park Memorial Institute (RPMI) 1640 medium was obtained from Sigma-Aldrich (St. Louis, MO, USA).

1.2. Supplements and chemicals

Fetal bovine serum (FBS), Penicillin/Streptomycin (Pen/Strep), L-glutamine and nonessential amino acids (NEAA) were purchased from Biochrom AG (Berlin, Germany). Geneticin 418 sulfate (G418) was obtained from Gibco/Calbiochem (Darmstadt, Germany). Poly-D-Lysine (PDL), trypsin-EDTA (ethylenediamine tetraacetic acid) solution (1X), Hoechst 33258 dye, bovine serum albumin (BSA), fluorescent latex beads 1 μm (2.5%), Coomassie Brilliant Blue R-250, naphtylethlenediamine (C₁₂H₁₄N₂), sulfanilamide (C₆H₈N₂O₂S), sodium nitrite (NaNO₂), phosphoric acid (H₃PO₄) and PKH67 Fluorescent Cell Linker Kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). Triton X-100 was obtained from Roche Diagnostics (Mannheim, Germany). Paraformaldehyde, hematoxylin and Cellular Senescence Assay Kit were purchased from Merck Millipore (Darmstadt, Germany). TRlzo™ reagent and primers for HMGB1, IL-1β, TNF-α and β-actin were purchased from Invitrogen Corporation™ (Carlsbad, CA, USA). SensiFAST™ cDNA Synthesis Kit and SYBR® Hi-ROX Kit were purchased from Bioline (London, UK). miRCURY™ RNA Isolation Kit and miRCURY LNA™ Universal RT microRNA PCR (Universal cDNA Synthesis Kit II and ExiLENT SYBR® Green master mix), were purchased from Exiqon (Vedbaek, Denmark), as well as the PCR primer mixes for miR-124, miR-146a, miR-155 and SNORD110. All the other common chemicals were purchased either from Sigma-Aldrich or Merck.

1.3. Antibodies

The primary antibodies used in this work were rabbit anti-ionized calcium-binding adaptor molecule 1 (Iba1) (1:250), purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan), and rabbit anti-NF-κB p65 subunit antibody (1:250), from Santa Cruz Biotechnology® (CA, USA). The secondary antibodies used were Alexa Fluor® 488 and Alexa
Fluor® 594 goat anti-rabbit (1:1000), obtained from Invitrogen Corporation™ (Carlsbad, CA, USA).

1.4. Equipment

Fluorescence microscope (model AxioScope.A1) coupled with an AxioCam HR camera and AxioScope HBO50 microscope with an AxioCam 105 color camera were purchased from Carl Zeiss, Inc. (North America). The optical microscope with phase-contrast equipment was from Olympus (model CK2-TR).

For exosome isolation, we used a Beckman Optima™ L-100 XP ultracentrifuge, with a type 90 Ti rotor (fixed angle) and centrifuge bottles of polycarbonate, from Beckman Coulter, Inc. (Fullerton, CA, USA). For zymography assays, a Mini-PROTEAN® Tetra Cell system was used and, for spectrophotometric measurements of nitrites, a microplates reader (PR 2100 Microplate Reader). Metalloproteinases gels photos were obtained by using the ChemiDoc™ XRS, from Bio-Rad Laboratories (Hercules, CA, USA). Total RNA was quantified using NanoDrop® ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). For determination of mRNA and miRNA expression, by quantitative Real-Time PCR, it was used the 7300 Real-Time PCR System, from Applied Biosystems™ (Foster City, CA, USA). Eppendorf 580R (Eppendorf, Hamburg, Germany) and Sigma 3K30 centrifuges were used for different experimental procedures.

To ensure a stable environment to optimal cell growth (37°C and 5% CO₂), cell cultures were maintained in a HERAcell™ 150 incubator (Thermo Fisher Scientific, Waltham, MA, USA) and the experimental work was performed in sterile conditions using a Holten Lamin Air HVR 2460 (Allerod, Denmark).

1.5. Cell lines

For in vitro studies, two different cell lines were used during the experimental work: (i) NSC-34 cell line, resulting from hybridization between murine neuroblastoma and motor neurons obtained from mouse spinal cord (Cashman et al., 1992); and (ii) N9 cell line that was developed by immortalization of microglia cells obtained from CD1 mouse cortex and that shows features similar to microglia in primary cultures such as phagocytosis and inflammation-related features (Righi et al., 1989; Bruce-Keller et al., 2000; Fleisher-Berkovich et al., 2010). Both cell lines are currently used in the lab and results obtained so far, with different experimental models, evidence that they well reproduce primary cultures and are suitable for the general purpose of the present thesis.
2. Methods

2.1. Cell lines

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\textsuperscript{wt} (wt MNs) or NSC-34/hSOD1\textsuperscript{G93A} (mSOD1 MNs), respectively], were a gift from Júlia Costa, Instituto de Tecnologia Química e Biológica (ITQB), Universidade Nova de Lisboa, Portugal (Gomes et al., 2008). NSC-34/hSOD1\textsuperscript{wt} cells were used as controls. NSC-34 cells were grown in proliferation media (DMEM high glucose with glutamine, w/o pyruvate, supplemented with 10% of FBS and 1% of Pen/Strep) and selection was made with G418 at 0.5 mg/ml, as usual in our lab (Vaz et al., 2015). Medium was changed every 2 to 3 days. Cells were seeded in 6- and 12-well culture plates, at a concentration of 5x10\textsuperscript{4} cells/ml, and maintained at 37°C in a humidified atmosphere of 5% CO\textsubscript{2}. Culture plates were coated with PDL (10 μg/ml) before plating the cells.

2.1.2. N9 cell line
N9 cell line was a gift from Teresa Pais, Instituto de Medicina Molecular (IMM), Lisboa, Portugal. Cells were cultured in RPMI medium supplemented with 10% of FBS, 1% of L-glutamine and 1% of Pen/Strep, grown to confluency and splitted every 2 to 3 days, as described by Cunha and colleagues [submitted]. For incubation and characterization, cells were seeded in 6- and 12-well culture plates, at a concentration of 1x10\textsuperscript{5} cells/ml, and maintained at 37°C in a humidified atmosphere of 5% CO\textsubscript{2}. No coating was required.

2.2. Cell treatments

2.2.1. NSC-34 cell line
After 48 hours in proliferation medium, differentiation was induced by changing medium for DMEM-F12 plus 1% of FBS-exosome depleted (removed by differential ultracentrifugation, Section 2.3), 1% of NEAA, 1% of Pen/Strep and 0.1% of G148, as indicated by Cho and colleagues (2011). Cells were maintained in culture with differentiation medium for 4 days in vitro (DIV) to induce SOD1 accumulation (Vaz et al., 2015) and, after that, exosomes were isolated from extracellular media by differential ultracentrifugation (Section 2.3). After obtaining exosomes from NSC-34 cells, exosomes were resuspended in RPMI medium and incubated in N9 microglia cells, in a ratio of 1:1.

2.2.2. N9 cell line and incubation with exosomes from NSC-34 cells
N9 cells were plated 24 hours before incubation with exosomes from NSC-34 cells, at a concentration of 1x10\textsuperscript{5} cells/ml, in order to maintain the usual proportion of microglia and
neurons in the CNS (ratio 1:1), as in previous studies of the group with mixed neuron-microglia cultures (Silva et al., 2011). To determine the different effects of exosomes on microglia, cells were incubated with RPMI, either alone (control) or containing exosomes from NSC-34/hSOD1<sup>wt</sup> or from NSC-34/hSOD1<sup>G93A</sup>. Microglia response was evaluated upon incubation with exosomes during 2, 4 and 24 hours, as described in Figure II.1A.

These different time-points of incubation were performed in order to see the outcomes of a short (2 and 4 hours) and a prolonged (24 hours) exposure to exosomes in healthy microglia. At the end of each incubation period, medium free of cellular debris was collected to evaluate soluble factors, while attached cells were (i) fixed for 20 min with freshly prepared 4% (w/v) paraformaldehyde in phosphate-buffer saline (PBS), for immunocytochemical studies; (ii) fixed with Fixing Solution for cellular senescence assays; or (iii) was added TRIzol, for RNA isolation.

**2.2.3. NSC-34-N9 co-cultures**

In parallel studies, NSC-34 cells and N9 cells were grown separately, as described for mono-cultures. At 4 DIV of NSC-34 cells, N9 cells were co-cultured with them and grown in RPMI medium for another 24 hours, at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. After these 24 hours, exosomes were isolated from extracellular media of NSC-34-N9 co-cultures by differential ultracentrifugation, as described in Figure II.1B.

The isolated exosomes were then labelled with the PKH67 Fluorescent Cell Linker Kit to assess their distribution (Section 2.4), resuspended in RPMI medium and incubated in a new co-culture, grown in the same conditions as the first one, for another 24 hours. After incubation, cells were (i) fixed for 20 min with freshly prepared 4% (w/v) paraformaldehyde in PBS, for immunocytochemical studies; or (ii) was added TRIzol, for RNA isolation.
Chapter II. Materials and Methods

2.3. Differential ultracentrifugation

For exosome isolation, extracellular media of wt MNs and mSOD1 MNs, in mono- and co-cultures, were collected and centrifuged as described by Wang and colleagues (2010), with some minor modifications. Briefly, cell supernatant was centrifuged at 1,000 g for 10
min, to pellet cell debris. Then, the supernatant was transferred to another tube and centrifuged at 16,000 g for 1 hour, to pellet microvesicles. The supernatant was filtered in a 0.22 μm pore filter, transferred to an ultracentrifuge tube and centrifuged at 100,000 g for 2 hours, to pellet exosomes. The pellet of exosomes was then resuspended in PBS and centrifuged one last time at 100,000 g for 2 hours, in order to wash the pellet. All centrifugations were performed at 4°C. For mono-cultures, the final pellet was resuspended in RPMI medium and stored overnight at 4°C, for further experiments. For co-cultures, exosomes were resuspended in PBS and labelled with a PKH67 fluorescent probe.

Figure II. 2 – Schematic procedure for the isolation of exosomes from the extracellular media. (1) Cell supernatants are centrifuged at 1,000 g for 10 min, to pellet cell debris; (2) supernatant is transferred to another tube and centrifuged at 16,000 g for 1 hour, to pellet microvesicles; (3) supernatant is filtered in a 0.22 μm pore filter, to remove the remaining particles larger than 200 nm, and transferred to an ultracentrifuge tube; (4) supernatant is centrifuged at 100,000 g for 2 hours, to pellet exosomes; (5) the pellet of exosomes is resuspended in PBS and, finally, (6) exosomes are centrifuged one last time at 100,000 g for 2 hours, in order to wash the pellet.

2.4. Labelling with PKH67 fluorescent probe

To study the uptake of exosomes by MNs and microglia, exosomes were labelled with a PKH67 fluorescent probe, using the PKH67 Fluorescent Cell Linker Kit (Sigma-Aldrich). Labelling was performed as described by Dutta and colleagues (2014). Briefly, we diluted the
PKH67 dye in Diluent C, all supplied with the kit. Then, equal volumes of exosomes resuspended in PBS and the PHK67 solution were mixed (1:1 v/v) and incubated for 5 min at room temperature (RT). The labelling reaction was stopped by addition of PBS and labelled exosomes were incubated in a new MN-microglia co-culture for 24 hours, as described in Section 2.2.3.

2.5. Determinations

2.5.1. Microglial phagocytosis

To evaluate the phagocytic ability of N9 microglia cells, after each period of incubation with exosomes, cells were incubated with 0.0025% (w/w) green fluorescent latex beads (diameter 1 μm) for 75 min at 37°C and fixed with 4% (w/v) paraformaldehyde in PBS. For the immunostaining, cells were first permeabilized with 0.1% Triton X-100, for 30 min at RT, and then incubated with blocking solution (1% BSA, 0.4% Triton X-100, 4% FBS), for 30 min at RT. The primary antibody used was rabbit anti-Iba1 and cells were incubated overnight at 4°C. The secondary antibody was goat anti-rabbit Alexa Fluor 594 and the incubation was performed for 2 hours at RT. Cell nuclei were stained with Hoechst 33258 dye (1:1000, Sigma-Aldrich). Finally, coverslips were mounted onto uncoated glass blades using PBS:glycerol (1:1) and stored at 4°C, until acquisition of images.

Fluorescence was visualized using a fluorescence microscope (model AxioScope.A1) coupled with an AxioCam HR camera (Carl Zeiss). UV, green and red fluorescence images of ten random microscopic fields, under 400X magnification, were acquired per sample. The number of cells with ingested beads and total cells were counted to determine the percentage of phagocytic cells. At least 100 cells were counted for each independent condition.

2.5.2. Microglial senescence

Microglial senescence was evaluated by determining the activity of senescence-associated β-galactosidase (SA-β-gal). The senescence phenotype has been associated with changes in cellular morphology and increased activity for SA-β-gal (Sikora et al., 2011). SA-β-gal is present only in senescent cells and not in presenescent, quiescent or proliferating cells. In this assay, SA-β-gal catalyzes the hydrolysis of X-gal, which results in the accumulation of a distinctive blue color in senescent cells. Microglial SA-β-gal activity was determined using the Cellular Senescence Assay Kit (Millipore), as usual in our lab (Caldeira et al., 2014). Briefly, cells were previously fixed with a Fixing Solution diluted in PBS (1:100), supplied with the kit. Then, cells were incubated overnight at 37°C, protected from light, with a detection solution. Afterwards, microglial nuclei were counterstained with
Chapter II. Materials and Methods

hematoxylin and coverslips were mounted onto uncoated glass blades using 70% glycerol in PBS.

Brightfield microscopy images were obtained using an AxioScope HBO50 microscope with an AxioCam 105 color camera (Carl Zeiss). Ten random microscopic fields, under 400X magnification, were acquired per sample. The number of turquoise stained cells (SA-β-gal-positive cells) and total cells were counted to determine the percentage of senescent cells. At least 100 cells were counted for each independent condition.

Furthermore, to quantitatively characterize microglia morphology, we evaluated the transformation index, first defined by Fujita and colleagues (1996) as \[ \frac{[\text{perimeter of cell (µm)}]^2}{4\pi [\text{cell area (µm}^2)]} \], which categorizes microglia ramification status. A cell with long processes and a small soma exhibits a large index that is dependent on cell shape but independent of cell size. Image analysis was made using ImageJ software (National Institutes of Health, USA).

2.5.3. NF-κB activation

NF-κB is found in the cytosol of unstimulated cells bound to an inhibitory molecule, the IκB. However, when inflammatory mediators bind their respective receptors, a signaling cascade is initiated that leads to phosphorylation and degradation of IκB. Then, NF-κB translocates to the nucleus where it binds to DNA elements and induces gene expression (Ghosh et al., 1998). For immunofluorescence detection of the translocation of NF-κB from the cytoplasm to the nucleus, N9 cells were fixed and an immunocytochemistry was performed as explained above, using a rabbit anti-NF-κB p65 subunit antibody as the primary antibody, and a goat anti-rabbit Alexa Fluor 488 as the secondary antibody. Microglial nuclei were stained with Hoechst 33258 dye.

UV and green fluorescence images of ten random microscopic fields, under 400X magnification, were acquired per sample. NF-κB positive cells were identified by the ratio between the mean gray value of the nucleus and the mean gray value of the whole cell. A threshold was defined for each individual experiment and cells above that value were considered positive for NF-κB. Positive cells and total cells were counted to determine the percentage of NF-κB positive nuclei. At least 200 cells were counted for each independent condition. Image analysis was made using ImageJ software (National Institutes of Health, USA).
2.5.4. Extracellular nitric oxide

NO levels were estimated by measuring the concentration of nitrites (NO\textsubscript{2}^\textsuperscript{-}), a product of NO metabolism, in the extracellular media of N9 cells incubated with RPMI, either alone or containing exosomes from wt MNs or from mSOD1 MNs. Cell supernatants free from cellular debris were mixed with Griess reagent [1% (w/v) sulphanilamide in 5% H\textsubscript{3}PO\textsubscript{4} and 0.1% (w/v) N-1-naphthylethylenediamine, in a proportion of 1:1 (v/v)] in 96-well culture plates for 10 min in the dark, at RT. Nitrites react with the Griess reagent, producing a red-violet color. The absorbance at 540 nm was determined using a microplate reader (Bio-Rad Laboratories) and a calibration curve of standard nitrites was used for each assay. All samples were measured in duplicate and the mean value was used (Vaz et al., 2011).

2.5.5. Gelatin zymography

MMP-2 and MMP-9 quantification was performed in the extracellular media of N9 cells incubated with RPMI, either alone or containing exosomes from wt MNs or from mSOD1 MNs, through the gelatin zymography assay. With this method it is possible to detect the protease activity in the running gel based on the absence of color (white bands), at the particular site of protease action.

The assay was performed as usual in our lab (Silva et al., 2010). Cell supernatants free from cellular debris were used in SDS-PAGE zymography in 0.1% gelatin-10% acrylamide gels, under non-reducing conditions, at 30 mA/gel. After electrophoresis, gels were washed for 1 hour at RT with 2.5% Triton X-100 (in 50 mM Tris pH 7.4; 5 mM CaCl\textsubscript{2}; 1 \textmu M ZnCl\textsubscript{2}) to remove SDS and renature the MMP species in the gel. Then, to induce gelatin lysis, the gels were incubated overnight at 37°C in the developing buffer (50 mM Tris pH 7.4; 5 mM CaCl\textsubscript{2}; 1 \textmu M ZnCl\textsubscript{2}). For enzyme activity analysis, gels were stained with 0.5% Coomassie Brilliant Blue R-250 for 30 min and destained in 30% ethanol/10% acetic acid/H\textsubscript{2}O to see the bands. Gelatinase activity, detected as a white band on a blue background, was photographed in ChemiDoc™ (Bio-Rad Laboratories) and measured using the Image Lab™ software.

2.5.6. Quantitative Real-Time PCR

Determination of TNF-\alpha, IL-1\beta and HMGB1 mRNA expression, as well as miR-124, miR-146a and miR-155 expression, was performed by quantitative Real-Time PCR (qRT-PCR). Total RNA was extracted from N9 cells using TRIzol® reagent, according to the manufacturer instructions. RNA from exosomes was extracted using the miRCURY™ RNA Isolation Kit (Exiqon). Total RNA was quantified using a Nanodrop® ND-100 Spectrophotometer (NanoDrop Technologies) (Caldeira et al., 2014).
Chapter II. Materials and Methods

For mRNA expression, aliquots of 500 ng/μl of total RNA were reverse transcribed into cDNA using SensiFAST™ cDNA Synthesis Kit (Bioline), under the recommended conditions. qRT-PCR was performed on a 7300 Real-Time PCR System (Applied Biosystems), using a SYBR® Hi-ROX Kit (Bioline). The sequences listed in Table II.1A were used as primers. qRT-PCR was performed in 96-well plates, with each sample performed in duplicate, and under optimized conditions: 50°C for 2 min, 95°C for 2 min, followed by 40 amplification cycles at 95°C for 5 seconds and 62°C for 30 seconds. In order to verify the specificity of the amplification, a melting curve analysis was performed, immediately after the amplification protocol. qRT-PCR was performed using β-actin as an endogenous control, to normalize the expression level of TNF-α, IL-1β and HMGB1, and fold change was determined by the $2^{-\Delta\Delta CT}$ method.

Expression of miR-124, miR-146a and miR-155 was also performed by qRT-PCR. After RNA quantification, cDNA conversion was performed with the Universal cDNA Synthesis Kit II (Exiqon), using 5 ng/μl of total RNA according to the following protocol: 60 min at 42°C followed by heat-inactivation of the reverse transcriptase for 5 min at 95°C. For miRNA quantification, the ExiLENT SYBR® Green master mix was used in combination with pre-designed primers (Exiqon) for miR-124, miR-146a, miR-155 and SNORD110 (reference gene). The sequences used are listed in Table II.1B. The reaction conditions consisted of polymerase activation/denaturation at 95°C for 10 min, followed by 50 amplification cycles at 95°C for 10 seconds and 60°C for 1 min (ramp-rate of 1.6°/second). Quantification of target miRNAs was made in comparison to the reference gene (SNORD110) and fold change was determined by the $2^{-\Delta\Delta CT}$ method.

Table II.1 – List of primer sequences used in qRT-PCR.
(A) Primers used in gene expression. (B) Primers used in microRNA expression.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>5'-TACTGAACTTCGCGGTGATTGTCCTCC-3' (fwr)</td>
</tr>
<tr>
<td></td>
<td>5'-CAGCCCTTGCTCCCTGAAGAGAACC-3' (rev)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5'-CAGGCTCCAAGCATCAAC-3' (fwr)</td>
</tr>
<tr>
<td></td>
<td>5'-GGTGGAGAGCTTCAGCTCATA-3' (rev)</td>
</tr>
<tr>
<td>HMGB1</td>
<td>5'-CTCAGAGAGGGGAAGACCATGT-3' (fwr)</td>
</tr>
<tr>
<td></td>
<td>5'-GGGATGGTTTTTCATTTCTCTCTT-3' (rev)</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-GCTCCGGCAGTGCGCA-3' (fwr)</td>
</tr>
<tr>
<td></td>
<td>5'-AGGATCTCTGAGGTAGT-3' (rev)</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td>miRNA sequences</td>
</tr>
<tr>
<td>miR-124</td>
<td>5'-UAAGGGCAAGCGCGAGACG-3'</td>
</tr>
<tr>
<td>miR-146a</td>
<td>5'-UGAGAAGCUGUCAUUCAUGGGU-3'</td>
</tr>
<tr>
<td>miR-155</td>
<td>5'-CTCAGAGAGGGGAAGACCATGT-3'</td>
</tr>
<tr>
<td>SNORD110</td>
<td>Reference gene</td>
</tr>
</tbody>
</table>


2.5.7. **Exosome distribution**

To evaluate the cellular distribution of exosomes derived from NSC-34 cells alone or from NSC-34-N9 co-cultures, after incubation with labelled exosomes, cells were fixed with 4% (w/v) paraformaldehyde in PBS and cell nuclei were stained with Hoechst 33258 dye. Fluorescence was visualized using a fluorescence microscope (model AxioScope.A1) coupled with an AxioCam HR camera (Carl Zeiss). UV and green fluorescence images, under 630X magnification, were acquired per condition.

2.6. **Statistical analysis**

Results of at least seven independent experiments were expressed as mean ± SEM. Comparisons between the different parameters evaluated in wt and mSOD1 MNs were made using one-tailed Student’s *t*-test for equal or unequal variance, as appropriate. In the characterization of N9 microglia cell line, after incubation with exosomes obtained from wt or mSOD1 MNs, comparisons between the different groups for the assessed parameters were done by one-way ANOVA followed by multiple comparisons Bonferroni *post-hoc* correction. *p*<0.05 was considered statistically significant and *p*<0.01 very significant. Statistical analysis was made using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA).
III. RESULTS

1. NSC-34 inflamma-miR profiling indicates that only miR-124 is upregulated in mSOD1 MNs, which is recapitulated in cell-derived exosomes

In this thesis, we started by characterizing the inflamma-miR profile in the NSC-34 MN-like cell line and in exosomes derived from these cells. As detailed in the Methods section, we used an in vitro model of MN degeneration in ALS by using NSC-34 cells stably transfected with either human wild-type SOD1 or mutant in G93A [NSC-34/hSOD1<sup>wt</sup> (wt MNs) or NSC-34/hSOD1<sup>G93A</sup> (mSOD1 MNs), respectively]. Differentiation was induced and exosomes were isolated from extracellular media after 4 DIV, to induce SOD1 accumulation, as previously reported (Vaz et al., 2015).

Recent data from our lab, obtained by DLS and TEM, indicated that the nanoparticle size of exosomes in the extracellular media of NSC-34 cells was approximately 140 nm, corroborating the existence of exosomes released from the cells (Vaz et al., 2016). Since exosomes are known to contain miRNAs that can be transferred to recipient cells and influence their functionality (Valadi et al., 2007; Alexander et al., 2015), we compared the exosome content from the wt NSC-34 cells with that from mSOD1 ones. As indicated in Figure III.1, mSOD1 MNs presented an increased expression of miR-124, in comparison with wt MNs, and no expression of miR-146a or miR-155 was detected. Interestingly, the exosome content reflected this same pattern, since we could only detect the upregulation of miR-124 in exosomes from mSOD1 MNs.

![Figure III. 1 – Exosomes from mSOD1 MNs contain an increased expression of microRNA (miR)-124, reflecting their cells of origin. MicroRNA expression in cells and exosomes was evaluated by quantitative Real-Time PCR, as indicated in Methods. Exosomes were isolated from extracellular media of NSC-34 cells expressing either human wild-type SOD1 (wt MNs) or mutated in G93A (mSOD1 MNs), after 4 days in vitro. Results are mean (± SEM) from at least five independent experiments and fold changes are relative to the wt MNs. *p<0.05 vs. wt MNs.](image-url)
2. Exosomes from mSOD1 MNs induce loss of microglia phagocytic ability and increase the number of senescent cells

After confirming the presence of exosomes in extracellular media of NSC-34 cells and their specific miRNA inflammatory-related cargo, the main focus of the present thesis was to evaluate the changes conferred by exosomes derived from both wt and mSOD1 MNs over healthy microglia, in terms of microglia activation and dysfunction. For this, we used the same model described in III.1. Healthy N9 microglia cell line was incubated with NSC-34-derived exosomes for 2, 4 and 24 hours and phagocytic ability and cellular senescence were evaluated.

Phagocytosis is a characteristic of an activated microglia, being essential in the clearance of cellular debris, as well as of pathogenic organisms (Nakamura et al., 1999; Kloss et al., 2001). Phagocytic cells are generally associated with the M2 (anti-inflammatory) phenotype, to repair the damage and restore homeostasis. As depicted in Figure III.2, the number of phagocytic cells decreased after incubation with exosomes from mSOD1 MNs in all time-points, in comparison with cells exposed to exosomes from wt MNs (~36% vs. 19%, p<0.05).
Figure III. 2 – Exosomes from mSOD1 MNs produce a lasting decrease in the microglia phagocytic ability. Phagocytosis was evaluated by the capacity of cells to engulf beads, after being exposed to exosomes, as indicated in Methods. N9 cells were incubated for 2, 4 and 24 hours with exosomes from wt MNs and mSOD1 MNs (MG+wt Exos or MG+mSOD1 Exos, respectively). Non-treated cells (MG) served as control. (A) Representative results of one experiment, with ingested latex beads (green). Microglia were stained with an antibody raised against ionized calcium binding adaptor molecule 1 (Iba1) (red) and nuclei were stained for Hoechst dye (blue). (B) Results are expressed as the percentage of total cells showing ingested beads, and are mean (± SEM) from eight independent experiments. Scale bar represents 40 μm. *p<0.05 vs. exosomes from wt MNs (MG+wt Exos).

Therefore, the decrease in the phagocytic ability of microglia does not derive from exosomes per se, but from a specific cargo delivered by the mSOD1 exosomes.

Microglia have been described to acquire multiple phenotypes, with overactivated, senescent and atypical subtypes at the end-stage of ALS disease (Brites and Vaz, 2014). Actually, chronic activation of microglia cells can lead to overactivation followed by degeneration (Nikodemova et al., 2014). So, we evaluated if N9 cells displayed an increased number of cells with typical signs of senescence in the presence of exosomes from mSOD1 MNs. Quantitative assay of SA-β-gal activity revealed that the percentage of positively stained cells increased after 24 hours of incubation with exosomes from mSOD1 MNs, when compared with non-treated cells (26% vs. 37%, p<0.05), as evidenced in Figure III.3A and 3B. Again, such effect was only produced by the mSOD1 exosomes. In addition, looking at the cellular morphology (Figure III.2A and 3A), we saw that after 24 hours of incubation microglia change from a ramified to a more rounded morphology, indicative of an activated cell. This is reflected in the transformation index that was decreased after 24 hours of incubation in all conditions (Figure III.3C). To note, however, that dystrophic microglia (deramified, increased loss of fine processes and presence of spheroids at the end of the cytoplasmic process) was more apparent in cells that were treated for 24 hours with exosomes derived from mSOD1 MNs.
Figure III. 3 – Microglia exposed to exosomes from mSOD1 MNs display signs of dysfunction, with increased SA-β-gal activity and dystrophic features. Microglia senescence was evaluated by determining the activity of senescent-associated β-galactosidase (SA-β-gal), using a commercial kit, as indicated in Methods. N9 cells were incubated for 2, 4 and 24 hours with exosomes from wt MNs and mSOD1 MNs (MG+wt Exos or MG+mSOD1 Exos, respectively). Non-treated cells (MG) served as control. (A) Representative results of one experiment showing SA-β-gal staining and dystrophic microglia at 24 hour incubation with MG+mSOD1 Exos. (B) Results are expressed as the percentage of SA-β-gal positive cells (turquoise staining). (C) Results are expressed as the transformation index defined by Fujita and colleagues (1996). Results are mean (± SEM) from at least five independent experiments. Scale bar represents 40 μm. *p<0.05 vs. non-treated cells (MG). $p<0.05, $$p<0.01 vs. respective condition.
3. Evaluation of microglial inflammatory response to exosomes released by wt MNs and mSOD1 MNs

Neuroinflammation has been known as a component of ALS pathology, with activation and proliferation of microglia observed at sites of MN injury (Hall et al., 1998; Alexianu et al., 2001; Turner et al., 2004). Thus, after assessing the alterations induced by exosomes in microglia performance and cellular senescence, we considered relevant to evaluate whether microglia response in terms of the produced and released inflammatory mediators differed when treated with the wt or the mSOD1 exosomes.

3.1. Exosomes from mSOD1 MNs lead to an early microglial production of inflammatory mediators

One player in ALS-mediated neurotoxicity is NO, which contributes for oxidative stress and neuroinflammation (Drechsel et al., 2012). As evidenced in Figure III.4A, after 2 hours of incubation with exosomes from mSOD1 MNs, we observed a markedly increase of NO levels, when compared with non-treated cells and cells incubated with exosomes from wt MNs (1.9-fold, p<0.01 vs. treatment with exosomes from wt MNs). However, such effect disappeared at 4 hours of incubation and even was downregulated after 24 hours (0.8-fold, p<0.01 vs. non-treated cells). Such finding requires further investigation.

Activation of MMPs is another marker of neuroinflammation, with elevation of MMP-9 and MMP-2 expression found in the spinal cord of SOD1G93A mice, as well as that of MMP-9 in NSC-34 cells (Fang et al., 2010; Vaz et al., 2015). As shown in Figure III.4B and 4C, we were able to detect two different bands by gelatin zymography, at 67 kDa and 92 kDa, corresponding to MMP-2 and MMP-9, respectively (Silva et al., 2010). After 2 hours of incubation, we observed an increase in the activity of MMP-9, in the presence of exosomes from mSOD1 MNs (1.5-fold, p<0.05 vs. non-treated cells), and MMP-2, in the presence of both exosomes from wt and mSOD1 MNs (1.6- and 1.8-fold respectively, p<0.01). However, similarly to NO, this increase ceased over time, staying approximately in the same level of non-treated cells. To note, however, that MMPs activation appears to be a little higher with the mSOD1 exosomes than with the wt ones.
Figure III. 4 – Microglia exposure to exosomes from mSOD1 MNs lead to an immediate microglial release of NO, MMP-9 and MMP-2. Nitric oxide (NO) production and matrix metalloproteinases (MMPs) activity was determined in the extracellular media of microglia cells, as indicated in Methods. N9 cells were incubated for 2, 4 and 24 hours with exosomes from wt MNs and mSOD1 MNs (MG+wt Exos or MG+mSOD1 Exos, respectively). Non-treated cells (MG) served as control. (A) NO production was assessed by Griess reaction. (B-C) Activation of MMP-9 and MMP-2, respectively, was assessed by gelatin zymography assay. Representative results of MMPs activity are shown. The intensity of the bands was quantified using computerized image analysis (Image Lab™ software). Results are mean (± SEM) from at least seven independent experiments and presented as fold change relatively to non-treated microglia. *p<0.05, **p<0.01 vs. non-treated cells (MG); ##p<0.01 vs. treatment with exosomes from wt MNs (MG+wt Exos).

3.2. Microglia increasingly release pro-inflammatory cytokines upon interaction with exosomes from mSOD1 MNs

NF-κB is one of the most important regulators of inflammation and is found to be activated in the SOD1G93A mice model, specifically in glia (Frakes et al., 2014). Activation of NF-κB leads to the expression of pro-inflammatory cytokines, such as TNF-α and IL-1β, which are also upregulated in ALS (Johann et al., 2015; Van Dyke et al., 2016). To further confirm the activation of microglia cells exposed to exosomes from mSOD1 MNs, we
investigated NF-κB activation and cellular expression of TNF-α and IL-1β. As shown in Figure III.5A and 5B, NF-κB translocation into the nucleus was chronically increased in cells exposed to exosomes from mSOD1 MNs, when compared with non-treated cells. Thus, we were expecting that the pro-inflammatory cytokines were also increased along the 24 hour incubation. However, we saw that the expression of the pro-inflammatory cytokines TNF-α and IL-1β was significantly upregulated in cells exposed to exosomes from mSOD1 MNs only for the first 4 hours of incubation (Figure III.5C and 5D). We hypothesize that feedback mechanisms may become activated in order to counteract microglia reactivity. Again, additional studies should be undertaken to explore if they are due to secondary cytokines overexpression, such as IL-6 and IL-10 (Minogue et al., 2012), also explaining what we have observed for NO and MMPs. Most important, as previously noticed, microglia reactivity was mainly observed with exosomes derived from mSOD1 MNs.
Chapter III. Results

Figure III. 5 – NF-κB is activated in the microglia exposed to exosomes from mSOD1 MNs, leading to increased expression of TNF-α and IL-1β. Nuclear factor-kappa B (NF-κB) activation was determined by the immunocytochemical assessment of the cytoplasmic or nuclear localization of the NF-κB p65 subunit, and cytokine mRNA expression in cells was evaluated by quantitative Real-Time PCR (qRT-PCR), as indicated in Methods. N9 cells were incubated for 2, 4 and 24 hours with exosomes from wt MNs and mSOD1 MNs (MG+wt Exos or MG+mSOD1 Exos, respectively). Non-treated cells (MG) served as control. (A) Representative results of NF-κB translocation into the nucleus. (B) Activation of NF-κB after exosomal treatment. The fluorescence intensity of cells was quantified using the ImageJ software. (C-D) Relative tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) mRNA levels, respectively, determined by qRT-PCR in total RNA. Results are mean (± SEM) from at least seven independent experiments and fold changes are relative to the non-treated microglia. Scale bar represents 40 μm. *p<0.05, **p<0.01 vs. non-treated cells (MG); #p<0.05 vs. treatment with exosomes from wt MNs (MG+wt Exos).

We also studied the expression of HMGB1 by microglia, when exposed to exosomes from mSOD1 MNs, since HMGB1 was suggested to be involved in inflammatory processes and was identified in the cytoplasm of microglia in spinal cord samples from ALS patients (Casula et al., 2011; Gao et al., 2011). However, it was not possible to detect any change in its gene expression (Figure III.6), indicating that the activation of microglia is not mediated by its autocrine signaling, but by the interaction of exosomes per se.

Figure III. 6 – Microglial HMGB1 expression is maintained unchanged upon incubation with exosomes from wt and mSOD1 MNs. High mobility group box 1 (HMGB1) expression in cells was evaluated by quantitative Real-Time PCR, as indicated in Methods. N9 cells were incubated for 2, 4 and 24 hours with exosomes from wt MNs and mSOD1 MNs (MG+wt Exos or MG+mSOD1 Exos, respectively). Non-treated cells (MG) served as control. Results are mean (± SEM) from ten independent experiments and fold changes are relative to the non-treated microglia.
3.3. Exosomes from mSOD1 MNs trigger a delayed upregulation of inflammatory miRNAs

Recently, inflamma-miRs have been proposed to have a role as modulators of microglial activation, namely miR-124, miR-146a and miR-155 (Ponomarev et al., 2011; Cardoso et al., 2012; Saba et al., 2012). Therefore, we assessed the expression of these miRNAs in microglia treated with exosomes from wt MNs and mSOD1 MNs. Although miRNA fine tuning on microglia is a complex issue, recent evidences suggest that the most abundant brain-specific miRNA, miR-124, correlates inversely with the activation state of microglia, while promoting M2 polarization, and increases the rehabilitation opportunity (Ponomarev et al., 2011; Liu and Abraham, 2013). However, miR-124 that has mainly a neuronal localization was also suggested to activate microglia via neuronal-microglial signaling, enhancing microglia activation and inflammatory cytokines in epileptogenesis (Brennan et al., 2016). MiR-146a downregulates microglia activation by acting as a negative feedback effector of the inflammatory signaling pathway initiated by NF-κB (Taganov et al., 2006) and is associated with M2 polarization (Li et al., 2016). Nevertheless, it was also found upregulated during senescence and associated with a pro-inflammatory status (Bhaumik et al., 2009; Olivieri et al., 2013b).

As depicted in Figure III.7A and 7B, after 2 hours of incubation with exosomes from both wt and mSOD1 MNs, the expression of both miR-124 and miR-146a was significantly decreased, when compared with non-treated cells (0.37-fold and 0.43-fold respectively, p<0.01), reinforcing the idea that the presence of exosomes induces an early activated M1 (pro-inflammatory) microglia as a response to their presence in the milieu. However, exosomes further triggered their increased expression 24 hours later (2.5-fold and 1.9-fold, for miR-124 and miR-146a respectively, p<0.01). We may hypothesize that it can result from miRNA direct passage as exosomal cargo into microglia, or that exosomes are inducers of such expression. However, as previously noticed only miR-124 is increased in exosomes from mSOD1 MNs. Thus, its major representation in microglia may result from its passage from exosomes into microglia and, additionally, due to specific microglial upregulation. As for miR-146a, such upregulation derive from exosomal interaction with microglia, is not exclusive of mSOD1 MN-derived exosomes and may also result from an exosomal unknown factor. Looking at these results, we may then assume that, at this stage, some microglial cells are polarized as M2 subtypes.

Regarding miR-155, it is accepted to have a pro-inflammatory role in microglia and to be related with the M1 phenotype (Cardoso et al., 2012; Ponomarev et al., 2013). As seen in Figure II.7C, miR-155 only became significantly upregulated after 24 hours of incubation with exosomes from mSOD1 MNs (1.6-fold, p<0.01). Therefore, we may conclude that exosomes
Chapter III. Results

from mSOD1 MNs promote the representation of several microglia subclasses whose effects for ALS pathogenesis deserve to be investigated.

Figure III. 7 – Expression of microRNA (miR)-124, miR-146a and miR-155 in microglia cells after 24 hours of incubation indicate the acquisition of several microglia phenotypes. MicroRNA expression in cells was evaluated by quantitative Real-Time PCR (qRT-PCR), as indicated in Methods. N9 cells were incubated for 2, 4 and 24 hours with exosomes from wt MNs and mSOD1 MNs (MG+wt Exos or MG+mSOD1 Exos, respectively). Non-treated cells (MG) served as control. (A-C) Relative miR-124, miR-146a and miR-155 levels were determined by qRT-PCR in total RNA. Results are mean (± SEM) from eight independent experiments and fold changes are relative to the non-treated microglia. *p<0.05, **p<0.01 vs. non-treated cells (MG); ##p<0.01 vs. treatment with exosomes from wt MNs (MG+wt Exos).

4. Exosomes from MN-microglia donors preferentially distribute in microglia when both recipient cells are considered

An additional and ongoing goal of this thesis was to understand whether exosomes released by both MNs and microglia move with selective specificity, preferentially triggering one cell type over the other, when using cell type co-culturing models. For this, we have recently implemented a model using co-cultures of the NSC-34 cell line (either wt or mSOD1) and healthy N9 microglial cell line. Exosomes were isolated from extracellular media of these co-cultures, as described in Methods (Section II). Many studies have demonstrated the release and uptake of exosomes by neuronal and glial cells, and that these exosomes have the ability to modulate the recipient cells through their cargo (Valadi et al., 2007; Fitzner et al., 2011). Therefore, isolated exosomes from both cultured cell types were incubated in a
novel similar co-culture, for 24 hours, to assess their distribution and subsequent effects produced.

In order to prove that isolated exosomes are internalized by our cells, we first performed the evaluation of the distribution of exosomes in N9 cells alone and, then, in both MNs and microglia when in co-culture, by using a PKH67 fluorescent probe (green). As shown in Figure III.8A, we saw that microglia (recipient cells) are capable of receiving exosomes derived from both wt and mSOD1 MNs (donor cells), which sustain their cytoplasmic localization after 24 hours of incubation. When in the co-culture system, it is also clear that exosomes are preferentially taken-up by microglia, suggesting that these cells are more likely to incorporate and to be functionally influenced by exosomes than MNs, as depicted in Figure III.8B.

Figure III. 8 – Exosomes released from motor neurons (MNs) and microglia are preferentially sorted in microglia that in MNs. Exosomes were isolated from NSC-34 cells (wt and mSOD1), stained with the PKH67 Fluorescent Cell Linker Kit (green) and incubated with N9 cells for 24 hours. In parallel studies, exosomes were isolated from co-cultures of NSC-34 and N9 cells, stained as before and incubated for 24 hours in a new co-culture, grown in the same conditions as the first. (A) Representative results of one experiment demonstrating the distribution of NSC-34-derived exosomes in N9 cells alone. (B) Representative results of one experiment demonstrating the distribution of exosomes in NSC-34 and N9 cells, when in co-culture. Nuclei were stained with Hoechst dye (blue). Scale bar represents 20 μm.
After demonstrating that exosomes can be internalized by microglia, we evaluated their effects in the glial cells when in the presence of MNs. Our most recent data indicate that exosomes enhance the microglial expression of TNF-α and IL-1β (Table III.1). It was a surprise to see that HMGB1 upregulation in microglia is induced by mSOD1 MNs (but not by their derived exosomes), pointing to an effect of HMGB1 as a soluble factor released by the ALS MNs. Moreover, we further confirmed this finding with enhanced values when the exosome-treated microglia was incubated with mSOD1 MNs, attesting HMGB1 as a neuronal alarmin released by the mSOD1 MNs. The next step will be to study the inflamma-miR content of these exosomes.

Table III. 1 – Expression of TNF-α, IL-1β and HMGB1 in microglia co-cultured with wt and mSOD1 motor neurons (MN), in the presence and absence of added exosomes isolated from microglia-MN co-cultures (either wt or mSOD1). Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and high mobility group box 1 (HMGB1) expression in cells was evaluated by quantitative Real-Time PCR, as indicated in Methods. Results are mean (± SEM) from at least four independent experiments and fold changes are relative to N9 cells when in co-culture with wt MNs (without exosomes).

<table>
<thead>
<tr>
<th></th>
<th>N9 cells (without exosomes)</th>
<th>N9 cells (incubated with exosomes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ wt MNs</td>
<td>+ mSOD1 MNs</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.00 ± 0.003</td>
<td>0.91 ± 0.17</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1.00 ± 0.004</td>
<td>0.64 ± 0.22</td>
</tr>
<tr>
<td>HMGB1</td>
<td>1.00 ± 0.003</td>
<td>1.52 ± 0.26</td>
</tr>
</tbody>
</table>
Understanding the role of exosomes, either in physiological or in pathological conditions, as well as their involvement in the spreading of disease, is essential for a complete comprehension of the intra- and extracellular signaling involved in ALS and as clues for the development of more effective therapeutic strategies in this pathology. Recently, it was demonstrated that exosomes carrying mSOD1 are released from MNs and are subsequently taken-up by neighboring cells, propagating SOD1 toxicity (Gomes et al., 2007; Grad et al., 2011, 2014b). However, what is lacking so far is to understand the effect of the uptake of these exosomes by neighbor cells, namely microglia, and their contribution for the disease progression and spread, since microglia has been known to be a key player in ALS pathology (Alexianu et al., 2001; Turner et al., 2004). Therefore, in the present study, we investigated the specific role of exosomes derived from MNs expressing either wt or mSOD1 in the activation and dysfunction of microglia. For this, we isolated exosomes from NSC-34 cells, a MN-like cell line harboring a well-established causative mutation of fALS, a glycine-to-alanine substitution in position 93 (SOD1<sup>G93A</sup>) (Rosen et al., 1993; Gomes et al., 2008), and then evaluated their effect on healthy N9 cells, a microglial cell line.

We started by characterizing the inflamma-miR profile in NSC-34 cells and in exosomes derived from these cells. In our group, it was previously demonstrated that NSC-34/hSOD1<sup>G93A</sup> cells accumulate SOD1 after 4 DIV, while presenting more cell dysfunction in comparison with NSC-34/hSOD1<sup>wt</sup> cells, what may mimic the progression of MN degeneration in fALS (Vaz et al., 2015). Thus, we purified exosomes from the extracellular media after MN maturation and, by differential ultracentrifugation, we isolated particles with a size of approximately 140 nm (determined by DLS and TEM), which are compatible with exosomal formation. Looking at the miRNA content of exosomes, we noticed a major representation of miR-124 and no detectable expression of both miR-155 and miR-146a, in both mSOD1 MNs and respective exosomes, in comparison with wt MNs. In fact, miR-124 has been found to be the most abundant miRNA expressed in neurons (Mishima et al., 2007), unlike miR-146a and miR-155 that are associated with inflammatory processes (Cardoso et al., 2016). Its increased presence in exosomes is not without precedence due to their endosomal origin and as a result of inward budding during the formation of the intraluminal vesicles, reason why it is considered that exosomes reflect partially the cell of origin (Simons and Raposo, 2009; Felicetti et al., 2016).
After confirming the presence of exosomes and their inflamma-miR cargo, we tried to understand the toxicity conferred by these exosomes from mSOD1 MNs, as compared to those from wt MNs, over healthy microglia. Microglia (dys)functional response was evaluated after a short (2 and 4 hours) or a prolonged (24 hours) exposure to exosomes. For this, we looked for microglia basic functions like phagocytic ability and cell-associated senescence, as a dysfunctional property. In a quiescent state, microglia acquire a ramified appearance, continually surveying the surrounding environment, and lack phagocytic ability (Nimmerjahn et al., 2005). However, when facing a stimulus, microglia become activated and highly phagocytic, in order to avoid damage and clear the debris of the dead cells and pathogens (Nakamura et al., 1999). Phagocytosis is then associated with a M2 microglia, since it promotes tissue repair and restores homeostasis (Zimmer et al., 2003; Chhor et al., 2013). In our results, we observed that microglia phagocytic ability, induced by the presence of exosomes from wt MNs, was compromised when exposed to exosomes from mSOD1 MNs, even after a short period of time. Thus, wt exosomes produced a switch from the quiescent to the activated M2 phenotype, a finding not observable with the mSOD1 exosomes and with the neuroprotective response by microglia. Therefore, we decided to evaluate if microglia cells displayed signs of cellular senescence, compatible with the atypical/dysfunctional phenotype already observed at the end-stage of ALS disease (Nikodemova et al., 2014). Changed morphology and increased activity of SA-β-gal are considered cellular senescence markers (Sikora et al., 2011; Caldeira et al., 2014) and our results evidenced that, after a prolonged exposure to exosomes from mSOD1 MNs, the number of SA-β-gal positive cells increased when compared with non-treated cells. This observation may suggest that chronic activation of microglia cells by exosomes from mSOD1 MNs could lead to overactivation followed by degeneration. Indeed, it has been shown that repeated stimulation of microglia cells lead to cell senescence (Yu et al., 2012). However, the activity of SA-β-gal has also been associated with senescence-unrelated settings, such as contact inhibition and serum starvation (Severino et al., 2000; Cho and Hwang, 2012). In our cultures, cells did not reach confluence until 24 hours of incubation. However, they were incubated in media without serum, which can explain the slightly high percentage of SA-β-gal positive cells, especially at short periods of incubation. It should also be taken into account that contact inhibition and serum starvation may also be responsible for the altered morphology of cells observed after 24 hours of incubation. Nevertheless, since our control condition (microglia exposed to exosomes from wt MNs) was also grown in the same conditions, we can assume that the increase of SA-β-gal activity after a prolonged exposure to exosomes from mSOD1 MNs results from a senescent phenotype associated with the mSOD1 exosomes interaction.
Next, we focused on the inflammatory response of microglia to the presence of MN-derived exosomes, since neuroinflammation has been known as a major component of ALS pathology, with activation and proliferation of microglia observed at sites of MN injury (Hall et al., 1998; Alexianu et al., 2001; Turner et al., 2004). To verify the existence of oxidative stress and neuroinflammation, we analyzed the production of NO and activation of MMPs, respectively, and our results showed a markedly increase of NO levels and MMP-2 and MMP-9 activation, upon a short exposure to exosomes from mSOD1 MNs. The increase in NO levels only occurred in cells exposed to exosomes from mSOD1 MNs, suggesting that such exosomes induce microglial oxidative stress. Interestingly, extracellular mSOD1 has been shown to activate microglia through the CD14/TLR pathway, leading to the activation of NF-κB and, consequently, to the upregulation of iNOS and release of NO from these cells (Zhao et al., 2004, 2010). Since it is also believed that exosomes can trigger TLR-dependent signaling pathways (Bretz et al., 2013), this leads to the possibility that these exosomes may be activating the same pathways that mSOD1 or may even contain the protein in their lumen/membrane that can be responsible for this specific reaction. Indeed, Gomes and colleagues (2007) showed that NSC-34 cells secrete wt and mSOD1 via exosomes, but we were not able to confirm its presence in our model. The same does not occur with MMP-2, where exposure to exosomes from wt MNs also produces activation. Whether such effect results from TLR-dependent activation requires further investigation, specially directed towards TLR2 and TLR4. In what concerns MMP-9 activity, it has been found primarily in ALS-associated MNs (Lim et al., 1996; Vaz et al., 2015). Interestingly, MMP-9 was suggested to be shed as membrane vesicle-associated components by endothelial cells (Taraboletti et al., 2002). It was also previously shown that neuronal cells release MMP-2 and MMP-9 in vesicles of 160-200 nm in size, which are proposed to have a role in synaptic plasticity (Sbai et al., 2008). Once ALS MNs release increased amounts of MMP-9 (Vaz et al., 2015), we hypothesize that the elevation of MMP-9 levels in microglia may result from their interaction with mSOD1 exosomes. Anyway, the release of MMPs by microglia upon exposure to exosomes is an acute effect and, to that, it may account the progressive dysfunction observed in microglia along the incubation time. Such finding was somehow corroborated by the profile of pro-inflammatory cytokines, but not by the NF-κB activation that showed to be sustained. We observed that NF-κB was translocated into the nucleus from 2 to 24 hours of incubation with both types of exosomes, but only significantly with those from mSOD1 MNs. NF-κB is found in the cytosol of unstimulated cells bound to an inhibitory molecule, the IκB. However, when inflammatory mediators bind their respective receptors, a signaling cascade is initiated that leads to phosphorylation and degradation of IκB. Then, NF-κB translocates to the nucleus and induces gene expression, increasing the production of
pro-inflammatory mediators (Ghosh et al., 1998). Accordingly, the expression of pro-inflammatory cytokines like TNF-α and IL-1β was also upregulated in microglia exposed to mSOD1 MNs, despite the lack of significance after 24 hours of incubation. So, no doubt that mSOD1 MN-derived exosomes cause the activation of inflammatory signaling mechanisms on microglia. Indeed, it is worthwhile to note that exosomes have been shown to activate the NF-κB signaling pathway (Matsumoto et al., 2005; Bretz et al., 2013), which seems to be particularly driven by the mSOD1 exosomes. The gradual loss of the effects produced may result from compensation between the induced reactivity caused by mSOD1 exosomes in microglia and the loss of cell function they trigger. Because activation of NF-κB in microglia cells was shown to induce gliosis and MN death, we may assume that exosomes from ALS MNs may have a role in neuroinflammation and neurodegeneration associated to ALS onset and progression (Frakes et al., 2014).

HMGB1 was shown to be secreted from cells in exosomes or insoluble fractions (Liu et al., 2006). However, when secreted by senescent cells, HMGB1 is predominantly soluble (Davalos et al., 2013). Since we were not able to observe any induction of the HMGB1 mRNA expression, we may consider that the majority of the released HMGB1 was solubilized. Actually, release of HMGB1 from the nucleus to the extracellular space represents a sign of cell injury, which triggers the release of pro-inflammatory factors through activation of their receptors (Zou and Crews, 2014). In ALS, HMGB1 has been found upregulated during disease progression and glial cells have been proposed as a major source of extracellular HMGB1 (Casula et al., 2011; Lee et al., 2015). In our case, it will be interesting to investigate, in the future, any changes in microglia cytosolic amount of the protein, in exosomes cargo and in the extracellular medium.

Finally, an important part of this work was to understand the effects produced by exosomes on microglial expression of inflamma-miRs, as an attempt to better understand the role of neuron-microglia communication through exosomes in the onset and progression of the disease. Thus, similarly to what we did for exosome cargo, we studied the expression of miR-124, miR-146a and miR-155 by microglia cells, upon exposure to exosomes derived from wt and mSOD1 MNs. Interestingly, we saw that microglia downregulated the expression of miR-124 and miR-146a upon exposure to exosomes, independently of their origin. This may translate in an induced microglial activation, as suggested by the increased release of inflammatory mediators and cytokine expression we also observed, though more notorious with those from mSOD1 MNs. Actually, miR-124 is thought to keep microglia in a quiescent state and its expression decreases when microglia is activated (Ponomarev et al., 2011), and miR-146a downregulates the production of pro-inflammatory cytokines by acting as a negative feedback effector of the inflammatory signaling pathway initiated by NF-κB.
(Taganov et al., 2006). Together with previous data, we may assume that exosomes from mSOD1 MNs trigger an immediate M1 polarization, with activation of the NF-κB signaling pathway, increased pro-inflammatory cytokines and release of toxic mediators. This may account, as shown by Liao and colleagues (2012), for microglia neurotoxicity and MN neurodegeneration. The upregulation of miR-155 after 24 hours of incubation may derive from the maintained NF-κB activation. As an attempt to promote its modulation, microglia later overexpresses miR-124 and miR-146a, namely in the ALS model, which contribute to switch M1 into the M2 phenotype (Ponomarev et al., 2011; Cardoso et al., 2012; Willemen et al., 2012). This indicates the presence of distinct microglia phenotypes at this stage, with subpopulations of pro-inflammatory microglia and anti-inflammatory microglia. Such combined populations justify the moderate inflammatory profiles we observed at 24 hours of treatment. Moreover, as stated before, miR-146a overexpression is likewise associated with increased senescence (Jiang et al., 2012; Olivieri et al., 2013b; Caldeira et al., 2014), accounting for the presence of this additional subtype, together with M1 and M2 phenotypes. However, one should also consider that the delayed miR-146a induction may be a compensatory response to restrain inflammation, although accounting to sustain NF-κB activation in senescent cells, as previously observed (Bhaumik et al., 2009; Jiang et al., 2012). These observations justify the complexity of neuroinflammation in the ALS disease.

Regulatory mechanisms in neuroinflammation and increased expression of miR-155 and miR-146a by pro-inflammatory cytokines like TNF-α (Taganov et al., 2006; O’Connell et al., 2007) contribute to the difficulty of understanding the signaling mechanisms and define a target to develop medicines, including the tentative usage of anti-inflammatory therapies. Independent of that, exosomes from mSOD1 MNs trigger the overexpression of miR-124 in a much high degree than the wt ones. The high expression of miR-124 found on microglia may be a direct consequence of its increased content in exosomes from the mSOD1 MNs we observed. Indeed, many studies have demonstrated the uptake of exosomes and modulation of cells through their miRNA cargo (Valadi et al., 2007; Iguchi et al., 2010). Others have shown that exosomes from oligodendrocytes were selectively transferred to microglia by macropinocytosis and that uptake by neurons or astrocytes was minimal (Fitzner et al., 2011). In our case, selective transfer of exosomes from mSOD1 MNs into microglia was demonstrated by using a fluorescent probe bound to exosomes. Since neuronal miR-124 is thought to keep microglia in a quiescent state, it will be interesting to evaluate whether alterations on miR-124 expression on MNs trigger different microglia activated states and consequent neurodegeneration. If so, we may wonder how and when miR-124 is downregulated in ALS MNs? Such aspect surely deserves further investigation. Another interesting aspect to be further explored would be to look for targets of this miRNA, to see if it
is functional and capable of producing a response in recipient cells (Valadi et al., 2007; Montecalvo et al., 2012) or if, by contributing to microglial maturity, is directly associated with the increased number of senescent cells and reduced phagocytic ability (Svahn et al., 2016).

After studying the inflamma-miR content of MN-derived exosomes and looking at microglial inflammatory response to those exosomes, an additional and ongoing goal of the present thesis was to evaluate the influence of MN-microglia cellular communication on exosome distribution and cellular reactivity. Cellular communication between neurons and microglia is a complex process, responsible for shaping microglia responses and restrain their activation. To study this interaction, we used a co-culture system from where exosomes were isolated and re-incubated for 24 hours in a new co-culture. The preliminary results achieved clearly indicate that exosomes from MNs are preferentially taken-up by microglial cells and that they regionally influence microglia functionality. This observation corroborates previous findings and may relate with the phagocytic property of the cell (Fitzner et al., 2011; Adams et al., 2015). Similarly to what we did for microglia in mono-culture, we started by addressing the effects of exosomes on microglia reactivity in a co-culture system. Expression of pro-inflammatory cytokines like TNF-α and IL-1β was upregulated when in the presence of exosomes, mainly from mSOD1 MNs, reinforcing the activation of microglia. Such activation may also be related with the mSOD1 protein in the extracellular medium, known to be recognized and to activate microglia through the TLR pathway, triggering NF-κB pro-inflammatory cytokine signaling (Zhao et al., 2010; Roberts et al., 2013).

More notorious than the overexpression of pro-inflammatory cytokines, is that of HMGB1 in microglia exposed to mSOD1 MNs, and even more to simultaneous exosomes. HMGB1 was shown to be released by neurons under different conditions (Sun et al., 2014; Zou and Crews, 2014) and to exist either in exosomes, solubilized or in insoluble fractions (Liu et al., 2006; Davalos et al., 2013). Therefore, it can be recognized and internalized by microglial cells and, if released, be again recognized by their receptors, leading to the activation of NF-κB pathway and forming a vicious inflammatory cycle (Casula et al., 2011; Gao et al., 2011). Such aspects may justify why we did not observe any increased HMGB1 mRNA expression in microglia cells and its protein content in exosomes, as well as extracellular medium, requires further studies.

Taken together, the results obtained in this thesis suggest that exosomes released from MNs are internalized by microglia and modulate their response in different ways, depending on the chronicity of exposure. As schematically represented in Figure IV.1, the presence of mSOD1 exosomes provokes an acute inflammatory response with production of pro-inflammatory mediators and cytokines, indicative of a major population of reactive cells. However, afterwards, there seems to be distinct phenotypes (pro- and anti-inflammatory),
with a subpopulation of senescent/irresponsive microglia. Therefore, we may summarize our findings as follows: exosomes from mSOD1 MNs (i) impair microglia's function, impeding them to repair and maintain cellular homeostasis; (ii) contribute to the early overactivation of microglia and, consequently, to neuroinflammation and MN death; and (iii) trigger different microglial subpopulations that include M1, M2 and senescent subtypes overtime.

Although the communication between MNs and microglia and their influence in exosome cargo needs to be further explored and unraveled, we propose that mSOD1 MNs have a key role on microglia dysfunction in ALS and that miR-124 may constitute a promising target to be modulated in mSOD1 MNs.
Figure IV. 1 – Schematic representation of the major findings in this thesis. NSC-34 inflamma-
miR profiling indicates that only microRNA (miR)-124 is upregulated in mutant SOD1 motor neurons
(mSOD1 MNs), which is recapitulated in cell-derived exosomes. When incubated with microglia cells,
MN-derived exosomes appear to be internalized by these cells. Looking at microglia response to the
presence of exosomes, we can conclude that exosomes from mSOD1 MNs impair microglia's
function, with loss of their phagocytic ability. After a short exposure, microglia presents a M1
phenotype, with downregulation of miR-124 and miR-146a. Downregulation of miR-124 is probably
promoting the expressing of M1-associated markers and downregulation of miR-146a is promoting
NF-κB activation, with the production of pro-inflammatory mediators (NO, MMP-2 and MMP-9) and
cytokines (TNF-α and IL-1β). After a long exposure to exosomes, there seems to be distinct
phenotypes, with upregulation of miR-124, miR-146a and miR-155. Upregulation of miR-155 is
contributing to a M1 microglia, with production of pro-inflammatory cytokines, while upregulation
of miR-124 is contributing to a M2 microglia, antagonizing the effect of miR-155. Regarding miR-146a, its
upregulation has been described in aged cells that, together with the increase in cellular senescence
verified after a prolonged exposure to mSOD1 exosomes, suggest the presence of a subpopulation of
irresponsive/senescent microglia at this stage. Furthermore, miR-146a in aged cells has been
reported to not be capable of downregulate the NF-κB pathway, which may contribute to the sustained
activation of this pathway and promotion of mild degree of inflammation. Consequently, pro-
inflammatory cytokines like TNF-α may be inducing the expression of miR-155 and miR-146a. Finally,
we hypothesize that upregulation of miR-124 is a result of the transfer of content from mSOD1
exosomes into microglia, which is capable of incorporating exosomes and utilizing their cargo. NF-κB,
nuclear factor-kappa B; NO, nitric oxide; MMP, matrix metalloproteinase; TNF-α, tumor necrosis
factor-alpha; IL-1β, interleukin-1beta.
Concluding remarks

- MiR-124 increases in exosomes from mSOD1 MNs and may account to its increased content in microglial cells by selective transfer;
- Exosomes from mSOD1 MNs lead to an early reactive response by microglia, with production and release of inflammatory mediators;
- Microglia respond to MN-derived exosomes (either wt or mSOD1) by lowering miR-124 and miR-146a expression, favoring microglia activation;
- Increased HMGB1 mRNA in microglial cells may derive from mSOD1 MNs via exosomes or be induced by soluble factors;
- Exosomes are selectively transferred to microglia, than to MNs, suggesting a crucial role of microglia in ALS neuroinflammation and propagation.

New perspectives and approaches

In the present study we discussed the role of exosomes in ALS pathogenesis and we were able to identify exosomes derived from mSOD1 MNs as inducers of microglia activation, as well as dysfunction. Further investigation will add on the clarification of the importance of the interplay between MNs and microglia, in order to evaluate if soluble factors released by MNs can have some supplementary effects on microglia, additionally to exosomes. Thus, it would be interesting to evaluate if microglia react differently when incubated with conditioned media of MNs after exosomal depuration.

It would be also interesting to explore through which route exosomes enter the cells. One possible approach would be to inhibit all the possible pathways in recipient cells and see if the reaction induced is the same as we observed in the present study. Moreover, it would be important to identify more molecular components contained in exosomes, including mutant and misfolded SOD1, to further unravel the mechanisms involved in microglia activation and dysfunction in the disease.

The final purpose of understanding the influence of exosomes in ALS pathogenesis would be to develop an effective therapy by using modulation of the exosome cargo to (i) prevent the transfer of pathogenic proteins or molecules, as well as miRNAs, to other cells, avoiding propagation of toxicity and neuroinflammation; or (ii) deliver therapeutic agents to target cells, in order to confer them protection.


Cozzolino, M.; Pesaresi, M. G.; Amori, I.; Crosio, C.; Ferri, A.; Nencini, M.; Carri, M. T. Oligomerization of Mutant SOD1 in Mitochondria of Motoneuronal Cells Drives Mitochondrial Damage and Cell Toxicity. *Antioxid Redox Signal* 2009, 11, 1547–1558.


Van Damme, P.; Braeken, D.; Callewaert, G.; Robberecht, W.; Van Den Bosch, L. GluR2 Deficiency Accelerates Motor Neuron Degeneration in a Mouse Model of Amyotrophic


Fauré, J.; Lachenal, G.; Court, M.; Hirrlinger, J.; Chatellard-Causse, C.; Blot, B.; Grange, J.;
Chapter V. References


Hall, E. D.; Oostveen, J. a.; Gurney, M. E. Relationship of Microglial and Astrocytic Activation to Disease Onset and Progression in a Transgenic Model of Familial ALS. *Glia* 1998, 23, 249–256.


Chapter V. References


Kang, S.; Li, Y.; Fukaya, M.; Lorenzini, I.; Cleveland, D.; Ostrow, L.; Rothstein, J.; Bergles, D. Degeneration and Impaired Regeneration of Gray Matter Oligodendrocytes in


Krämer-Albers, E.-M.; Bretz, N.; Tenzer, S.; Winterstein, C.; Möbius, W.; Berger, H.; Nave,


Minogue, A. M.; Barrett, J. P.; Lynch, M. A. LPS-Induced Release of IL-6 from Glia Modulates Production of IL-1β in a JAK2-Dependent Manner. *J Neuroinflammation* 2012, 9, 629.


Momen-Heravi, F.; Bala, S.; Bukong, T.; Szabo, G. Exosome-Mediated Delivery of Functionally Active miRNA-155 Inhibitor to Macrophages. *Nanomedicine* 2014, 10,
80

Chapter V. References

1517–1527.


Chapter V. References


Chapter V. References


Yamauchi, J.; Miyamoto, Y.; Chan, J. R.; Tanoue, A. ErbB2 Directly Activates the Exchange
Chapter V. References


Annex VI. 1

Pathological role of exosomes in neuroinflammation and in ALS-disease mechanisms

Carolina Cunha¹, Cátia Gomes¹, Sara Pinto¹, Marta Barbosa¹, Ana Rita Vaz¹,², Dora Brites¹,²*

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

All neural cells, including neurons, astrocytes, oligodendrocytes and microglia, release EVs comprising exosomes and ectosomes, either in normal or pathological conditions. EVs are present in many if not all bodily fluids, and were shown to play a key role in intercellular communication and neuroinflammation. Molecular content of EVs is highly specific and includes proteins, lipids and nucleic acids, namely messenger RNAs and microRNAs (miRNAs). Exosomes are endocytic membrane-derived vesicles of small size (30–100 nm) that are originated from multivesicular bodies (MVB) and secreted upon MVB fusion with the plasma membrane. Recent evidences suggest an important role of exosomes in propagating neurotoxic misfolded proteins, or even specific miRNAs, from one cell to another. Indeed, secretion of exosomes derived from motor neuron(MN)-like NSC-34 cells overexpressing mutant hSOD1G93A was proposed as a mechanism of cell-to-cell transfer of mutant SOD1 toxicity. Lately, it was demonstrated that astrocyte-derived exosomes also efficiently transfer the mutant SOD1 protein associated to the amyotrophic lateral sclerosis (ALS) disease to spinal neurons causing selective MN death. Therefore, exosomes besides having a broad impact in disease dissemination, are suggested as target-driven therapies to modulate ALS disease and as promising biomarkers of this devastating disorder. New findings evidenced that miRNA(miR)-155 and miR-146a, two critical miRNAs that regulate inflammation, are present in exosomes released from dendritic cells, and while exosome-delivered miR-155 promoted endotoxin-induced inflammation, exosomal miR-146a down-regulated the inflammatory response. We recently showed that both exosomes and ectosomes (microvesicles with a larger diameter than exosomes resulting from plasma membrane budding) isolated from endotoxin-stimulated microglia exhibit the same inflamma-miRNA representation of the cell of origin. In latest studies, inhibition of miR-155 revealed to slow the disease progression in the SOD1G93A mouse model of ALS. In this work we evaluated the distribution of exosomes released from neurons and glia in neural coculture cell systems recapitulating ALS disease. Additionally we determined the spreading of the misfolded SOD1 and the exosomal differential inflammatory miRNA profiling composition. A special focus was given to the alterations produced by exosomes delivered from ALS-MNs on microglia activation in terms of inflammatory mediators’ expression and induced pathogenicity.
We used the MN-like NSC-34 cell line transfected with human SOD1, either wild-type (wt) or with G93A mutation (mSOD1) and mouse N9 microglial cell line. Primary cultures of astrocytes and microglia were isolated from mSOD1 mice pups and cells used in monocultures and cocultures, in the absence and in the presence of MNs. Exosomes were obtained from cell supernatants by differential centrifugation.

Exosomes from mSOD1-MN, when compared with those from wt, led to permanent loss of microglia phagocytic ability and increased MMP-2 activation from 2 until 24 h incubation. Acute exposure of microglia to exosomes resulted in the production of nitric oxide and pro-inflammatory cytokines, such as interleukin(IL)-1beta and tumor necrosis factor-alpha. At this stage a significant downregulation of miR-146a and miR-124 was observed, thus favoring the observed microglia activation. Remarkably, all the inflammation-related miRNAs, miR-146a, miR-124 and miR-155, were elevated at 24 h incubation relatively to the effects produced by the exosomes resulting from the wt MNs. These results suggest that exosomes from ALS dysfunctional MNs directly participate in microglia proinflammatory phenotype. Intriguingly, microglia also showed elevated markers of cell senescence and among the resulting microglia subtypes we observed cells that are suggestive of morphological alterations compatible with the hallmarks we used to characterize a dystrophic/senescent pool of microglia. Moreover, we observed an increased production of exosomes from ALS MNs, as compared with wt MNs. Such exosomes were preferentially taken-up by microglia and better transferred to the dysfunctional MNs than to the healthy ones.

It may be concluded that exosomes from ALS MNs may be involved in M1 microglia polarization and implicated in the cell-to-cell dissemination of inflammation, while triggering a subpopulation of dystrophic microglia. Clarification of the pathways involved in ALS-associated neuroinflammation will identify key aspects and targets that will contribute to a better understanding of the pathogenicity of glia reactivity in ALS, thus allowing the development of novel therapeutic and diagnostic biomarkers.

Correspondence:
*Dora Brites
Faculdade de Farmácia, Universidade de Lisboa, Avenida Professor Gama Pinto, 1649-003 Lisboa
217946450
dbrites@ff.ulisboa.pt
Annex VI. 2

Conferência Prof. Carlos Garcia
Alzheimer’s disease: mechanistic insights and biomarkers discovery
Paula Moreira, Centro de Neurociências e Biologia Celular, Universidade de Coimbra

14h00 - 15h00
Conferência Prof. Carlos Garcia
Alzheimer’s disease: mechanistic insights and biomarkers discovery
Paula Moreira, Centro de Neurociências e Biologia Celular, Universidade de Coimbra

15h00 - 16h00
Comunicações Orais (Sessão 2)
#Moderação: Odete Cruz e Silva, Paula Moreira

1 - Amyloid-β1-42-induced oxidative stress in mature hippocampal neurons - role of Src kinase (Lígia Fão, Sandra I. Moto, A. Cristina Rego)
2 - Exosomes as mediators of neuroinflammation and pathogenicity in ALS (Dora Brites, Sara Pinto, Carolina Cunha, Cátia Gomes, Marta Barbosa, Ana Rita Vaz)
3 - Bioenergetic defects in pre-symptomatic and symptomatic stages in Huntington’s disease YAC128 mouse model (Carina Marange, Mário Loco, Sandra Moto, Ildefete L. Ferreira, A. Cristina Rego)
4 - Compromised mitochondrial function in cell and animal models of Machado-Joseph disease (Filipa Almeida, Luana Neta, I. Luisa Ferreira, Sara Duarte-Silva, Patrícia Maciel, A. Cristina Rego)
5 - Modulating HMGB1 in microglia as a promising strategy to inhibit specific Aβ-induced inflammatory pathways (Ana Rita Vaz, Maria Carlos Nunes, Dora Brites)
6 - New BACE1 inhibitors decrease Aβ production in cellular and in vivo models of Alzheimer’s disease (Rosa Resende, Marisa Ferreira-Marques, Teresa Dinis, Cláudia Pereira, Arminda E. Santos)
7 - Neuroblastoma cells expressing Amyloid-beta peptide change human microglia microRNA profile and phenotype (A. Fernandes, M. Monteiro, C. Caldeira, D. Brites)
Introduction: Exosomes and microRNAs contribute to a panoply of diseases, including amyotrophic lateral sclerosis (ALS). Exosomes result from multivesicular bodies and are released upon exocytosis. MicroRNAs are small non-coding RNAs. Recent evidences indicate that exosomes-delivered microRNAs to recipient cells regulate the inflammatory response and gene expression.

Aims: Assess if: (i) exosomes from LPS-M1 N9 activated microglia evidence the same microRNA cargo of the cell of origin; (ii) motor-neuron (wild-type and ALS-SOD1G93A) derived exosomes trigger microglia activation; (iii) exosomes from motor-neurons (wild-type and ALS-SOD1G93A) plus/minus microglia distribute differently in recipient cells (motor-neurons alone or in co-culture with microglia).

Methods: M1 and M2 markers, inflamma-miRNAs and inflammatory mediators were assessed in microglia as described (Caldeira et al. 2014). Exosomes were obtained from cell supernatants by sequential centrifugations. PKH67 fluorescent dye was used for exosome identification.

Results: M1-microglia showed upregulation of iNOS and MHC-II (M1-markers) and downregulation of arginase 1 and FIZZ1 (M2-markers). Overexpression of microRNA(miR)-155 and miR-146, and subexpression of miR-124 were reproduced in microglia-derived exosomes. Exosomes from ALS-SOD1G93A motor-neurons, when compared with those from wild-type cells, highly decreased phagocytosis, increased NO and MMP-2 release, and enhanced TNF-α, IL-1β and miR-155 expression in microglia. Secreted exosomes were preferentially internalized by microglia than by motor-neurons, in which they better accumulated in ALS-SOD1G93A relatively to wild-type cells.

Conclusions: MiR-155 is transferred from M1-microglia into exosomes. Exosomes from ALS-SOD1G93A motor-neurons cause microglia activation with increased expression of inflammatory biomarkers, including miR-155, and decreased phagocytosis. In microglia/motor-neuron systems, released exosomes are preferentially taken-up by microglia and better transferred to ALS-SOD1G93A than to wild-type motor-neurons. We conclude that exosomes participate in dissemination of inflammation and may have a key role in motor-neuron degeneration in ALS.

Keywords: microRNAs, exosomes; motor-neurons; microglia, neuroinflammation, amyotrophic lateral sclerosis.

Annex VI. 3
Extracellular vesicles from ALS motor-neurons trigger microglia activation

*Neuron-Glia Biology in Health and Disease*

Pinto S* a, Barbosa M a, Cunha C a, Vaz A R a,b, Brites D a,b

a iMed.ULisboa – Research Institute of Medicines, Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal; b Department of Biochemistry and Human Biology, Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal.

* Email: sfcpinto@campus.ul.pt

Extracellular vesicles (EVs) are released by all cells, including neurons and microglia. EVs comprise exosomes (smaller) and ectosomes (larger) with a key role in intercellular communication, neuroinflammation and spread of neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS). Indeed, secretion of exosomes from motor neuron(MN)-like NSC-34 cells overexpressing mutant hSOD1G93A (mSOD1), was proposed as a mechanism of cell-to-cell transfer of mSOD1 toxicity. Hence, we determined the alterations produced by EVs from wild type (wt) MNs and from mSOD1 MNs, on microglia (dys)functional response at 2 and 24 h of incubation. EVs were obtained from cell supernatants by differential centrifugation and identified by the PKH67 fluorescent dye. Microglial response, inflammatory mediators and inflamma-microRNAs were assessed as usual in our lab.

Furthermore, EVs from monocultures of mSOD1 MNs and from cocultures of mSOD1 MNs+microglia (N9 mouse microglial cell line) were incubated in new cultures of MNs alone and in MNs+microglia cultures to determine their distribution.

EVs from mSOD1 MNs (diameter of ~140 nm by dynamic light scattering) led to an immediate generation of inflammatory mediators by microglia. Actually, we observed increased generation of NO (1.8-fold, p<0.05) and MMP2 (1.5-fold, p<0.01), as well as expression of IL-1β (6.2-fold, p<0.05) and TNF-α (2.7-fold, p<0.05), together with a decrease in microRNA(miR)-124 and 146a expression (respectively 0.3- and 0.4-fold, p<0.01), all suggesting a switch to an activated microglia. Delayed effects at 24 h comprised an elevation of miR-146a, miR-124 and miR-155 (respectively 1.9-, 2.6- and 1.6-fold, p<0.01), indicative of several microglia phenotypes. Loss of microglia phagocytic ability occurred from 2 to 24 h incubation, indicating cell dysfunction, also manifested by the increased number of dystrophic/senescent-like microglial cells (~35%, p<0.05) at 24 h incubation. Interestingly, EVs from cocultures of mSOD1 MNs+microglia (donors) revealed an increased density in recipient microglia, when compared with those from wt MNs+microglia, reinforcing the preferential microglia activation and dysfunction by EVs from mSOD1 MNs.

It may be concluded that EVs from mSOD1 MNs trigger a heterogeneous activated and dysfunctional microglia population, suggestive to be part of the dysregulated cell-to-cell communication, dissemination of inflammation and MN degeneration in ALS disease.

Acknowledgements:

We thank Santa Casa da Misericórdia de Lisboa for the ELA project (DB) and research grant (MB), and the Fundação para a Ciência e Tecnologia (UID/DTP/04138/2013 to iMed.ULisboa, SFRH/BPD/76590/2011 to ARV and SFRH/BD/91316/2012 to CC) for financial support.

References:
