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Dissertação no âmbito do Mestrado em Biotecnologia Farmacêutica orientada pelo Professor Doutor Luís Pereira de Almeida e pela Doutora Liliana Simões Mendonça e apresentada à Faculdade de Farmácia da Universidade de Coimbra.

Setembro de 2018

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O presente trabalho for desenvolvido no grupo de Vetores e Terapia Génica do CNC – Centro de Neurociências e Biologia Celular (Universidade de Coimbra, Portugal), liderado pelo Professor Doutor Luís Pereira de Almeida.

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Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.
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List of Abbreviations

Aβ – Amyloid beta

AD – Alzheimer's disease

Akt – Protein kinase B

APP – Amyloid precursor protein

ATG – Autophagy related protein

BCL-2 – B-cell lymphoma 2

BDNF – Brain derived neurotrophic factor

BSA – Bovine serum albumin

CAG – Cytosine-Adenine-Guanine

CASP – Caspase

CNS – Central nervous system

CSF – Cerebrospinal fluid

cAMP – Cyclic adenosine monophosphate

DMEM – Dulbecco's Modified Eagle's medium

DMEM/F12 – Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12

DTT – Dithiothreitol

DUB – Deubiquitination

ERK – Extracellular signal-regulated kinase

ESCs – Embryonic stem cells

ESCRT – Endosomal sorting complex required for transport

EVs – Extracellular vesicles

FGF2 – Fibroblast growth factor 2

GFP – Green fluorescent protein

HD – Huntington’s disease

Iba1 – Ionized calcium-binding adapter molecule 1

IL – Interleukin

ILV – Intraluminal vesicles

iPSCs – induced pluripotent stem cells

LC3-B – Light chain protein 3

MAP2 – Microtubule associated protein 2

MJD – Machado–Joseph disease

MVB – Multivesicular body

mHtt – Mutant huntingtin

miRNA – Micro RNA

mRNA – Messenger RNA

NESCs – Neuroepithelial stem cells

NGS – Normal goat serum

NK – Natural killer

NMDA – N-methyl-D-aspartate

NPC – Neural progenitor cell

NTA – Nanoparticle tracking analysis

PBS – Phosphate-buffered saline

PCR – Polymerase chain reaction

PD – Parkinson's disease

PFA – Paraformaldehyde

PMA – Purmorphamine

PMSF – Phenylmethane sulfonyl fluoride

PolyQ – Polyglutamine

PVDF – Polyvinylidene fluoride

p38 – Mitogen-activated protein kinase 14

p62 – Nucleoporin 62

ROS – Reactive oxygen species

SCA – Spinocerebellar ataxia

SCA3 – Spinocerebellar ataxia type 3

SDS – Sodium dodecyl sulfate

SHH – Sonic hedgehog

SOD-1 – Superoxide dismutase 1

TBS – Tris-buffered saline

TEM – Transmission electron miscroscopy

TNF – Tumor necrosis factor

TNT – Tunneling nanotubes

TSG – Tumor susceptibility gene

UPS – Ubiquitin-Proteasome System

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Resumo

A ataxia espinocerebelosa do tipo 3 (SCA3), também conhecida como doença de Machado-Joseph (MJD), é uma doença neurodegenerativa hereditária autossómica dominante. Esta doença é devida a uma mutação no gene *MJD1/ATXN3* envolvendo um aumento do número de repetições no codão CAG, que se traduz numa expansão de poliglutaminas na proteína Ataxina-3. O gene mutado apresenta 55-87 repetições CAG, enquanto o gene normal tem 10-51 repetições. A MJD caracteriza-se pela presença de inclusões intranucleares e extensa morte neuronal, afetando diversas regiões cerebrais, particularmente o cerebelo. Clinicamente, os doentes apresentam vários sintomas, como comprometimento da coordenação motora, disartria e disfagia. Atualmente, não há terapia disponível para interromper ou atrasar a progressão da doença.

Existem vários estudos que comprovam a disseminação de proteínas aberrantes associadas a doenças neurodegenerativas entre várias regiões do Sistema Nervoso Central. Vesículas extracelulares (EVs), como exossomas, microvesículas e corpos apoptóticos, demonstraram nos últimos anos envolvimento em várias patologias, em particular, doenças neurodegenerativas. As EVs têm sido associadas à disseminação de proteínas aberrantes em doenças como a doença de Creutzfeld-Jacob, a doença de Huntington ou a doença de Alzheimer. Foi proposto que a disseminação "infeciosa" de proteínas mutantes pode desempenhar um papel crucial na morte neuronal e progressão da doença.

Para estudar este processo, é atualmente possível utilizar Células-estaminais pluripotentes induzidas (iPSCs), obtidas pela reprogramação de células somáticas. As iPSCs revolucionaram o campo dos modelos de doença, na medida em que permitem o acesso a neurónios dos doentes, portanto com toda informação genética do doente, o que lhes confere particular relevância fisiológica. Neste trabalho as EVs testados foram obtidas de células-estaminais neuroepiteliais derivadas de iPSCs

(NESCs), e de culturas neuronais derivadas de NESCs de indivíduos controlo e de doentes de MJD.

O presente trabalho teve como objetivo investigar se a neuropatologia da MJD é disseminada pelo cérebro por meio de EVs, o que poderá promover a agregação de Ataxina-3 mutante, neuroinflamação, mediadores apoptóticos e alterações da autofagia, desencadeando morte neuronal em regiões distantes do cérebro, contribuindo para o agravamento da neuropatologia. Para testar esta hipótese, na primeira tarefa, EVs derivadas de NESCs controlo e MJD foram submetidas a uma caracterização física (tamanho, forma, concentração) e de conteúdo (tais como marcadores de autofagia, marcadores de inflamação, níveis de Ataxin-3 mutantes, marcadores apoptóticos), com o intuito de avaliar potenciais diferenças entre EVs derivados de células controlo e de células MJD.

A segunda tarefa teve como objetivo testar a hipótese das EVs funcionarem como veículos de propagação de Ataxina-3 mutante e de fatores agravantes da neuropatologia. Para isso, foram adicionadas EVs derivadas de NESCs de doentes de MJD a culturas neuronais derivadas de iPSCs de indivíduos controlo. Em seguida, os níveis de Ataxina-3 mutante, de autofagia e de mediadores apoptóticos foram avaliados nessas células neuronais, a fim de avaliar se as EVs de doentes MJD conseguem induzir neuropatologia em células controlo. Além disso, nesta tarefa, também averiguámos se o contato celular é necessário para a formação de inclusões de Ataxina-3 mutante e o agravamento da neuropatologia.

Na terceira tarefa, investigámos se a disseminação de Ataxina-3 mutante ocorre *in vivo*. Com esta finalidade, NESCs derivadas de iPSCs foram transplantadas no cerebelo de murganhos e foi avaliada a presença de inclusões de Ataxina-3 mutantes fora do enxerto.

O nosso trabalho sugere que poderão existir diferenças físicas e fisiológicas entre EVs derivadas de doentes de MJD e controlos. Observámos também diferenças em processos celulares relevantes após a incubação com EVs derivadas de doentes MJD e controlos. Finalmente, concluímos que a disseminação da Ataxina-3 mutante poderá ocorrer tanto *in vitro* como *in vivo*.

Palavras-chave: SCA3, Ataxina-3 Mutante, Doença Poliglutamínica, NESCs derivados de iPSCs, Vesículas Extracelulares, Disseminação.

Abstract

Spinocerebellar Ataxia Type 3 (SCA3), also known as Machado-Joseph Disease (MJD), is an inherited autosomal dominant neurodegenerative disease. This disease arises from a mutation in the *MJD1/ATXN3* gene, in which an increased number of CAG repeats result in an expanded polyglutamine tract in the Ataxin-3 protein. The mutated gene presents 55-87 CAG repeats, while the normal gene has 10-51 repeats. Pathologically, MJD is characterized by the presence of neuronal intranuclear inclusions and extensive neuronal death, affecting diverse brain regions, particularly the cerebellum. Clinically, the patients present several symptoms such as impaired motor coordination, dysarthria and dysphagia. Presently there is no therapy available to stop or delay the progression of this disease.

Evidence for the neuropathological spreading of aberrant, disease-causing proteins across the brain exist. Extracellular vesicles (EVs), such as exosomes, microvesicles and apoptotic bodies, rouse in recent years as playing an important role in several diseases, in particular, neurodegenerative diseases. EVs have been linked to the spreading of aberrant proteins in diseases such as Creutzfeld-Jacob disease, Huntington´s disease and Alzheimer´s disease. It is hypothesized that "infectious spread and seeding" of mutant proteins may play a crucial role in neuronal death and disease progression.

To test this hypothsesis, induced Pluripotent Stem Cells (iPSCs), obtained by reprogramming somatic cells, can be used, iPSCs came to revolutionize the field of disease modeling in a way that researchers have now access to patient's neurons and are able to work with all the genetic background related to the diseases. In this work the tested EVs were obtained from control and MJD-patient´s iPSCs-derived neuroepithelial stem cells (NESCs), and their differentiated neural cultures.

The present work aimed at investigate whether MJD neuropathology is spread through the brain via EVs, which may promote seeding of mutant Ataxin-3,

neuroinflammation, apoptotic mediators and autophagy alterations, triggering mutant Ataxin-3 aggregation and neuronal death in distant brain regions and so contributing for neuropathology aggravation. To test this hypothesis, in the first task, EVs derived from control NESCs and MJD NESCs were submitted to a physical (e.g. size, shape, concentration) and content (e.g. autophagy markers, inflammation markers, mutant Ataxin-3 levels, apoptotic markers) characterization in order to evaluate potential differences between control and MJD patient-derived EVs.

The second task aimed to test the hypothesis of EVs being the spreading vehicles of mutant Ataxin-3 and neuropathology aggravation factors. For this, MJD NESCs-derived EVs were added to control cultures of human iPSCs-derived neurons. Then, mutant Ataxin-3 inclusions, autophagy and apoptotic mediators levels were evaluated in these cells in order to assess if EVs from MJD patients can deliver disease causing factors to control "healthy" cells. Moreover, in this task we also investigated whether cellular contact is needed to seed mutant Ataxin-3 inclusions and aggravation of neuropathology.

In the third task, we investigated whether neuropathological seeding occurs in vivo. For this, MJD iPSCs-derived NESCs were transplanted into the cerebellum of mice and it was assessed the presence of mutant Ataxin-3 inclusions outside the graft.

In general, our research hint physical as well as physiological differences between MJD and control-derived EVs. We also observed differences in key cellular processes upon incubation with MJD and control-derived EVs. Finally we concluded that mutant Ataxin-3 spreading occurs both *in vitro* and *in vivo*.

Keywords: SCA3, Mutant Ataxin-3, Polyglutamine Disorder, iPSCs-derived NESCs, Extracellular Vesicles, Spreading.

.

CHAPTER 1

Introduction

Introduction

1.1. Machado-Joseph Disease

Machado-Joseph Disease (MJD), also known as Spinocerebellar Ataxia Type 3 (SCA3) is the most common form of SCA and was first described in Portuguese immigrants living in the USA, in 1972 (Nakano, Dawson, and Spence, 1972; Schöls et al., 2004). In fact, Portugal is one of the countries most affected by this disease and the Azorean island of Flores has the highest worldwide prevalence (1:239) (Bettencourt et al., 2008). Nevertheless, SCAs are rare diseases, with a worldwide prevalence ranging from 0.3 to 2 per 100,000 (Van De Warrenburg et al., 2002). The mean age of onset is 40, with a wide range between 4 (Carvalho et al., 2008) and 70 years (Coutinho, 1992). The mean survival time after diagnosis is 21 years (Kieling et al., 2007).

MJD is an inherited autosomal dominant neurodegenerative disease that arises from a mutation in the *MJD1* (or *ATXN3*) gene, present in the exon 10 (Ichikawa et al., 2001) of the chromosome 14q32.1, in which an increased number of the cytosine-adenine-guanine (CAG) trinucleotide result in an expanded polyglutamine (polyQ) tract in the Ataxin-3 protein, consequently causing protein misfolding and aggregation. The normal *ATXN3* gene has 10-51 CAG repeats and the mutated gene presents 55-87 repeats (Takiyama et al., 1993; Kawaguchi et al., 1994).

Although not yet fully clarified, it is believed that the physiological function of Ataxin-3 is involved in regulating the ubiquitin-proteosome system and, with the regulation of some transcriptional factors (Li et al., 2002; Evert et al., 2006; Nóbrega et al., 2018). Whereas, the mutant Ataxin-3 leads to a toxic gain of function, because this mutated protein has a tendency to aggregate and form insoluble oligomeric structures causing impairment of several cellular mechanisms (Doss-Pepe et al., 2003; Liu et al., 2016). As well as by the recruitment of other proteins into the inclusions, impairing their normal functions and causing cellular toxicity at several pathways (Donaldson et al., 2003).

1.1.1. Neuropathology of MJD

MJD is characterized by the presence of neuronal intranuclear inclusions and extensive neuronal death, affecting diverse brain regions (Paulson et al., 1997) such as the cerebellum, vermis, dentate nucleus, brainstem, basal ganglia, substantia nigra, thalamus and spinal cord (Figure 1.1); whereas the cerebral cortex seems to be spared from this atrophy (Rüb et al.,, Brunt, and Deller 2008; Schulz et al., 2010; Scherzed et al., 2012; Paulson, 2012). Despite being a hallmark of the disease, evidence suggests that no direct correlation exists between neuronal inclusions and neurodegeneration (Yamada et al., 2004; Paulson, 2012) and, in some studies with other polyQ diseases, it is even suggested that these aggregates may play a neuroprotective role (Arrasate et al., 2004). Interestingly, a direct correlation between the number of CAG repetitions (extension of the polyQ tract) and disease severity has been established, and an inverse correlation exists between the number of CAG repetitions and the age of onset, indicating that the higher the number of repetitions, the sooner the age of onset (Maciel et al., 1995).

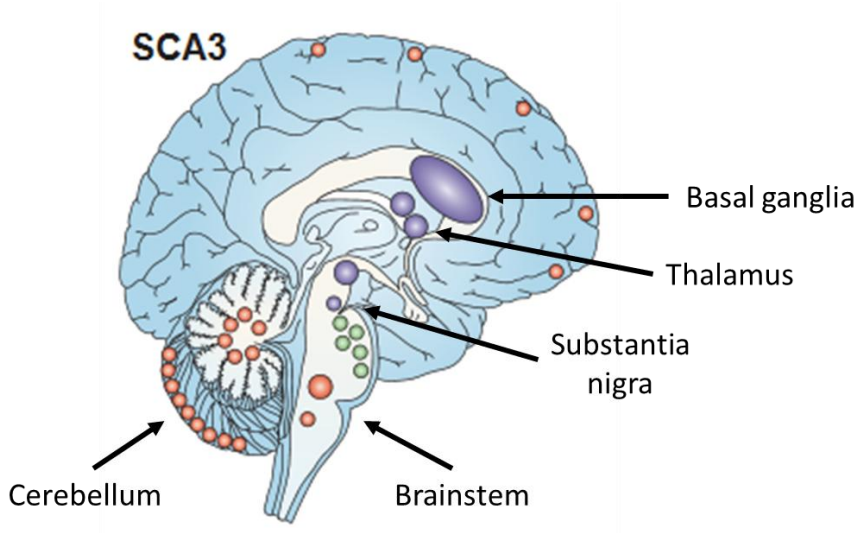

Figure 1.1. Brain regions affected in MJD. Large dots indicate severe neuronal loss. Blue dots indicate involvement of extrapyramidal nuclei. Green dots indicate cranial nerve involvement (Adapted from Taroni and DiDonato, 2004).

1.1.2.1. Proteolytic Cleavage of Mutant Ataxin-3

One of the molecular mechanisms associated to the neurodegeneration observed in MJD is the proteolytic cleavage of mutant Ataxin-3 protein (Figure 1.2). The "toxic fragment" hypothesis postulates that protein fragments are more toxic and aggregation prone than the full length mutant Ataxin-3 protein (Wellington et al, 1998). In fact, it was shown that mutant Ataxin-3 fragments increase in direct relation with disease severity in *post mortem* brains of MJD patients and transgenic animal model (Goti, 2004). These cleaved fragments are thought to be essential for inclusion formation and sequestration of other proteins, causing toxicity and neuronal loss (Goti, 2004; Jung et al., 2009). Particularly, the C-terminal fragment of Ataxin-3, where the expanded polyQ tract is found, was shown to be prone to aggregate in the nucleus, the main subcellular compartment where mutant Ataxin-3 accumulates (Breuer et al., 2010; Evers, Toonen, and van Roon-Mom, 2014). Caspases and calpains were shown to play important roles in this proteolytic cleavage (Shoesmith Berke et al., 2004; Hübener et al., 2013) and, their inhibition alleviated MJD neurodegeneration (Haacke, Hartl, and Breuer, 2007; Simões et al., 2012; Simões et al., 2014).

1.1.2.2. Impairment of Cellular Clearance Mechanisms (UPS and Autophagy)

Cellular clearance processes, like Ubiquitin-Proteosome System (UPS) and autophagy, are impaired in MJD (Figure 1.2). These two mechanisms are cellular "cleaning" systems responsible for the management of cellular waste. Ataxin-3 directly interferes with UPS by deubiquitination (DUB activity) of proteins before degradation (Doss-Pepe et al., 2003; Winborn et al., 2008). However, this is not completely clear because the DUB function seems not to be compromised by mutant Ataxin-3 (Burnett, Li, and Pittman, 2003). Even so, the presence of the polyQ tract and the proteins tendency to accumulate may impair the UPS, leading to deficient removal of misfolded proteins and an accumulation of ubiquitin tagged proteins (Bence, Sampat, and Kopito, 2001). Concerning to autophagy evidence suggests that it is impaired in MJD patients (Ravikumar, 2002; Onofre et al., 2016). It has been reported that proteins associated with autophagy machinery, like p62, LC3-B and Beclin-1, are entrapped within mutant

Ataxin-3 aggregates (Nascimento-Ferreira et al., 2011), which might explain the autophagy impairment given that these protein will not be available to carry out their functions. In this matter, induction of autophagy was shown to alleviate neuropathological and phenotypical hallmarks of MJD (Menzies et al., 2010; Nascimento-Ferreira et al., 2011).

1.1.2.3. Impaired Axonal Transport

Other cell function that might be affected in MJD is the axonal transport, given that the presence of intracellular inclusions may affect the normal transport of signaling molecules and organelles over the axon, leading to neuronal vulnerability and ultimately neuronal death, as was already shown for other polyQ diseases like Huntington's Disease (HD) (Szebenyi et al., 2003). This axonal transport anomaly could help to explain some of the symptomatic manifestations presented by MJD patients (Klockgether et al., 1999) (Figure 1.2). But ultimately no direct correlation could yet be established between impaired axonal transport and MJD neuropathology.

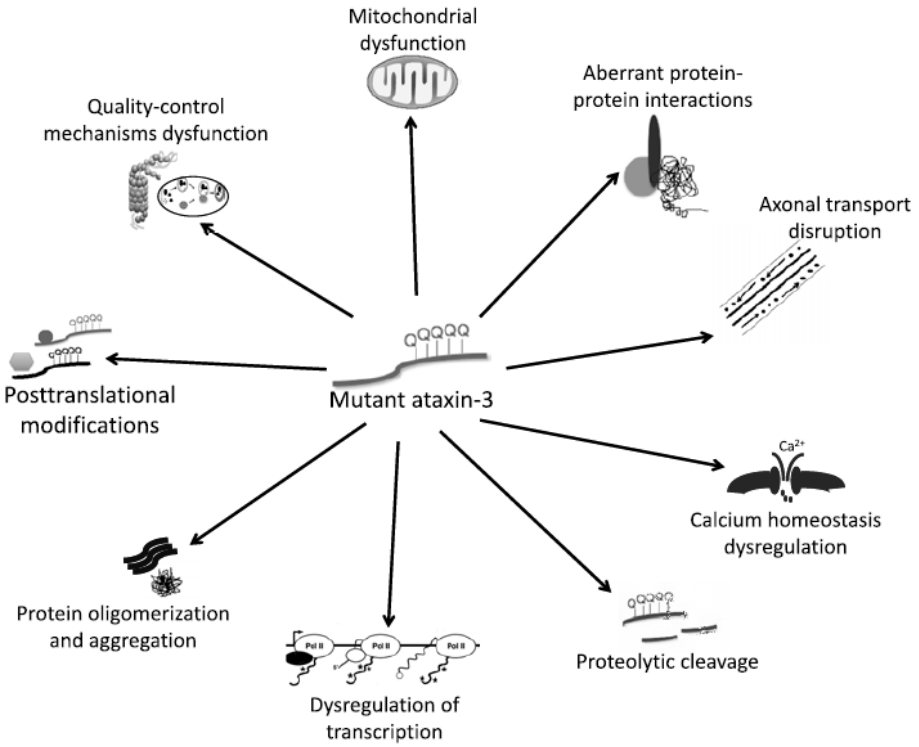

Figure 1.2. Altered cellular mechanisms in MJD. Adapted from Nóbrega, and Pereira de

Almeida, 2012.

1.1.2.4. Dysregulation of Calcium Homeostasis

Calcium homeostasis is another neuronal mechanism that evidence suggests to be dysregulated in neurodegenerative diseases (Bezprozvanny, 2009). This cellular process is crucial for neural viability and synaptic activity and lack of equilibrium in intranuclear calcium concentration may be sufficient to cause toxicity and consequent neurodegeneration (Figure 1.2) (Brini et al., 2014). Evidence specifically linking calcium deregulation to MJD is lacking, however, one study showed motor coordination improvement and prevention of neuronal loss in a MJD transgenic mouse model with long-term treatment with dantrolene, a general calcium stabilizer (Chen et al., 2008).

1.1.2.5. Dysregulation of Transcription

Endogenous Ataxin-3 also presents transcription regulation activity by interacting with several transcription factors like cAMP-response element binding protein (CBP), p300 and their associated coactivators, repressing their transcriptional activity (Figure 1.2) (Li et al., 2002; Chou et al., 2008). The dysfunctional transcription caused by the mutant Ataxin-3 has been implicated in the overexpression of inflammatory signals that cause the characteristic neuroinflammation observed in MJD. Evert and colleagues found that cells expressing mutant Ataxin-3 had increased expression of matrix metalloproteinase 2 (MMP-2), transmembrane protein amyloid precursor protein (APP), interleukin-1 receptor-related Fos-inducible transcript, and the cytokine stromal cell-derived factor 1alpha (SDF1α) mRNA. In the same study, the referred cells also had an upregulation in proinflammatory cytokine interleukin-1beta (IL-1β) and the proinflammatory chemokine SDF1, as well as increased reactive astrocytes and activated microglia (Evert et al., 2001). Another study of the same authors identified several other genes which expression is dysregulated in MJD cells. One of these genes is Brain-Derived Neurotrophic Factor (BDNF), which they show to be downregulated in MJD neurons, resulting in the lack of the essential neurotrophic support provided by this factor (Evert et al., 2003). Additionally, the microRNA's

(miRNA) expression, which have important functions in gene expression regulation, is also dysregulated in MJD (Carmona et al., 2017).

1.1.2.6. Mitochondrial Dysfunction

Regarding mitochondria, evidence suggests that this organelle is highly dysfunctional in several neurodegenerative diseases such as MJD (Figure 1.2) (Johri and Beal, 2012). Particularly for MJD, some studies hinted a mitochondrial dysfunction. These studies showed that mutant Ataxin-3 induces mitochondria-associated apoptotic pathways by upregulating Bax protein and downregulating Bcl-x(L) protein expression, causing oxidative stress and promoting damage and reduction in mitochondrial DNA (mtDNA) copy number (Chou et al., 2006; Yu et al., 2009; Kazachkova et al., 2013; Ramos et al., 2014). Interestingly, fragments from proteolytic cleavage of mutant Ataxin-3 were found inside mitochondria, hinting a potential toxic role of these fragments in damaging this organelle (Pozzi et al., 2008).

1.1.3. Clinical Features of MJD

MJD is a very heterogeneous disease and patients present a wide range of symptoms (Bettencourt and Lima, 2011). Among these symptoms, progressive ataxia is one of the most common, leading to motor incoordination which influences gait, balance and speech (dysarthria and dysphagia) (Jacobi et al., 2015; Lo et al., 2016). Other described symptoms are pyramidal syndrome, spasticity, peripheral neuropathy (affecting both motor- and sensatory- neurons), oculomotor abnormalities, fascial and lingual fasciculation, extrapyramidal signs, including dystonia and rigidity, and parkinsonism (tremors) (Bettencourt and Lima, 2011). Besides these movement impairments, MJD patients also usually present other non-motor symptoms like sleep disorders, cognitive impairment, psychiatric symptoms (including depression and anxiety), fatigue, cramps and chronic pain (D'Abreu et al., 2009; Pedroso et al., 2011; Kawai et al., 2004; Braga-Neto et al., 2012; Cecchin et al., 2006; Schmitz-Hübsch et al., 2010; Friedman and Amick, 2008; Kanai et al., 2003; Franca et al., 2007).

Because of this wide range of symptoms presented by patients and, regarding their non-specificity, for correct diagnosis a combination of the evaluation of these symptoms with imagiological exams (to assess cerebellar atrophy) may be helpful (Mendonca et al., 2018). Ultimately, the genetic test confirms the presence of the disease.

To this day, no disease modifying treatment, capable of stopping or delaying MJD progression, is available. However, some therapeutic approaches may help reducing the associated symptoms. Regular physical activity and physiotherapy can substantially improve patient's motor coordination and balance. Regular speech therapy and occupational therapy are also desirable (D'Abreu et al., 2010; Mendonca et al., 2018).

1.2. iPSC-derived Neural Progenitors (NPCs) as Disease Model for MJD

1.2.1. Disease Models

Animal models (such as mice and rats) and immortal cells lines (e.g. N2A, HEK293, etc.) are by far the disease models most used in R&D. However practical, these models are very limited. Particularly for neurodegenerative disease is important taking into account the higher complexity of the human brain as compared to rodents´ brains, which might explain the failure of several clinical trials (Bart van der Worp et al., 2010; Sayed, Liu, and Wu, 2016; Liu et al., 2018). As for commercial available cells lines as disease models, they lack essential genetic background of the diseases, particularly for neurodegenerative diseases. Also, the specific mechanistic of a given disease, very often not completely known to the scientific community, may not be present (Grskovic et al., 2011; Maury et al., 2011; Xu and Zhong, 2013). Taken together, a need for better models for diseases seems imperative as we render towards personalized medicine (Younesi and Hofmann-Apitius, 2013). As so, induced Pluripotent Stem Cells (iPSCs), with their capacity of originating all type of cells of an organism, present themselves as attractive alternative in disease modeling field, especially for neurodegenerative diseases.

1.2.2. Induced Pluripotent Stem Cells (iPSCs)

The induction of human somatic cells into iPSCs was first demonstrated by Takahashi and colleagues, over a decade ago. In this groundbreaking work, they obtained iPSCs by the retroviral transduction of fibroblast with four transcription factors: Oct3/4, Sox2, Klf4, and c-Myc (Takahashi et al., 2007). The obtained iPSCs resembled human Embryonic Stem Cells (hESCs) in morphology, proliferation, telomerase activity and pluripotency status. Furthermore, this experiments showed the ability of iPSCs to differentiate into any cell from the tree germ layers (ectoderm, mesoderm and endoderm), including neuronal cells. This pioneering work opened the avenue for researchers to gain access to disease- and patient-specific cell lines of any kind. In fact, disease-specific motor neurons of Amyotrophic Lateral Sclerosis (ALS) patients were one of the first disease models to be generated (Dimos et al., 2008). Since then, several other models for neurodegenerative diseases based in iPSCs have been created (Figure 1.3) (Bahmad et al., 2017; Poon et al., 2017).

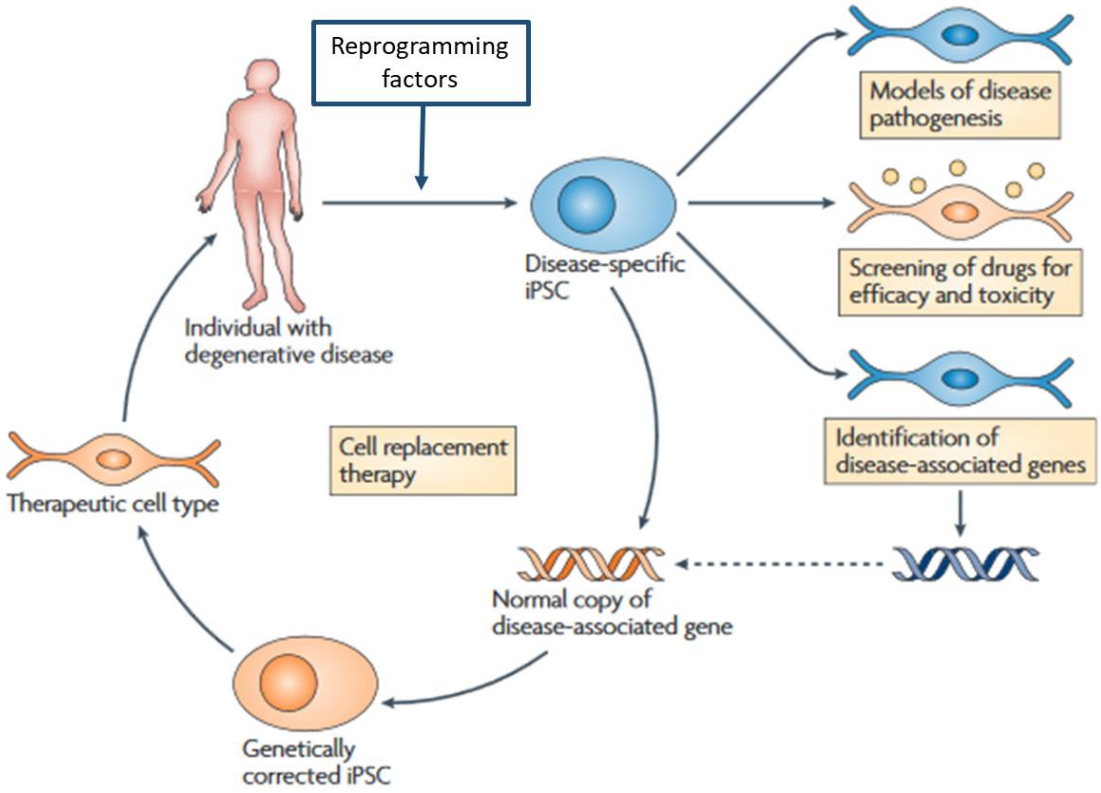

Figure 1.3. Cell reprogramming and potential applications of iPSCs as disease models, drug screening platform and, source of cells for cell replacement therapies (adapted from Fairchild, 2010).

1.2.3. Applications of iPSCs

The potential applications of iPSCs are outstanding and the advantages of their use as disease-models undoubtful. iPSCs have three main applications: i) a source of cells for regenerative therapy, ii) to be used in drug screening and iii) development of disease models (Ebert, Liang, and Wu, 2012; Singh et al., 2015). For cell therapy applications, these cells hold the promise of allowing the implementation of patient-specific cellular therapy and transplantation with minimal probability of immune rejection, as they can be generated from the patient to be transplanted (Liu et al., 2017). In the drug screening field, the exact response from a patient to a drug might be predicted with the use of these cells, avoiding cases of non-responsiveness or severe side effects (Efthymiou et al., 2014). Finally, as a disease model, reprogramming cells from patients will provide iPSCs with the cellular and molecular mechanistic of the disease, then iPSCs can be differentiated in any cell of interest (for example neurons) (Onder and Daley, 2012; Xu and Zhong, 2013; Xie and Zhang, 2014). These features make them a strong model to study pathogenesis and response to treatment. Beside this, their use is not associated to the ethical controversies that the use of hESCs in research triggered. However, the use of iPSCs also presents some limitations. The more frequently used protocol of iPSCs generation requires viral mediated integration of genes in the cells genome. This may result in genome instability, caused by the not controlled integration of the genes that can activate oncogenes or suppress important genes, leading to mutations and aberrant phenotypes (Yoshihara, Hayashizaki, and Murakawa, 2017)**.** Additionally, usually the induction efficiencies are very low, about 1%, and the obtained cells may present epigenetic memory from their progenitor cells (Robinton and Daley, 2012; Xu and Zhong, 2013), contributing to cell genomic instability. Nevertheless, iPSCs are not to be considered as models to be use for all type of diseases. In fact, in case of sporadic diseases, iPSCs may not be able to completely mimic the phenotype since some important environmental key factors may not be present. It is also important to have in mind that the culture system also affects the ability of the cells to model the disease. Given that 2D model does not mimic the complexity of a diseased organ,

with several cell types associated and many supporting structures that may be disease causing. This last limitation however, can be rendered obsolete in a near future with the creation of 3D organoids and organ-on-a-chip platforms (Poon et al., 2017; Liu et al., 2018).

1.2.4. iPSCs-derived Neural Cells

To study the disease mechanisms and potential therapeutic approaches for neurodegenerative diseases, mature neurons, preferentially derived from patients, are the ideal research tools. As so, after the reprogramming of adult somatic cells into iPSCs, a cell stage transition mimicking the embryo neural tube development must occur. Thus, following induction for neural differentiation iPSCs become neuroepithelial cells (NESCs) and, then they might be differentiated into distinct neural lineages such as neurons, astrocytes and oligodendrocytes (Conti and Cattaneo, 2010). In the embryo development NESCs are the first primitive form of neuronal progenitor cell (NPC) to be formed, which can be recreated *in vitro* with the aid of specific morphogenic factors (Reinhardt et al., 2013).

Early approaches in the neural induction field were made in embryoid bodies derived from hESCs that when cultivated with FGF2 gave rise to Nestin-positive neural rosettes, an early structure in human neurodevelopment (Okabe et al., 1996; Zhang et al., 2001). These colonies however, showed limited expansion potential and high genetic variation, as well as required laborious culturing techniques. Later on, observations showed that it is possible to obtain robust and immortal NPCs, without manual culture or selection steps, using only small molecules that activate the neuroectoderm inducing Wnt and SHH signaling pathways (Reinhardt et al., 2013). The obtained NPCs were efficiently differentiate in neural lineage and neural crest (peripheral neurons and mesenchymal cells) progenitors and exhibited useful features as disease model (Reinhardt et al., 2013).

Koch and colleagues successfully isolated from ESCs a type of NESCs that exhibited long-term self-renewing capacity and termed them lt-NES cells (Koch et al., 2009). Lt-NESCs could derive functional neurons that showed ability to undergo synaptic

integration into the host's brain. Furthermore, the same group observed that lt-NESCs could also be derived from iPSCs, with the same self-renewing, expansion abilities (Falk et al., 2012) and ease to differentiate in neuronal cells, demonstrating that the formation of a robust line of NESCs is independent of the type of PSCs line from which is derived. The fact that NESCs represent an early developmental stage, their excellent expansion and differentiation abilities, and, consequently, their less expensive culturing requirements as compared to other sources of human neural progenitors, make them ideal tools in basic research. Moreover, NESCs were already used to model neurodegenerative diseases (Koch et al., 2011; Koch et al., 2012), and more recently were used as cell therapy tool to treat adenosine-deficient brain diseases (Poppe et al., 2018)

1.2.5. iPSCs-derived Neural Cells in the Context of MJD

The first MJD patient-specific iPSCs-derived neuronal model was published in 2011 by Koch and associates (Koch et al., 2011). In this work, they were able to differentiate MJD patient-specific neurons with the expanded CAG triplet and, under calcium excitation, promoted the formation of Ataxin-3 insoluble aggregates. These aggregates were only found in iPSCs-derived neurons and not in the original fibroblasts nor in the progenitor iPSCs, demonstrating a neuron specificity of mutant Ataxin-3 aggregates. Furthermore, they did not observed any relevant neuronal death related to these aggregates, hinting that this may be an early disease stage phenomenon. Since then, several other groups successfully created iPSCs-based neuronal models for MJD, with specific protein markers, morphological, physiological and pathological characteristics presented by typical NSCs and neurons and from diverse progenitor cells (Hansen et al., 2016; Soong et al., 2017). However promising, the cells produced in these experiments appear to be quite immature, which can represent a problem given that MJD is a late-onset disease (Miller and Studer, 2014). So, there is an urge to induce aging in these neurons. The aging-related protein progerin has successfully been able to induce this necessary aging (Miller et al., 2013),

nevertheless this might be a too artificial approach, thus, this is an issue that still needs to be overwhelmed.

Transplantation of patients-specific iPSCs-derived neuronal cells to promote the replacement of dead neurons is the ultimate goal in neuroregeneration approaches for neurodegenerative disease treatment. This kind of therapy ultimately replaces the lost neurons and gives important neurotrophic support to the damaged ones. Mendonça and colleagues successfully demonstrated behavioral impairments alleviation and reduction of typical neuropathological signs in a transgenic MJD mouse model upon transplantation of cerebellar neural stem cells (NSCs). The injected NSCs were able to differentiate into neurons, astrocytes and oligodendrocytes and provide neurotrophic support to the diseased cerebellum as well as promoting reduction in inflammation levels (Mendonca et al., 2015). This work has opened the avenue for the transplantation of iPSCs-derived neural progenitors in MJD patients.

The recent development of precise and very efficient gene editing tools, such as the CRISPR-Cas9 system, opened another interesting avenue in iPSCs-based research for MJD. It is expected in the near future that the two technologies may be combined for the correction of the disease causing mutation, rendering the patient specific neurons ready for transplantation (Bassett, 2017; Ouyang et al., 2018).

1.3. Extracellular vesicles (EVs)

Extracellular vesicles (EVs) are organelles with membrane origin, which have been associated with several physiological and pathological mechanisms. The term extracellular vesicles is a broad terminology that englobes exosomes, microvesicles, ectosomes, apoptotic bodies and other similar structures that have been described over the years. In the dawn of their discovery it was usual to give different names to the same type of vesicles, this fact causes some confusion due to a lack of terminology consensus that is only now been reached. For example, given their described characteristics, microvesicles and ectosomes may be considered the same

kind of EVs. To simplify the nomenclature, the term extracellular vesicles (EVs), englobing the entire set of described vesicles, is considered the most correct terminology (Gould and Raposo, 2013; Raposo and Stoorvogel, 2013; Cocucci and Meldolesi, 2015; van Niel, D'Angelo, and Raposo, 2018).

EVs are phospholipid bilayers enriched in cholesterol, sphingomyelin and ceramide, with the capability of transporting several cargoes such as proteins, lipids and RNA in their aqueous lumen or in the membrane (Budnik, Ruiz-Cañada, and Wendler, 2016). Interestingly, EVs react to stimuli, so their cargo is highly dependent on the cell context (Lötvall et al., 2014). Consequently, the scientific community has been exploring the potential use of EVs as biomarkers for diagnostic, evaluation of disease progression and, measure of response to therapy (Thompson et al., 2016; Croese and Furlan, 2017). In fact it is believed that this vesicles can transport pathologic molecules (proteins and RNAs) between different cells, leading to disease spreading. Nevertheless, EVs may also display a neuroprotective role, namely in AD, Yuyama and colleagues, described that neuronal EVs, when injected into the brain of APP transgenic mice, act as scavengers for toxic Aβ protein (Yuyama et al., 2015). This neuroprotective role for EVs in AD is reinforced by the presence of Cystatin C, which is thought to be neuroprotective in AD, in EVs derived from primary mouse neurons cultures (Ghidoni et al., 2011). MicroRNA miR-193b is believed to target APP mRNA, blocking its translation. In EVs, this miRNA was observed to be decreased in blood and CSF of AD patients as compared to patients in early stages of the disease and controls (Liu et al., 2014). These finding reinforce the notion that EVs have potential to be used as biomarkers in AD. Finally, EVs have been evaluated as vehicles for therapeutic approaches in neurodegenerative diseases and cancer (Sarko and McKinney, 2017; Rufino-Ramos et al., 2017; Gilligan and Dwyer, 2017).

1.3.1. EVs Overview

References to a platelet "dust", described in the mid 1960's, can nowadays be considered as the first report of the pelleting of microvesicles, commonly shed by platelets (Wolf, 1967). As for exosomes, the first concrete report that describes them

is from 1983, when two different publications described them as a consequence of reticulocyte maturation. In these works, reticulocyte were shown to use these structures to recycle transferrin receptors and eliminate its excess from cell membrane (Pan and Johnstone, 1983; Harding, Heuser, and Stahl, 1983). Initially they were thought as cellular waste and with no part to play in relevant biological processes. Consequently, the scientific community practically ignored these particles for long decades. It was only in the late 1990's that two publications sparked a new interest regarding extracellular vesicles. These works described, for the first time, the role of EVs in intercellular communication, as they were shown to be released by B lymphocytes and Dendritic Cells (DC's) (Raposo and Nijman, 1996; Zitvogel et al, 1998). These publications hinted a role for EVs in immune modulation as the presence of MHC-II in the vesicles stimulate specifically $CD4^+$ T cells in vitro and, further the ability to suppress tumor growth *in vivo*. A decade later, the discovery that EVs can also transport functional RNA, such as miRNA, reinforced the interest in these structures (Ratajczak et al., 2006; Valadi et al., 2007). Recently, references to structures called exophers have been made. These are extremely large vesicles (4000 nm in size), described to be released in *C. elegans* under neurotoxic stress conditions and carry protein aggregates and damaged mitochondria (Melentijevic et al., 2017). However interesting, this information requires further investigation to evaluate if exophers are also present in human cells.

The fact that EVs are present in all eukaryotic, as well as prokaryotic, organisms, as communication or defense system (Deatheragea and Cooksona, 2012), shows that these vesicles are an evolutionary conserved mechanism. EVs are constitutively released by virtually all cell types and can be found in any biofluid, including breast milk, saliva, blood, urine and semen (Raposo and Stoorvogel, 2013; Yáñez-Mó et al., 2015; van der Merwe and Steketee, 2017; Paolicelli, Bergamini, and Rajendran, 2018).

1.3.2. EVs Cargo and EVs-Cell Interaction

Cargo transport between cells via EVs is important because it protects molecules that otherwise would be degraded by the extracellular conditions (like pH or enzymes)

(Keller et al., 2011). Consulting online databases like ExoCarta (Available online: http://www.exocarta.org) and Vesiclepedia (Available online: http://microvesicles.org), where researchers add information about their findings concerning EVs cargo; it can be appreciated that thousands of molecules were already described to be carried by EVs. Over 40 000 proteins, 7000 RNA and 1000 lipids were already associated to EVs (Keerthikumar et al., 2016) and the number is increasing every day, due to the extensive research developed in this filed in recent years. Interestingly, using next-generation sequencing, it has been described that a large number of RNA molecules carried by EVs are non-coding RNAs, underlining the regulatory gene expression exercised by EVs in target cells (Nolte'T Hoen et al., 2012).

Nevertheless, for EVs to execute their biological functions they need to be taken up, or at least have some sort of contact, with recipient cells. It has been reported that this process is not random, instead results from a specific interaction of EVs with target cells (Figure 1.4) (Fitzner et al., 2011; Montecalvo et al., 2012; Prada et al., 2016); whereas the docking and cellular uptake of EVs can be mediated by several pathways with multiple key players. In some cases, the docking of EVs surface proteins or lipids with specific cell receptors is enough to induce a cascade of events leading to a biological response (Zhang et al., 2006); Prada et al., 2016). In other pathways, internalization and delivery of EVs content into the target cell are crucial (Valadi et al., 2007; Fitzner et al., 2011; Montecalvo et al., 2012). This protein interaction has been shown to be orchestrated by families of proteins like tetraspanins, integrins, proteoglycans and lectins. The main internalization mechanisms involved in EVs uptake are endocytosis (clathrin-dependent and independent, pinocytosis, macropinocytosis and calveolin-mediated internalization) and plasma membrane fusion with EVs (Mulcahy, Pink, and Carter, 2014; French, Antonyak, and Cerione, 2017). Evidence suggests that EVs internalization is a quick process (as fast as 15 minutes after incubation), energy-dependent, as demonstrated by the reduced internalization verified when cells are kept at 4ºC, and highly dependent on cytoskeleton dynamics (Feng et al., 2010; Fitzner et al., 2011; Fabbri et al., 2012; Montecalvo et al., 2012; Tian et al., 2013; Christianson et al., 2013).

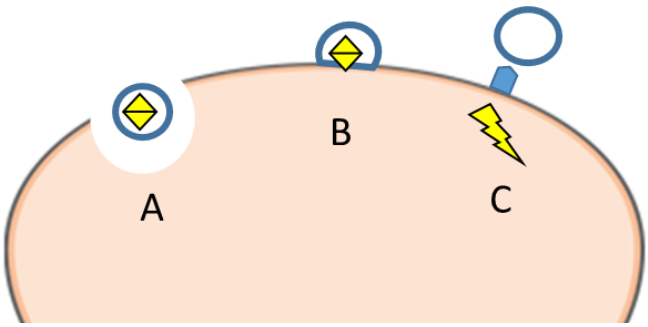

Figure 1.4. Signaling/uptake pathways for extracellular vesicles (EVs). (A) EVs can be internalized by endocytosis, which is believed to be the main uptake pathway. EVs can also be internalized by (B) direct fusion with the plasma membrane of the target cell releasing their cargo into the lumen. Finally, (C) EVs can interact with the target cells without being internalized via surface receptor interaction and activation of signaling pathways.

1.3.3. Physiological Functions of EVs

Among the outstanding functions that have been associated with EVs are cellular waste management and regulation of inflammation, cellular differentiation, coagulation and cell adhesion and communication (van der Pol et al., 2012). The first described immune modulation is particularly important in several aspects. EVs have been shown to present on their surface Fas ligand (FasL), a death receptor agonist. These EVs are able to interact with T cells, causing apoptosis and, consequently, immune suppression. This mechanism is used by tumor cells to evade the immune system and proliferate and, is also important in pregnancy, so that the mother's immune system does not recognize and attacks the forming embryo (Frängsmyr et al., 2005; Abrahams et al., 2003; Jeong et al., 2005). Tumor-derived EVs are also able to inhibit NK cells proliferation, again suppressing the immune response against cancer cells (Liu et al., 2006; Berchem et al., 2016). The role of EVs in cancer progression was empathized by Chalmin and colleagues that observed an increased efficacy of the chemotherapeutic drug cyclophosphamide when co-administered with dimethyl amiloride, a drug that inhibits EVs production (Chalmin et al., 2010). The opposite is also valid, since EVs derived from synovial fibroblasts of rheumatoid arthritis patients expose in their surface TNF-α. This delays T cell mediated apoptosis, prolonging cell survival (Zhang et al., 2006). This mechanism was already shown in cancer and infectious diseases (viral and bacterial) (Robbins, Dorronsoro, and Booker,

2016). These observations lead to the idea that EVs, depending on the physiological or pathological state of their progenitor cell, may have different cargo and, therefore, display different activities when interacting with acceptor cells. EVs derived from Antigen Presenting Cells (APCs), such as Dendritic Cells (DCs), macrophages and B cells, have also been shown to carry factors that modulate the immune system in both, stimulatory and suppressor ways (Bobrie et al., 2011; Robbins and Morelli, 2014; Lindenbergh and Stoorvogel, 2018). The fact that EVs derived from these cells express the same surface proteins as their progenitor cells reinforces the hypothesis that they may assume similar functions as their progenitor APCs in terms of immunomodulation (Robbins and Morelli, 2014). Due to EVs mobility, this immunomodulatory effect may occur in cells that are distant from APCs, leading to a long distance immunoregulation via EVs. Examples of factors present in these EVs are the Major Histocompatibility Complex (MHC) I and II and adhesion molecules (Raposo and Nijman, 1996; Zitvogel et al., 1998; Segura, Amigorena, and Théry, 2005), which indicates a potential role of EVs in antigen presentation and, thus, immune system modulation when internalized or in contact with T cells, NK cells, macrophages or, most importantly, other APCs (Raposo and Nijman 1996; Monleon et al., 2001; Bhatnagar et al., 2007; Munich et al., 2012).

The removal of unwanted molecules via EVs is also a key factor. This cellular pathway is apparently crucial in cell homeostasis and response to internal and external stress. Cancer cells have been shown to release EVs enriched in anticancer drugs (Aubertin et al., 2016). Caspase-3, an inductor of cell apoptosis, was also enriched in EVs as a mechanism of cell survival (Böing et al., 2013). EVs have the ability to mediate inflammation by transporting key inflammatory mediators, such as Interleukin-1β (IL-1β) (MacKenzie et al., 2001), and carry in their surface tumor necrosis factor (TNF) receptor 1 (Hawari et al., 2004) and also carry functional enzymes for the production of leukotrienes (Esser et al., 2010). The lipid components of EVs are also able to activate inflammation through activation of Toll-like receptor 4 (TLR4) (Manèek-Keber et al., 2015). Interestingly, mesenchymal stem cells-derived EVs, enriched in miR-133b, were capable to promote neural regeneration and neurite outgrowth in

neurons and astrocytes (Xin et al., 2012). Taken together, the above mentioned observations hint important functions for EVs in the organism homeostasis and pathological processes.

1.3.4. EVs in the CNS

The Central Nervous System (CNS) is based in the cellular communication between neurons, microglia, oligodendrocytes and astrocytes. This communication is essential to maintain the homeostasis in this organ and, its response to stimuli and developmental signals. The mechanisms underlying the many ways of interneural communication have been widely studied. Over the past few years, EVs secreted by brain cells (Fauré et al., 2006) have risen as a key component in several CNS stages of development, functions and, its response to stimuli and pathology. It is clear that neural cells-derived EVs are able to specifically recognize other brain cells (Chivet et al., 2013). The information carried by EVs may promote a series of functions in the CNS, including neuronal plasticity, synaptic activity, signaling or even the CNS biogenesis (Budnik et al., 2016).

Wnt is a family of morphogens critical in processes of brain development, cell migration and proliferation, as well as cell differentiation and plasticity (Mulligan and Cheyette, 2012). Evidence suggests that the transmembrane protein type II known as Evenness Interrupted/Wntless/Sprinter (Evi/Wls/Srt) is crucial for Wnt1 secretion and, therefore, to correct synapse formation in the neuromuscular junction of *D. melanogaster* (Bartscherer et al., 2006; Korkut et al., 2009). The Evi protein was found associated with EVs in synapse formation and neuronal network development processes. Given this, it can be assumed that both, Evi and Wnt, are released from presynaptic junctions via EVs. This work also demonstrated that this transport can occur in a trans-synaptically fashion (Korkut et al., 2009).

Additionally, glutamate release at the synaptic level was demonstrated to be influenced by microglia-derived EVs in a dose-dependent way, independently of EVs cargo. In this work, the sole contact of the vesicles with the neuron's surface was enough to increase synapse activity, via promotion of sphingolipid metabolism,

indicating that this excitatory effect is promoted by EVs surface components. This work is another proof of the complex network of communication between neurons and glia using EVs as message carrier (Antonucci et al., 2012). Microglia-derived EVs carry inflammatory signals (such as IL-1β and COX2), which can promote reactivity of other uptaking microglial cells, leading them to produce also inflammatory signs, contributing to the inflammation in the brain (Frühbeis, Fröhlich, Kuo, and Krämer-Albers, 2013). Interesting work showed that oligodendrocytes, upon glutamate stimulation, were able to release EVs, in a calcium-dependent manner (using NMDA and AMPA receptors) that were internalized by neurons, which protected them under conditions of oxidative stress and starvation (Frühbeis, et al., 2013). Indeed, in neuronal activity upon glutamate stimulation, the calcium equilibrium of the cell and particularly the NMDA receptors seem to be key mediators in the mechanism of EVs release by neural cells as shown by the experiments where NMDA inhibition or calcium depletion at the synapse was sufficient to abruptly reduce EVs release. These results indicate that oligodendrocyte-derived EVs are active and display a neuroprotective role when internalized by primary cultured mouse cortical neurons. Although interesting, this work did not explore the nature of the oligodendroglial-derived EVs content responsible for the neuroprotection observed.

It is well known that oligomannosidic glycans play interesting roles in brain development and brain function maintenance, promoting neurite outgrowth. Wang and colleagues found that the protein Synapsin I, which is implicated in neural development and synaptic transmission because it is an oligomannose-binding protein, and that the presence of oligomannose is essential to promote neurite outgrowth and synaptogenesis. Synapsin I was further demonstrated to be released from cultured cortical astrocytes via EVs in conditions of glucose depravation or oxidative stress, in a selective fashion, indicating a neuroprotective function for these EVs (Wang et al., 2011).

These studies strengthen the hypothesis that EVs represent an active way of communication in the brain. Remarkably, it has been reported that glial cells release EVs enriched in factors that are specific of their cellular function. For example, a

notable work showed that oligodendrocytes release EVs enriched in myelin and other trophic factors responsible for axon support (Krämer-Albers et al., 2007).

Interestingly, as EVs are able to cross the Blood-Brain Barrier (BBB), their effects may not be exclusive to the CNS, as neural-derived EVs can be found in peripheral compartments (Zhuang et al., 2011).

In conclusion, these works emphasized the role of EVs in the CNS as vehicles for important factors, contributing for neural development and neuroprotection under stress conditions.

1.3.5. EVs Biogenesis

1.3.5.1. Exosomes Biogenesis

Exosomes are the most studied EVs regarding biogenesis. Exosomes are derived from late endosomes that, by inward budding of the cell membrane, form small structures called Intraluminal Vesicles (ILV) inside a larger structure, the Multivesicular Bodies (MVB). MVB then undergo one of two possible faiths: they fuse with the plasma membrane, releasing ILV as exosomes into the extracellular medium, or they fuse with lysosomes, leading to degradation of their content (Hanson and Cashikar, 2012). How MVB chooses one of these faiths remains undisclosed but some evidence suggests that cholesterol presence may play an important role in this choice (Möbius et al., 2003). The formation of MVB can be mediated by two distinct pathways: the *Endosomal Sorting Complex Required for Transport* (ESCRT)-dependent pathway and the ESCRT-independent pathway (Figure 1.5).

The ESCRT-dependent pathway is a highly complex multimolecular machinery composed of four sub-units: ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III. In this process, ubiquitinated cargo is recognized by ESCRT-0, -I and –II, and invagination is promoted by curvature-inducing factors present in ESCRT-I and ESCRT-II. Finally, the binding of ESCRT-III to ESCRT-II leads to deubiquitination of the cargo and consequent abscission, creating ILV (Williams and Urbé, 2007; Hurley and Hanson, 2010; Rusten, Vaccari, and Stenmark, 2012).

The ESCRT-independent pathway accounts for structural lipid alterations in the membrane of late endosomes. In specific regions of the membrane, a lipid raft occurs, with the substitution of sphingomyelin by ceramide, promoted by the enzyme sphingomyelinase 2, inducing the inward budding of the region and forming ILV (Trajkovic et al., 2008). Both mechanisms seem to interfere in exosome biogenesis and the input of both for the end result depends on the cell type and state (van Niel et al., 2018). The final step in exosome formation is the docking and fusion of the MVB with the plasma membrane. In this regard Rab GTPases, such as Rab27a, Rab27b and Rab35 have been shown to be crucial (Ostrowski et al., 2010; Hsu et al., 2010). Once in the extracellular medium, ILV are termed exosomes (Colombo et al., 2014).

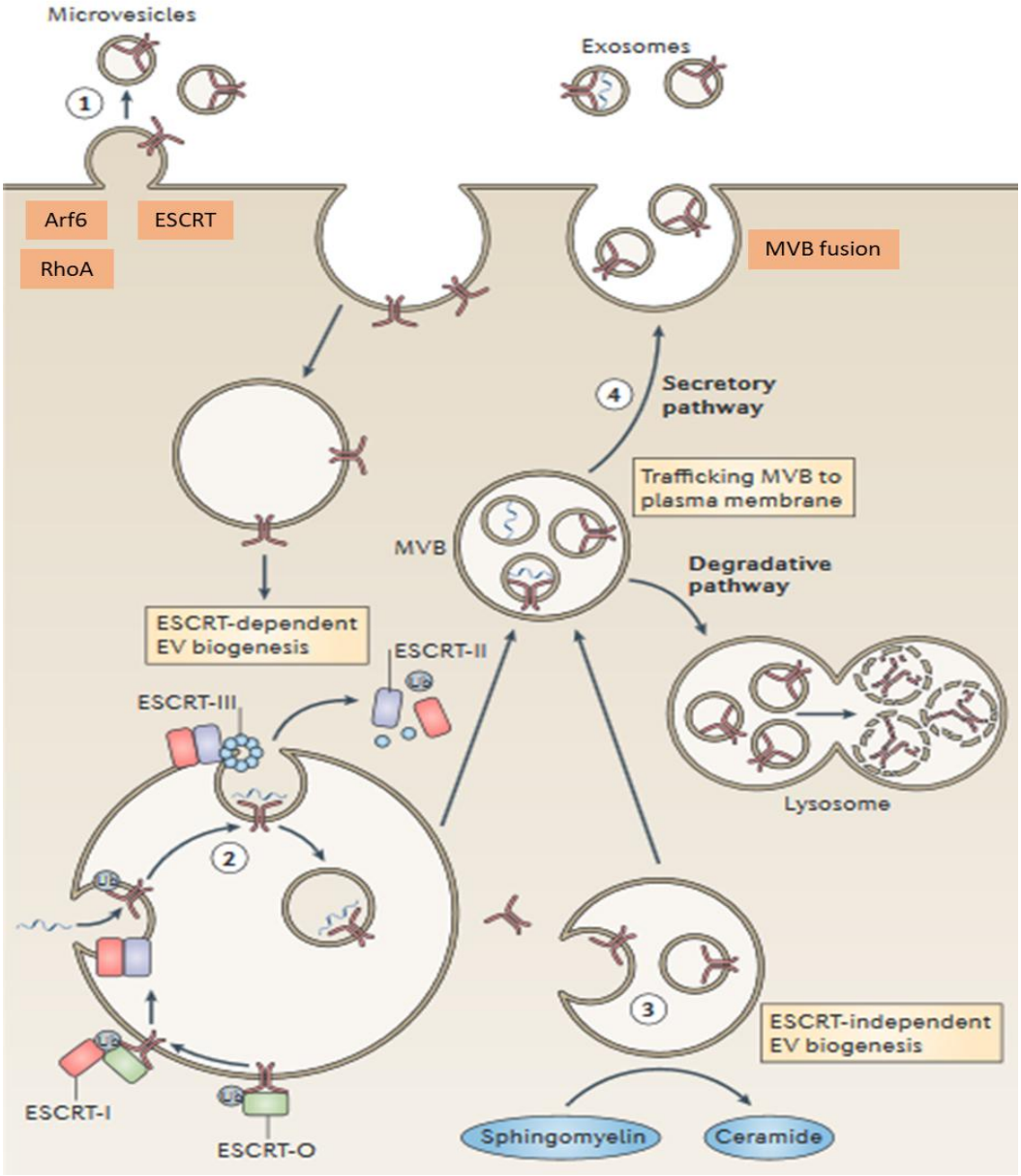

Figure 1.5. Extracellular vesicles biogenesis. (1) Biogenesis of microvesicles/ectosomes by direct pinching off from the plasma membrane. Biogenesis process of exosomes (2) via ESCRT-dependent and (3) ESCRT-independent pathways. (4) Biogenesis process resulting from fusion with the plasma membrane and ILV release as exosomes. Adapted from Thompson et al., 2016.

1.3.5.2. Microvesicles/Ectosomes Biogenesis

The biogenesis of microvesicles/ectosomes is a highly irregular process that begins with the gathering of the cargo in the inside surface of the plasma membrane in specific regions. Then, by physical reorganization of the actin cytoskeleton involving Arf6 and RhoA, an outward budding occurs leading to the pinching off of microvesicles (D'Souza-Schorey Crislyn and Clancy, 2012; Li et al., 2012). These plasma membrane regions are enriched in cholesterol and glycosphingolipids, and are called lipid rafts (Budnik et al., 2016). Again, hydrolysation of sphingomyelin by the enzyme sphingomyelinase originate ceramide, as well as some components of the ESCRT machinery, which appear to be crucial in the curvature and scission of the blebs (Raposo and Stoorvogel, 2013).

1.3.5.3. Apoptotic Bodies Biogenesis

Apoptotic bodies are the largest type of extracellular vesicle (1–5 μm in diameter) (Poon et al., 2014; Atkin-Smith and Poon, 2017). These particles share a similar biogenesis process with microviscles/ectosomes, in a way that both kind of vesicles bleb from the plasma membrane due to molecular rearrangements (Akers et al., 2013). Nevertheless, apoptotic bodies are generated from apoptotic cells through a process termed apoptotic cell disassembly (Atkin-Smith et al., 2015), giving rise to EVs that carry intact organelles and complete pieces of DNA from the dying cell (Holmgren et al., 1999; Elmore, 2007; Taylor, Cullen, and Martin, 2008). As so, apoptotic bodies are often characterized by the presence of histones. Additionally, they are highly irregular in shape and size when visualized by TEM due to this biogenesis process (van der Pol et al., 2012). Interestingly, recent data hints that apoptotic bodies share surface markers from their cell of origin, linking possibly their

function with their origin, and that their content highly depends on their biogenesis process (Jiang et al., 2017). In Table 1.1 are summarized the main physical characteristic of the aforementioned EVs.

Table 1.1. Physical and biological characteristics of Exosomes, Microvesicles/Ectosomes and apoptotic bodies.

	Size (nm)	Biogenesis	Enriched markers	Sedimentation*	Origin
Exosomes	**30-150**	**ESCRT-dependent or independent; lipid raft**	**Alix, CD63, CD81, TSG101 and flotilin-1**	**100,000g**	**Late endosome MVB**
Microvesicles/ Ectosomes	**50-1000**	**Cytoskeleton reorganization and pinching off**	**CD40, PS**	**20,000g**	**Plasma membrane**
Apoptotic Bodies	**1000-5000**	**Cytoskeleton reorganization and disassembly of apoptotic cells**	**Histones, DNA**	**10,000g**	**Cells undergoing apoptosis**

*centrifugation acceleration. PS: phosphatidylserine; ESCRT: *Endosomal Sorting Complex Required for Transport*; MVB: Multivesicular Body. The displayed data was adapted from: Basso and Bonetto, 2016; Cocucci and Meldolesi, 2015; Raposo and Stoorvogel, 2013; van Niel, D'Angelo and Raposo, 2018; Thompson et al., 2016; van der Pol et al., 2012.

1.3.6. EVs Isolation Methods

Often, it is difficult to distinguish between the different types of EVs due to some size overlap and presence of the same cell surface makers. Thus, there is an increasing demand for strategies that can correctly distinguish them. Noteworthy, presently there are no specific markers for the different EVs types. What is observed is an

enrichment in some markers, characteristic of the different EVs types (Kowal et al., 2016). These markers are often related to the biogenesis process of the vesicles above explained. As so, EVs are typically enriched in ESCRT machinery-related components such as ALIX, Flotilin-1, TSG-101 and tetraspanins (CD9, CD63) and lipid raft associated glycoproteins such as phosphatidylserine (Raposo and Stoorvogel, 2013; Colombo, Raposo, and Théry, 2014; Thompson et al., 2016).

Given the complexity associated with EVs, there is no consensus regarding the best isolation method (Lötvall et al., 2014). The most used isolation method for EVs is differential centrifugation (Théry et al., 2006). This method consists in a series of centrifugations, followed by a filtration step, to clean up the vesicles from cell debris and, a final ultracentrifugation to pellet the EVs. However, this method is not ideal in the way that it is very difficult to discriminate between different types of vesicles and other molecules that can be trapped in the pellet, consequently leading to overestimation of the cargo content of the vesicles. Additionally, EVs functionality may also be compromised by this isolation method. Other isolation methods consist in approaches such as density gradient centrifugation, size exclusion chromatography, immune affinity based methods, ultrafiltration and commercially available EVs precipitation kits (for example Exo Quick). Konoshenko and associates recently made a detailed and accurate review of all EVs isolataion methods available (Konoshenko et al., 2018).

Thus, having this in mind, the isolation method adopted should take into account the downstream application of the EVs (van Deun et al., 2014; Lötvall et al., 2014; Szatanek et al., 2015).

To minimize the discrepancy of isolation methods used and to standardize the criteria applied in terms of positive and negative controls used to identify EVs, the International Society for Extracellular Vesicles (ISEV) published a set of minimal experimental requirements (Lötvall et al., 2014) that should be followed to avoid substantial differences in experimental results from similar experiments. This position paper recommends that research articles exploring EVs functions and biogenesis

should be extensively detailed in the methodologies used and correct use of all appropriate negative and positive controls.

1.4. Neurodegenerative Disease Seeding and Spreading

1.4.1. Common Features of Neurodegenerative Diseases

Neurodegenerative diseases share a number of clinical features and neuropathological molecular processes. Loss of autonomy, dementia, and movement incoordination are common clinical characteristics of patients suffering from neurodegenerative diseases. Neuropathologically, protein-specific inclusions, brain inflammation and oxidative stress are found in the great majority of cases. Particularly the protein aggregates represent a relevant hallmark of neurodegenerative diseases and are characteristic for each disease; for example, amyloid-β and tau are hallmarks in Alzheimer's disease (AD), α-synuclein in Parkinson's disease (PD), mutant huntingtin (mHtt) in Huntington´s disease, and mutant Ataxin-3 in MJD. Interestingly, these aggregates share an abnormal enrichment in β-sheet structures, filamentous physical characteristics, and may suffer proteolytic cleavage, which results is even more aggregation prone oligomers (Guo and Lee, 2014). Another interesting common characteristic is the fact that disease onset affects primarily one specific brain region. Apparently, from this focal point, occurs a spread of the pathology, topographically predictable, to other brain areas according to the disease and its progression as demonstrated in Figure 1.6. (Goedert, 2015).

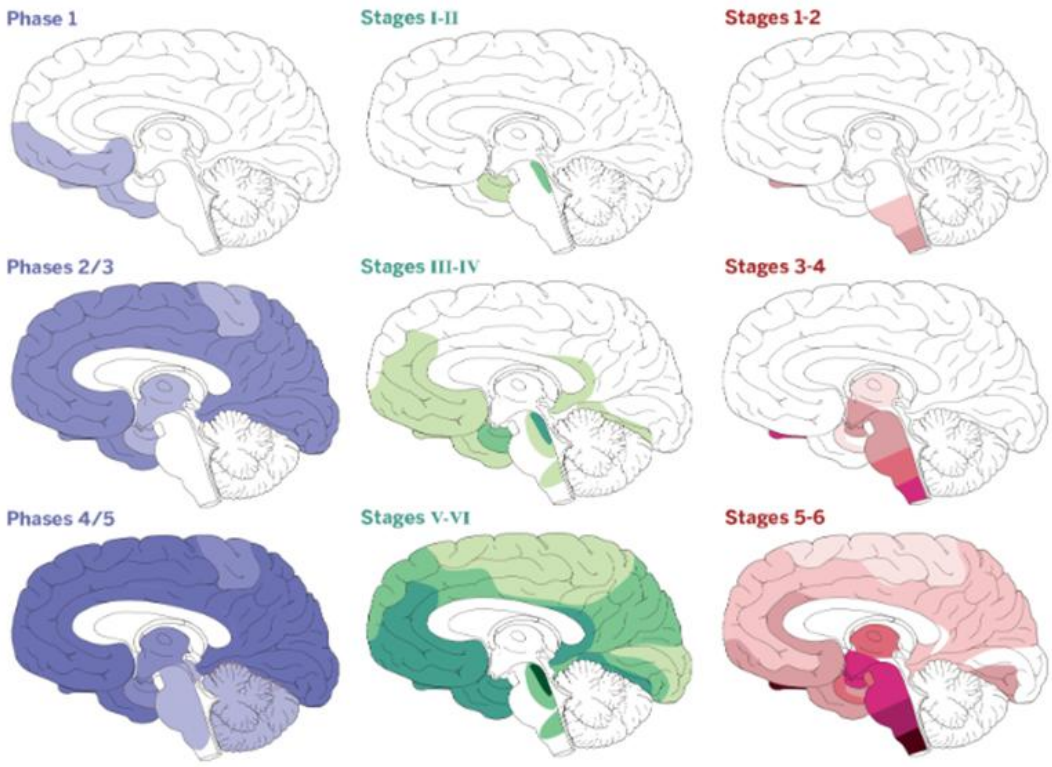

Figure 1.6. Disease spreading across the brain of pathology-associated proteins: β-amyloid (blue), tau (green) and α-synuclein (red). Spreading occurs with an initial focal point and in late-stage disease cases is present in most brain areas. Adapted from Goedert et al., 2015.

1.4.2. Creutzfeld-Jacob Disease

Nevertheless, only Creutzfeld-Jacob disease is proven to be "infectious". In this type of disease, the normal prion protein can be corrupted by the misfolded pathogenic form (seeding process) (Figure 1.7.), and then, spread from cell to cell (spreading process), between individuals and even between different species, as the transmission prions from cattle to humans by consumption of contaminated meat is well documented (Prusiner, 1998; Aguzzi, Montrasio, and Kaeser, 2001). The "infectious" inter-species and inter-individuals spread of the pathogenic protein, to date, is exclusive to Creutzfeld-Jacob disease (Guest et al., 2011). However some evidence points out a possible similar mechanism of spreading and seeding of disease-specific

misfolded proteins in other neurodegenerative diseases. Over the last decade, the possibility of a prion-like spreading and seeding of disease-related pathogenic proteins from cell to cell in the CNS has been risen (Guo and Lee, 2014; Thompson et al., 2016). This phenomenon spiked the interest of the researchers given the possibility of delaying or even stopping disease progression by arresting the transmission of the aberrant disease causing proteins between cells.

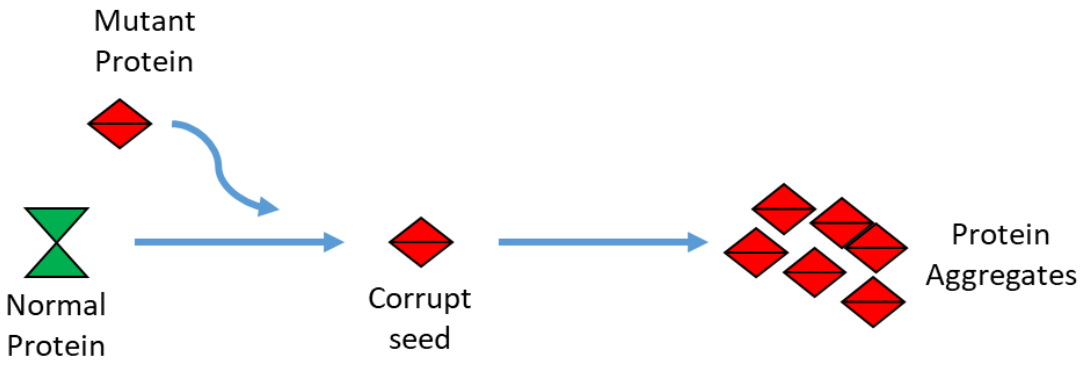

Figure 1.7. The prion-like protein "seeding" hypothesis. This theory states that like prions, misfolded disease-specific proteins are prone to corrupt normal proteins and induce disease propagation via aggregation.

1.4.3. Parkinson's Disease

The first evidence that a toxic protein might spread between cells came from the post-mortem analysis of PD patients who had received an embryonic stem cell-derived transplant of neurons. It was observed that grafted healthy neurons presented PD characteristic inclusions, called Lewy bodies, which demonstrated the transmission between the host neurons and the grafted ones (Kordower et al. 2008a, 2008b; Li et al., 2008). Furthermore, this hypothesis was tested in several *in vitro* and *in vivo* experiments (Desplats et al., 2009; Hansen et al., 2011; Angot et al., 2012). Desplants and colleagues were among the first to show that transgenic mice, overexpressing human α-synuclein, when transplanted with mouse stem cells, are able to transfer human α-synuclein to the grafted cells. Also, they demonstrated in vitro that fluorescently tagged human α-synuclein can be internalized by cultured mouse cortical neuronal stem cells and that in co-culture conditions, human α-synuclein is transferred from neuronal cells to mouse stem cells (Desplats et al., 2009).

The seeding properties of α-synuclein have also been explored with several studies reporting a cytoplasmatic spread of the disease causing protein and an induction of α-synuclein aggregation in the recipient cells (Luk et al., 2009; Volpicelli-Daley et al., 2011; Sacino et al., 2013). Interestingly, there are experiments that show that a single intracerebral inoculation of brain tissue from old transgenic PD mice or synthetic α-synuclein fibrils is enough to seed aggregation in young asymptomatic PD mice and in wild-type mice, proving the possibility of a prion-like transmission in PD (Luk et al., 2012a, 2012b; Mougenot et al., 2012; Osterberg et al., 2015).

1.4.4. Alzheimer's Disease

AD is characterized by extracellular β-amyloid (Aβ) plaques and intraneuronal hyperphosphorylated tau in the form of neurofibrillary tangles. Evidence of a seeding mechanism of tau aggregates were developed by Clavaguera and associates, which performed intracerebral injections of brain extracts from transgenic mice expressing mutant human tau into the brain of transgenic mice expressing wild-type human tau, and observed the formation of inclusions of wild-type tau. Moreover, these inclusions where able to travel to brain areas distant from the injection site (Clavaguera et al., 2009). Furthermore, the intracerebral injection of human brain homogenates from patients with tauopathies into wild-type mice also seeded the inclusion of tau. Interestingly, the species barrier was not present, since human mutant tau was able to aggregate wild-type mice tau (Liu et al., 2012; De Calignon et al., 2012; Clavaguera et al., 2013). Similar results were also obtained with intraperitoneal injection of tau inclusions in pre-symptomatic transgenic mice, albeit with less inclusion formation (Clavaguera et al., 2014). This data is supported by in vitro experiments that show the ability of aggregated tau to be internalized by cultured cells, possibly via endocytosis, and seed the fibrillation of endogenous tau. The *de novo* formed aggregated proteins where posteriorly able to transfer between cells. Interestingly, it was observed that only the assembled tau and, not the monomeric form, where internalized by the cells (Frost, Jacks, and Diamond, 2009). Moreover, in the early 90's, it was observed transmission of β-amyloidosis in marmoset monkeys upon intracerebral injection of

brain homogenates of early AD patients (Baker et al., 1993). This first observation paved the way for further studies regarding prion-like spread of Aβ protein. Similar to the experiments performed for tau, several studies demonstrated the formation of Aβ plaques in the brain of transgenic mice expressing human mutant amyloid precursor protein (AAP) after intracerebral injection of AD brain homogenates, hinting a possible "infectious" function of Aβ (Kane et al., 2000; Meyer-Luehmann et al., 2006; Langer et al., 2011). In these studies observations indicated that AD brain homogenates were able to induce disease progression in young asymptomatic transgenic mice. Additionally, it was demonstrated that the intraperitoneal inoculation of Aβ enriched extracts could induce the formation of Aβ plaques in the brain of transgenic mice, with less aggregation efficiency that intracerebral inoculation, as observed for tau (Eisele et al., 2010). It was also observed that amyloidosis can be induced in transgenic mice expressing wild-type human APP by injection of post-mortem brain extracts of AD patients. Interestingly, amyloidosis spread to regions anatomically distant from the injection site (Morales et al., 2012). *In vitro*, Wang and colleagues observed a transfer of Aβ oligomers between primary co-cultures of rat astrocytes and neurons, indicating that pathological spread can occur between different neural cell types (Wang et al., 2011). Accordingly, in a donor-acceptor cell model it was observed a transmission of Aβ oligomers, dependent on direct cellular connections, with considerable accumulation of Aβ oligomers and cellular damage in the acceptor cells (Nath et al., 2012). This data indicates that, unlike tau, it is not the fibrillary aggregates of Aβ that are damaging the cells, but their oligomeric form, which is more aggregation prone.

1.4.5. Polyglutamine Diseases

Both HD and SCAs are genetic neurodegenerative disorders caused by an overexpression of ploy glutamines (polyQ) in a specific protein, which aggregate and accumulate mostly in the neurons´ nucleus. It was demonstrated that several cell types are able to internalize synthetic mutant huntingtin aggregates. Once in the cells, these aggregates can travel to the nucleus, where they trigger their pathogenic

functions, or stay in the cytoplasm and serve as seeds for new aggregates (Yang, 2002; Ren et al., 2009).

hESCs- derived neurons were transplanted into a transgenic mouse model of HD and, it was observed a transynaptical spread of mHTT (Pecho-Vrieseling et al., 2014). Moreover, it was also observed an accumulation of mHTT in the synaptic terminals of a *Drosophila* model of HD. These aggregates were shown to travel and accumulate into other neurons causing neuropathology (Babcock and Ganetzky, 2015). Unlike the observations for PD, *postmortem* analysis of HD patients transplanted with fetal grafts of stratial tissue did not show any signs of mHTT aggregation, despite considerable neurodegeneration in this area (Cicchetti et al., 2009).

Regarding SCAs there is an in *vitro* study demonstrating that cultured cells are able to internalize synthetic polyQ fragments. These fragments were capable of seed aggregation of homologous proteins and interact with cytosolic chaperones and UPS constituents, leading to their co-aggregation. The aggregates persisted over several cell generations, leading to the hypothesis that there is a self-sustaining seeding mechanism (Ren et al., 2009). Nevertheless, there are no substantial studies regarding this hypothesis for SCAs and no information concerning disease spreading in SCA3 is available.

1.4.6. Spreading Mechanisms in Neurodegenerative Diseases

Despite all the excitement promoted by these discoveries, the precise way, how these seeds spread from cell-to-cell is still controversial. Nevertheless, it is accepted that for this cytotoxic spread to occur, the disease-causing species need (I) a mean for exiting the diseased cell and (II) an internalization mechanism into a healthy acceptor one. In fact, the presence of α-synuclein, Aβ and tau in CSF is proof that these proteins are able to escape the cells (Blennow et al., 1995; Tapiola et al., 2009; Mollenhauer et al., 2013; van Dijk et al., 2014; Mo et al., 2015). Therefore, several studies pointed out some strong evidences for the transcellular transfer of these "prion-like" seeds. There are several possible routes for these seeds to travel and reach the target cells, namely through soluble oligomers, synaptic connection, tunneling nanotubes and

extracellular vesicles (Figure 1.8). Nevertheless, further investigation elucidating the exact transfer mechanism of these seeds is required.

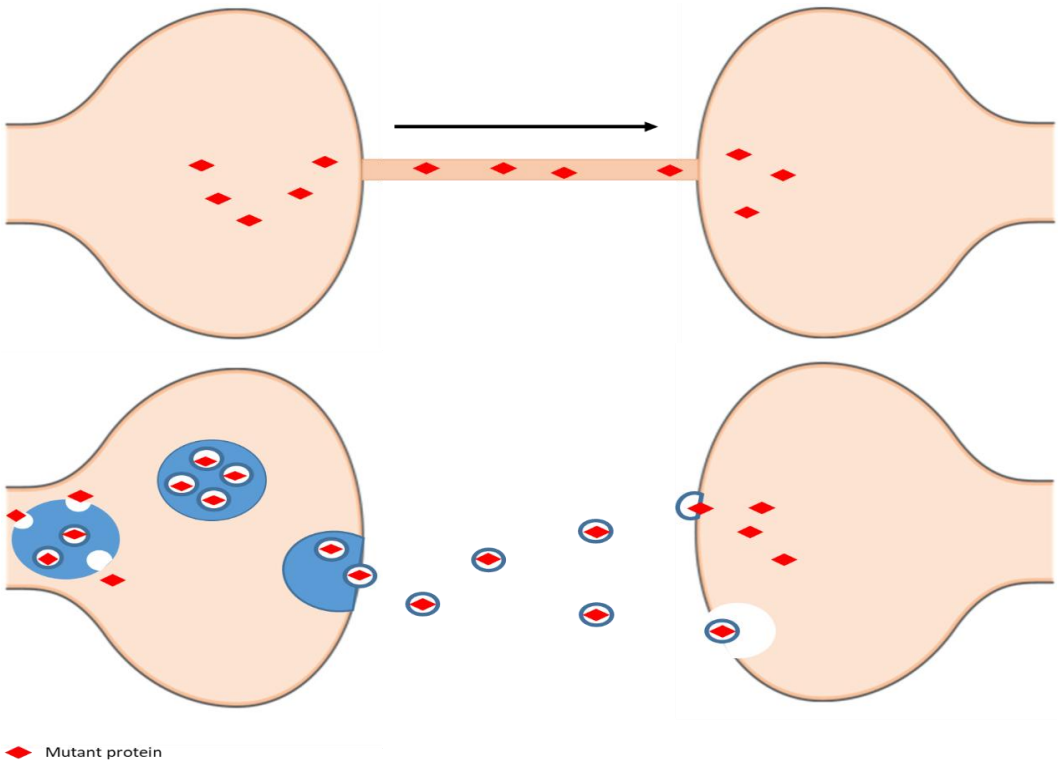

Figure 1.8. Two of the mechanisms of pathological spreading of neurodegenerative disease related proteins. Upper panel: cell-to-cell transmission via Tunneling Nanotubes (TNT) and Down panel: spreading via Extracellular Vesicles (EVs).

1.4.6.1. Soluble Oligomers

Soluble oligomers are able to disseminate into the extracellular medium and freely travel to adjacent cells, where they display their toxic functions (Nath et al., 2012). But for large, insoluble protein aggregates the direct dissemination into the extracellular milieu seems quite unlikely.

1.4.6.2. Synaptic Connection

One possible route for transneural spread of toxic aggregates is through synaptic connections. Ahmed and associates showed, by infusion of hyperphosphorylated fibrillary tau tangles in the hippocampus of a transgenic mouse model for human tau, a strong and fast propagation of the aggregates preferentially between neurons that

were synaptically connected, rather than between the spatially close ones (Ahmed et al., 2014). Similarly, oligomeric Aβ transmission in co-cultured primary rat neurons and human neuronal cells, with rat neurons being the donor of oligomeric Aβ and the human neurons as acceptor cells, showed a direct spread through the synaptic cleft, which resulted in time dependent cytotoxicity in the human neurons (Nath et al., 2012). Aggregates of α-synuclein were also shown to be taken up in nerve terminals, hinting a possible synaptic connection underlying the transfer. Furthermore once internalized, these aggregates were able to induce impairment in synapse connectivity and promote neuron death (Volpicelli-Daley et al., 2011).

1.4.6.3. Tunneling Nanotubes (TNT)

Tunneling Nanotubes are tunnel-like structures that connect two cells, acting as a natural "highway" for the transfer of molecules between cells. TNT have been shown to mediate prions transfer for its dissemination between neural cells (Gousset et al., 2009). An interesting study observed an increase in TNT formation in a co-culture of rat hippocampal astrocytes and neurons upon oxidative stress. Furthermore, Aβ was transferred from astrocytes to neurons via TNT. This work highlights the hypothesis that under stress cells promote the toxic transfer of Aβ (Wang et al., 2011). It was also demonstrated that assembled tau protein is transferred between neurons in primary cell cultures via TNT. Interestingly, the internalization of the assembled tau promoted the creation of new TNT, increasing the spreading capacity of the system (Abounit et al., 2016b; Tardivel et al., 2016).

Moreover, lysosomes loaded with α-synuclein fibrils are also involved in the spreading of the pathogenic protein to target cells via TNT. It is proposed that the diseased cell, in an attempt to dispose of the α-synuclein aggregates, loads them into lysosomes. However, instead of being degraded, they display the ability to spread to adjacent neurons via TNT. Additionally, these fibrils, when in the cytosol of the acceptor neurons were able to seed the aggregation of α-synuclein (Abounit et al. 2016b). The transfer of α-synuclein via TNT in the cell model SH-SY5Y overexpressing α-synuclein and primary human pericytes derived from post-mortem PD patients was

also assessed. In this work, α-synuclein was able to quickly transfer between cells via TNT. Interestingly, this work demonstrated that TNT are not an exchange system exclusive to neural cells and that it can transfer different types of molecules in a non-selective way (Dieriks et al., 2017).

Regarding mHTT, a rapid transfer of polyQ aggregates between cells was observed in a neuronal cell line and also between primary cultured neurons. This spread was not dependent on aggregates secretion but rather occurred via TNT. Noteworthy, it was observed that the mutant fragments, but not the wild-type ones, induced the formation of new TNT, which further promoted the pathological transfer of the fragments to neighbor neurons (Costanzo et al., 2013).

1.4.6.4. Extracellular Vesicles (EVs)

Extracellular vesicles (EVs) have several biological functions; among these is the ability to incorporate into their cargo and transfer pathological seeds, spreading them into other cells, often very distant between each other, contributing to disease spreading and progression. Given their physical and physiological characteristics, EVs represent the perfect 'infection' method as they are endogenous and, as so, the immune system does not recognize them as a hazard. Additionally, they protect their content from extracellular degradation pathways (e.g. low pH, proteases, etc.), and they can spread to long distant cells (Thompson et al., 2016).

The fact that EVs are naturally occurring structures and that their content reflects the state of the cell of origin makes EVs good biomarker candidates for disease progression and diagnose establishment. Nowadays, the gold standard in disease progression assessment is brain imaging, such as MRI or PET. This is not an ideal method considering that it is not suitable for early-stage diagnosis of neurodegenerative disease (Croese and Furlan, 2017). As so, the presence of CNS derived EVs in peripheral fluids (blood and CSF) represents a potential source of disease information that can be used as biomarkers to establish the disease diagnosis long before the first symptoms and to supply information regarding the disease progression during clinical trials.

1.4.6.4.1. EVs in Creutzfeld-Jacob Disease

The first evidence that EVs could shuttle pathological proteins come from an interesting study where EVs were associated to the infectious prion protein. Furthermore, the prion bearing EVs were able to induce pathology in recipient cells, contributing to the infectious spreading (Fevrier et al., 2004). Years later, it was shown that neuron-derived EVs are able to induce pathology in healthy cells, and that non-neural cell derived EVs bearing the infectious form of the prion protein were also able to induce pathology in healthy neurons. The same EVs were then able to induce prion disease when inoculated in mice (Vella et al., 2007). Interestingly, evidence exists that by inhibiting the EVs biogenesis mechanisms (namely the ESCRT-dependent and -independent pathways), the secretion of the pathogenic form of the prion protein could be decreased, and consequently, prion infectivity (Vilette et al., 2015). Even more surprising is the fact that plasma-derived EVs from mice bearing prion disease are infectious, and can transmit the pathology upon injection into other animals (Cervenakova et al., 2016).

1.4.6.4.2. EVs in Alzheimer's Disease

For AD, the association of the disease causing mechanisms with EVs is long known (Rajendran et al., 2006; Sharples et al., 2007). In these works, EVs were shown to transport APP, a key player in AD and a characteristic EVs protein was found in Aβ plaques, hinting a role for EVs in the physiopathology of AD. Also tau was found to be present and transmit pathology via EVs. Moreover, by inhibiting EVs biogenesis, it was also observed a reduction of tau propagation both *in vitro* and *in vivo* (Asai et al., 2015). Additionally, phosphorylated tau was observed associated to EVs in CSF of mild AD patients, reinforcing the potential use of EVs as biomarkers for AD diagnose and progression assessment (Saman et al., 2012). Of note is the fact that EVs enriched in Aβ (specially on the surface) are capable of disturbing Ca^{2+} homeostasis, impairing mitochondrial function, and making neurons vulnerable to excitotoxicity; thus contributing to neuronal damage (Eitan et al., 2016).

1.4.6.4.3. EVs in Parkinson's Disease

Emmanouilidou and colleagues demonstrated, in a cell model of PD, that prefibrilar oligomers of α-synuclein are also secreted in EVs. In these experiments, EVs secretion was dependent on Ca^{2+} stimulation and further incubation of these EVs with naïve neurons induced α-synuclein aggregation and neuronal death (Emmanouilidou et al., 2010). This work was the first report that described the cell secretion of α-synuclein via EVs. Later on the ability of α-synuclein oligomers to be internalized by recipient cells and display toxic processes was assessed. Interestingly, a higher uptake of EVs-associated α-synuclein oligomers, and consequent more cytotoxicity, by recipient cells was observed in comparison to free forms of the fragments (Danzer et al., 2012). Notably, the authors also described the presence of α-synuclein oligomers both in the lumen and on the surface of EVs. A mouse cell line of microglia was also shown to be stimulated to secrete EVs upon contact with α-synuclein. It was observed that the secreted EVs induced apoptosis, possibly by the transmission of inflammatory signals such as TNF-α (Chang et al., 2013). This work empathizes the role of EVs in α-synuclein-mediated neurodegeneration in PD.

Lipid extracts derived from EVs are also able to accelerate aggregation, leading to the hypothesis that EVs lipids alone are enough to produce this catalytic effect (Marie et al., 2015; Fernandes et al., 2016). Mutations in Leucine-Rich Repeat Kinase 2 (LRRK2) are associated with some forms of PD. EVs-associated LRRK2 is elevated in urine samples of PD patients and directly correlates with disease severity (Fraser et al., 2016). In another work, α-synuclein oligomers present in EVs extracted from CSF of PD patients was also correlated with disease severity. Furthermore, when administered to a reporter cell line, these EVs were able to induce oligomerization of soluble α-synuclein in a dose-dependent manner (Stuendl et al., 2016). Taken together, this data hints a role for EVs in PD pathology.

1.4.6.4.4. EVs in Polyglutamine Diseases

Regarding polyQ diseases, the information associating the spread of pathologic seeds and EVs is still very scarce. Concerning to HD, Zhang and collaborators showed the presence of the polyQ tract of mHTT and its mRNA in EVs *in vitro*. Furthermore, the derived EVs were internalized by cultured mouse striatal neurons, and polyQ mRNA was detected in cytoplasm. However, the presence of the polyQ tract in the neurons did not triggered toxicity over the short period of time of this experiment (Zhang et al., 2016). This pioneering work is the first to describe a potential pathologic transfer of mutant mRNA via EVs in HD. For SCAs, and in particular MJD, the issue of pathologic seeds transfer between neurons via EVs was still not addressed to this day.
If EVs are proved to play a critical role in disease progression, drugs that block their release or intake by target neurons may appear as an attractive therapeutic tool to treat proteinopathies. Moreover, its use as biomarkers might improve diagnose and evaluation of disease progression. This would be a major breakthrough in the quest of finding better biomarkers and new therapeutic options for misfolded protein-associated neurodegenerative diseases.
In the present work, we took advantage of already established lines of control and MJD-patient´s iPSCs-derived neuroepithelial stem cells (NESCs), which can be differentiated into neuronal cultures. Human NESCs and extracellular vesicles (EVs)-produced by human NESC or human neural cultures of diseased and control conditions were used in this work to further physical, cellular and molecular analysis, with the goal of evaluate differences between both normal and diseased cells-derived vesicles that could be used as biomarkers of MJD.

Objectives

The main goal of this work was to assess the potential of EVs in the neuropathological spreading of mutant Ataxin-3 and other mediators in the context of MJD. For this, the following aims were proposed:

- Characterization of EVs obtained from control and MJD iPSCs-derived NESCs, and control and MJD neuronal cultures of iPSCs-derived NESCs, and assessment of potential differences between control and MJD EVs;
- *In vitro* assessment of the effect of the EVs obtained from MJD iPSCs-derived NESCs and from MJD neuronal cultures of iPSCs-derived NESCs in MJD neuropathological players as mutant Ataxin-3 aggregation, and autophagy and apoptosis mediators;
- *In vitro* evaluation whether cell contact is required for neuropathological mediators spreading;
- *In vivo* assessment of mutant Ataxin-3 spreading.

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CHAPTER 2

Material and Methods

Materials and Methods

2.1. iPSCs-derived Neuroepithelial Stem Cells Culture

Human iPSCs-derived Neuroepithelial Stem Cells (NESCs) established in our lab, were obtained by reprogramming fibroblasts of Control and MJD-patients into iPSCs using lentivirus encoding for four reprogramming factors (Oct-4, Klf4, c-Myc and Sox-2) (Takahashi and Yamanaka 2006) (Takahashi et al. 2007) as previously described (Koch et al. 2011). IPSCs were later induced into NESCs (Reinhardt et al., 2013) and cultured as monolayers in Matrigel (Corning)-coated flasks at 37ºC with 5% CO2. Cells were maintained in culture medium composed of Neurobasal medium (Invitrogen) and DMEM/F-12 (Invitrogen), in a 1:1 ratio, 1% Penicillin/Streptomycin (Invitrogen), 2 mM L-Glutamine (Invitrogen), 1:200 N-2 supplement (Gibco), 1:100 B-27 without vitamin A (Gibco), 150 μM Ascorbic Acid (Sigma-Aldrich), 3 μM CHIR 99021 (Axon-Medchem), and 0.75 μM Purmorphamine (PMA) (Enzo) and were split every 5-7 days.

2.2. NESCs Differentiation in Neural Cells

NESCs were differentiated into neural cells in Matrigel-coated flasks and coverslips (MW6, MW12 and MW96), by changing the culture medium to Neurobasal medium (Invitrogen) and DMEM/F-12 (Invitrogen), in a 1:1 ratio, 1% Penicillin/Streptomycin (Invitrogen), 2 mM L-Glutamine (Invitrogen), 1:200 N-2 supplement (Invitrogen), 1:100 B-27 without vitamin A (Invitrogen), 0.25 μM Dibutyryl cyclic Adenosine Monophosphate sodium salt (cAMP) (Sigma-Aldrich), 5 μM Forskolin (Sigma-Aldrich), and 2 μM Retinoic Acid (Sigma-Aldrich). Differentiation media was changed every 3-4 days.

2.3. EVs Isolation

NESCs and differentiated neural cells culture medium was collected and stored at 20ºC until EVs isolation. EVs were isolated by defrosting the medium overnight followed by differential centrifugation. Firstly, the medium was centrifuged at 300 g (1300 rpm) for 10 minutes at 4ºC to pellet cells. Then, the supernatant was centrifuged at 2000 g (3400 rpm) for 10 minutes at 4ºC to remove remaining cells and cell debris. The resulting supernatant was filtered using a 0.8 µm filter to remove bigger vesicles. Finally, the medium was ultracentrifuged at 100 000 g (28 500 rpm) for 1 hour and 30 minutes at 4ºC, using the SW-41Ti rotator, in the Optima XE-100 ultracentrifuge (Beckman Coulter). The resulting pellet was resuspended according to the final use of the same: for *Western-Blot* analysis the pellet was resuspended in lysis buffer, for RNA analysis in lysis buffer (provided by miRCURY™ RNA Isolation kit, Quiagen) and 1% β-mercaptoethanol, and for NTA and functional analysis in sterilized PBS.

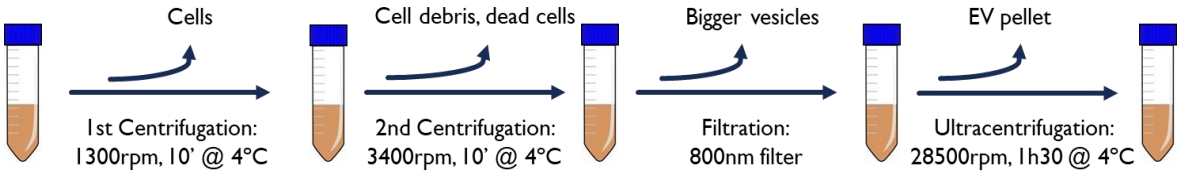


Figure 2.1. Differential centrifugation method used to isolate EVs.

2.4. Nanoparticle Tracking Analysis (NTA)

NTA was performed using NanoSight NS300 (Malvern) equipped with a syringe pump. EVs isolated as previously described were resuspended in 100 µL PBS after ultracentrifugation and diluted in water before loading in the syringe. Each sample was diluted accordingly for ideal reading. Pump speed was set to 20, camera level was 12-14, and detection threshold at 3. Five 60 second videos were recorded for each sample and analyzed using NTA software.

2.5. Transmission Electron Microscopy (TEM)

Transmission electron microscopy was performed according to Théry et al., 2006. Briefly, After isolation, EVs were fixed with 2% paraformaldehyde (PFA) and deposited on Formvar-carbon coated grids (TAAB Laboratories Equipment) for 20 min. Grids were washed with PBS 1X and fixed for 5min with 1% glutaraldehyde. Following a cycle of washes using distilled water, grids were contrasted with a uranyl-oxalate solution (pH=7) for 5 minutes, and transferred to methyl-cellulose-uranyl acetate for 10 minutes on ice. Images were obtained using a Tecnai G2 Spirit BioTWIN electron microscope (FEI) at 80kV.

2.6. Western-Blot

Cultured cells were washed twice with PBS and kept at -80ºC until processing. EVs were resuspended with lysis buffer and stored at -80ºC until further use. Both cells and EVs were disrupted using lysis buffer composed of 150 mM NaCl, 50 mM Tris, 5 mM EDTA, 1% Triton, 0.5% sodium deoxycholate and 0.1% SDS added freshly with protease inhibitor (Complete Mini), phosphatase inhibitor (PhosStop Easy, Roche), 1 mM Phenylmethane Sulfonyl Fluoride (PMSF) (Sigma-Aldrich) and 10 µg/mL Dithiothreitol (DTT) (Sigma-Aldrich), strong vortex and sonication for 3 cycles of 10 seconds at middle speed (40-60 kHz).

For each sample the amount of protein was quantified using Pierce BCA Protein Assay Kit (Thermo Fischer Scientific). The amount of protein loaded was 25 or 50 µg for cell lysates and 50 or 100 µg for EVs.

The sample was prepared with sample buffer composed of 0.5 M Tris-HCl pH: 6.8, 30% glycerol, 10% SDS, 0.6 M DTT and 0.1 mg/mL blue bromophenol, denatured by heating at 95ºC for 5 minutes and stored at -20ºC until use.

Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE), in 8 or 12% resolving acrylamide gels at 70V for 10-15 minutes and 100V for the rest of the run. After electrophoresis, proteins were transferred to polyvinilidene diflouride (PVDF) membranes (Millipore) previously activated by diving 1 minute in methanol, 5 minutes in water and 5 minutes in CAPS (N-cyclohexylamino-3-aminopropanesulfonic

acid)/10% methanol. Transference was performed at 0.75 A for 2-2:30 hours, with 4ºC cooling. Then, the membranes were blocked with 5% milk in TBS (Tris-Buffered Saline) with 0.1% Tween 20 (TBS-T) for 1 hour and incubated with primary antibodies overnight at 4ºC. Then, membranes were washed three times (5, 10 and 15 minutes) with TBS-T and incubated with alkaline phosphatase linked secondary antibodies (diluted 1:10000) for 2 hours at room temperature. Again, the membranes were washed with TBS-T three times (5, 10 and 15 minutes) and proteins were assessed using Enhanced Chemifluorescence substrate (ECF, Amersham Bioscences) in the Versadoc 3000 Imaging System (Bio-Rad). The signals obtained were quantified using ImageJ software (NIH, USA).

A complete list of the antibodies used is provided in Table 2.1. All antibodies used were diluted in 5% milk in TBS-T.

Table 2.1. Antibodies used in western-blot.

Antibody	Dilution	Manufacturer	Reference	Molecular Weight	Specie
p62	1:1000	Cell Signaling	5114S	62 kDa	rabbit
SOD-1	1:500	Santa Cruz	sc-17767	16-18 kDa	mouse
LC3-B	1:1000	Cell Signaling	2775S	18 kDa	rabbit
Alix (3A9)	1:1000	Cell Signaling	2171S	96 kDa	mouse
Spinocerebellar ataxin-3 (1H9)	1:1000	Millipore	MAB5360	26, 34, 50 and 66 kDa	mouse
Cleaved CASP3	1:1000	Cell Signaling	9661S	19 kDa	rabbit
β-tubulin	1:1000	Sigma	SAP.4G5	50 kDa	mouse
Calnexin	1:500	Santa Cruz	sc-11397	90 kDa	rabbit
HSP70	1:500	Santa Cruz	sc-24	70 kDa	mouse
CD81	1:500	Santa Cruz	sc-9158	26 kDa	rabbit
CD63	1:500	Santa Cruz	sc-15363	30-60 kDa	rabbit
Bcl-2	1:500	Cell Signaling	2876S	26 kDa	rabbit
Atg3	1:500	Cell Signaling	3415S	37 kDa	rabbit

Atg7	1:500	Cell Signaling	8558S	77 kDa	rabbit
Atg12	1:500	Cell Signaling	2010S	21 kDa	rabbit
Beclin-1	1:1000	BD Biosciences	612113	61 kDa	mouse
Akt1	1:500	Santa Cruz	sc-5298	62 kDa	mouse
p-ERK	1:500	Santa Cruz	sc-7383	43 kDa	mouse
HSP20	1:500	Santa Cruz	sc-51955	20 kDa	mouse
p-p38	1:500	Santa Cruz	sc-7973	38 kDa	mouse

2.7. Quantitative Reverse Transcriptase (RT) - Polymerase Chain Reaction (PCR) and Semi-Quantitative RT-PCR

After resuspension in RNA extraction lysis buffer with 1% β-mercaptoethanol, the RNA present in the samples was extracted using miRCURY™ RNA Isolation kit (Quiagen), according to manufacturer's recommendations. The RNA purity and concentration was measured using NanoDrop™ 2000 (Thermo Scientific). From this RNA, cDNA was synthesized with iScript™ cDNA Synthesis Kit (Bio-Rad), from 300 ng of total RNA, as instructed by the manufacturer. Quantitative real time PCR (qRT-PCR) was performed with SsoAdvanced™ SYBR® Green Supermix Kit (Bio-Rad). The cDNA obtained by reverse transcription was diluted (how any times) in DNase-free deionized water (Sigma). Briefly, qRT-PCR was performed with a single cycle of 95ºC for 30 seconds, followed by 45 cycles of two steps: first step of 5 seconds at 95ºC, and a second step of 15 seconds at temperature depending on the annealing temperature of each primer (Table 2.1.). The threshold cycle (CT) for each gene was generated automatically by the StepOne™ Software (Applied Biosystems). For all genes, a standard curve was performed, and quantitative PCR efficiency was determined by the software. Additionally, no template and no reverse transcriptase controls were performed. The primers used are indicated in Table 2.2.

Table 2.2. List of genes screened. Indicated are also the manufacturer of the primers, the reference of each primers, and the annealing temperature as well.

Gene	**Manufacturer**	**Reference**	**Annealing temperature**
CASP3	Sigma	KPP	60ºC
CASP7	Sigma	KPP	60.5ºC
CASP8	Sigma	KPP	58ºC
CASP9	Sigma	KPP	59ºC
CYCS	Sigma	KPP	59ºC
IL-8	Sigma	KPP	60ºC
LC3-B	Sigma	KPP	58ºC
Beclin-1	Quiagen	79237	60ºC
TNF-α	Sigma	KPP	60ºC
HPRT	Quiagen	QT00059066	60ºC
ATXN3	Invitrogen	R5569	58ºC

KPP: KiCqStart Pre-designed Primers.

Semi-quantitative RT-PCR was performed using 40 ng of cDNA obtained as described above. The reaction mix used was as follows: 10 % HF or GC buffer, 10 mM dNTP, 500 nM of each forward and reverse primers, 5% DMSO, 1% Phusion® enzyme (Quiagen) and DNase-free deionized water. The PCR was performed with a single cycle of 98ºC for 10 seconds, a second step composed of 35 cycles of: 98ºC for 10 seconds, variable temperature (according to primer´s annealing temperature) for 10 seconds and 72ºC for 30 seconds; and a third step of 72ºC for 30 minutes. After the reaction, samples were kept at 4ºC until agarose gel electrophoresis resolution. Thus, samples were loaded in 2% agarose gel and electrophoresis was performed at 90 V for 45 minutes to 1 hour. Images were taken using Image Lab software (Bio-Rad).

2.8. EVs Labeling

After isolation, as previously described, EVs pellet was resuspended in PBS. Then 10 μM of Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) (CellTrace, Invitrogen) in DMSO were added to fluorescently label the vesicles. Incubation of CFSE with the vesicles was done for 45 minutes, with shaking every 15 minutes, at 37ºC. Following, the marked EVs were diluted in 9.5 mL of PBS and ultracentrifuged at 100 000 g (28 500 rpm) for 1 hour and 30 minutes, at 4ºC, to remove the unbound dye. In parallel, a negative control (without EVs), composed of the same concentration of CFSE was added to 9.5 mL of PBS and submitted to the same steps as the EVs sample. After ultracentrifugation, both pellets were resuspended in 100 μL of sterile PBS.

2.9. Immunocytochemistry

NESC-derived neurons plated in coverslips were washed twice with PBS, fixed with 4% paraformaldehyde (PFA) (Sigma) for 20 minutes at room temperature, washed again twice with PBS and stored at 4ºC until use. Fixed cells were permeabilized with 1% Triton™ X-100 (Sigma), followed by 1 hour blocking with 3% bovine serum albumin (BSA)/PBS (Sigma) and overnight incubation with primary antibodies at 4ºC. The primary antibodies used were diluted in 3% BSA/PBS according to manufacturer instructions. In the following day, after washing twice with PBS, incubation with the secondary antibodies was performed for 2 hours at room temperature. The secondary antibodies were diluted in 1% BSA/PBS. Nuclear DNA was stained blue using 4', 6-diamidino-2-phenylindoline, DAPI (1:5000, Applichem).

Primary antibodies used: mouse anti-MAP2 (1:250), rabbit anti-β3-tubulin (1:400) and mouse anti-1H9 (1:1000).

Secondary antibodies used: Alexa 594 anti-mouse (1:250), Alexa 488 anti-rabbit (1:250) and Alexa 594 anti-rabbit (1:250).

2.10. Co-culture without Contact (indirect co-culture)

Control NESCs were plated in MW12 with 300.000 cells per well with differentiation medium. In half of the wells of each plate (6 per plate) cells were plated with cell

culture inserts (Millicell) containing 200.000 MJD NESCs also in differentiation-inducing medium. Cells were allowed to share medium through the 1 μm pore of the insert membrane. These experiments were carried for 1 or 3 weeks. After these periods, cells were immunostained for ataxin-3 and β3-tubulin or processed for Western-Blot.

2.11. Mixed Cultures (direct co-culture)

The lentiviral vectors encoding for green fluorescent protein (GFP, SIN-PGK-GFP-WHV) were produced in 293Tcells using a four plasmid system (de Almeida et al. 2001). Control NESCs were transduced with lentiviral vectors encoding GFP. Briefly, 4.5 million control NESCs were incubated in a T75 flask for 24h with lentiviral vectors encoding GFP (the equivalent to 100 ng of p24 antigen) 30 minutes after passage. Twenty-four hours later, the maintenance medium was changed. After 3-4 days NESCs were constitutively expressing GFP.

150.000 GFP-expressing control NESCs were mixed with MJD NESCs in a 1:1 ratio and plated in MW12 (300.000/per well in total) in differentiation-inducing media. This experiment carried for 2 weeks. After this period, the cells were fixed and immunostained for Ataxin-3 and β3-tubulin as described above.

2.12. Reactive Oxygen Species (ROS) Measurement

For the reactive oxygen species (ROS) quantification, control NESCs were plated in black/clear bottom MW96 (75.000 per well) and differentiated for one week. Then, differentiated neurons were washed twice with Hanks' Balanced Salt Solution (HBSS) (Sigma-Aldrich) and incubated with 100 μg of control and MJD NESCs-derived EVs for one hour for ROS induction. Reactive Oxygen Species Detection Assay Kit (Red Fluorescence) (Abcam) was used according to manufacturer´s instructions. The probe was incubated with the cells for two hours and fluorescence reading was performed in Cytation (BioTek) with an excitation wavelength of 520 nm, emission of 605 nm and cutoff of 590 nm.

2.13. Cell Viability

One week differentiated control NESCs were incubated for 3 days or 2 weeks with 50 μg/mL or 100 μg/mL EVs isolated from control NESCs or MJD NESCs culture medium. After this period cell viability was assessed by the resazurin reduction assay (O'Brien et al. 2000). The assay measures the chemical reduction of the resazurin dye resulting from cellular metabolic activity. For this, cells were incubated with 0.1 mg/mL resazurin, prepared in the differentiation culture medium, for 3-4 hours at 37ºC and 5% CO_2. The absorbance for the reduced and oxidized species of resazurin were read at 570 nm and 600 nm, respectively, using spectrophotometer (SpectraMax Plus 384, Molecular Devices). Cell viability was calculated according to the equation: $[(A_{570} - A_{600})\text{treated cells} * 100)/(A_{570} - A_{600})\text{control cells}]$.

2.14. Transplantation of iPSCs-derived NESCs

Immunodeficient NOD.CB17-Prdc scid/J mice (Charles River), 5-6 weeks old, received a single stereotaxic injection at postnatal days of 150 000 MJD or control human iPSCs-derived NESCs in lobule 5 of the cerebellum at 0.25 μL/min and were sacrificed 8 weeks after the transplantation. Mice were housed in sterile conditions (Ventiracks). Food and water were provided *ad libitum*. All experiments were conducted in accordance with the European Community Council directive (86/609/EEC) for care and use of laboratory animals.

2.15. Tissue Preparation for Immunohistochemistry

Animals were sacrificed by xylazine/ketamine overdosing and intercardically perfused with cold 4% paraformaldehyde (PFA). After, the brains were removed and kept in 4% PFA for 24h at 4ºC, then cryoprotected by incubation in 25% sucrose in PBS solution for 48h at 4ºC. Finally, the brains were frozen at -80ºC until further sectioning. The cerebellum was sliced in 25 μm sagittal sections using a cryostat (Thermo Fisher Scientific). Free-floating brain sections were collected in 48-well plates with PBS/0.05mM sodium azide and kept at 4ºC until processing.

2.16. Immunohistochemistry.

The brain sections were first washed three times with PBS, blocked and permeabilized for 2h in 0.1% Triton™ X-100 and 10% normal goat serum (NGS)/PBS at room temperature. After that, brain sections were incubated overnight at 4ºC with the following primary antibodies prepared in 10% NGS: mouse anti-1H9 (1:1000, Millipore), quail anti-ataxin-3 (1:1000) and rabbit anti-Iba-1 (1:500, Dako).

In the following day, sections were washed three times with PBS and incubated with secondary antibodies prepared in 2% NGS/PBS for 2h at room temperature. After this, brain sections were washed three times with PBS and nuclear DNA was stained using DAPI (1:5000). Sections were washed three times with PBS and mounted on gelatinized slides. After drying for 15 minutes at 37ºC, slides were mounted with Mowiol reagent (Sigma).

2.17. Microscopy

Widefield fluorescence images were acquired with a 10x and 20x objective on a Zeiss Axiovert 200 imaging microscope. Confocal fluorescence images were obtained using a 40x and 63x objective on a LSM Zeiss microscope.

CHAPTER 3

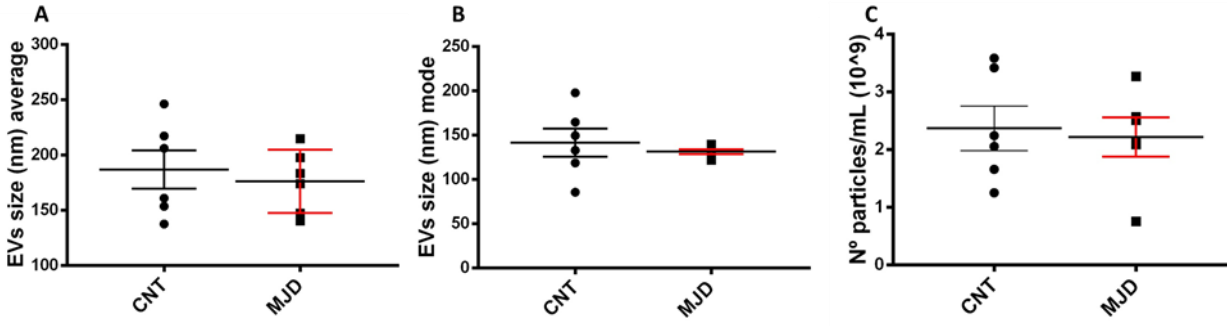
Results

Results

3.1. Extracellular Vesicles Characterization

3.1.1. Assessment of Size and Sample Concentration of EVs from Human iPSCs-derived NESCs

In the first part of this work, the physical characteristics of control and MJD iPSCs-derived NESCs EVs, henceforward designated as control and MJD NESCs-derived EVs, were assessed by NTA. Features such as particle size average and size mode, particles concentration and size distribution were analyzed (Figure 3.1). No statistically significant differences were observed between the two types of populations in terms of (Figure 3.1 A) particle size average, (Figure 3.1 B) particle size mode and (Figure 3.1 C) particle concentration. Being the mean average size of 186.96 ± 17.36 nm for control NESCs-derived EVs and 176.18 ± 11.72 nm for MJD NESCs-derived EVs. Which is in accordance with the accepted size for EVs. Moreover, particle concentration was of $2.37x10^9$ ± $0.39x10^9$ particles/ml for control NESCs-derived EVs and $2.22x10^9$ ± $0.34x10^9$ particles/ml for MJD NESCs-derived EVs. Additionally, no major differences in the size distribution profile between (Figure 3.1 D) control NESCs-derived EVs and (Figure 3.1 E) MJD NESCs-derived EVs were found. Control NESCs-derived EVs (Figure 3.1 A and B) appear to be slightly bigger but (Figure 3.1 D and E) with a more irregular size distribution.



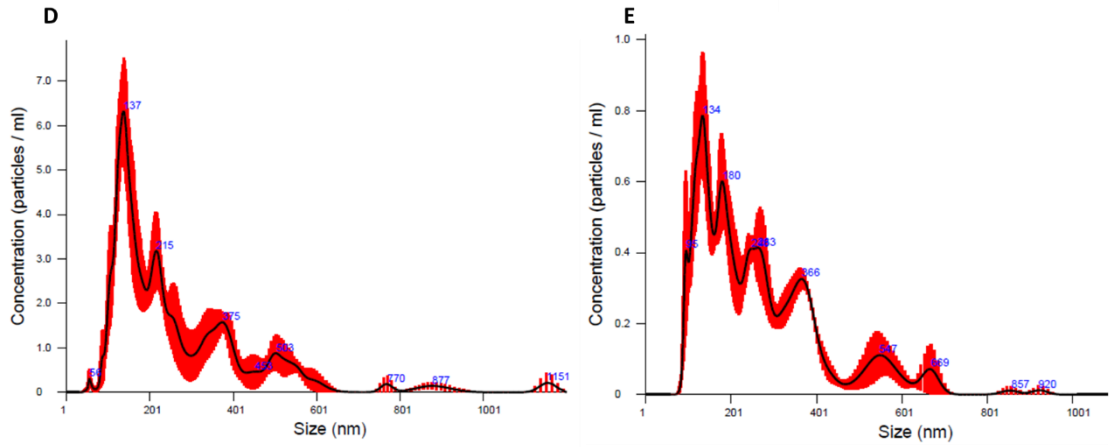


Figure 3.1. Physical characterization of control and MJD NESCs-derived EVs. Comparison of the (A) particle size average (B) particle size mode and (C) particle concentration. (D) Control NESCs-derived EVs and (E) MJD NESCs-derived EVs size distribution profile. Unpaired t test with Welch´s correction, n=6.

3.1.2. Assessment of Size and Sample Concentration of EVs from Human Neuronal Cultures of iPSCs-derived NESCs

The NTA analysis was also made to EVs derived from control and MJD neuronal cultures differentiated from iPSCs-derived NESCs, henceforward designated as control and MJD neuronal cultures-derived EVs (Figure 3.2). No statistically significant differences were observed between control and MJD neuronal cultures-derived EVs in terms of (Figure 3.2 A) size average and (Figure 3.2 B) size mode. The mean average size of control neuronal cultures-derived EVs is 123.87 ± 24.04 nm, and for MJD neuronal cultures-derived EVs of 154.57 ± 3.99 nm. Regarding the concentration values, the two populations are statistically different, control neuronal cultures-derived EVs present $2.27x10^{9}$ ± $0.09x10^{9}$ particles/ml, while MJD neuronal cultures-derived EVs have a concentration 2 times less particles/ml ($1.14x10^{9}$ ± $0.16x10^{9}$ particles/ml). Additionally, the size distribution profile of (Figure 3.2 D) control neuronal cultures-derived EVs appears to be more heterogeneous as compared to (Figure 3.2 E) MJD neuronal cultures-derived EVs. Thus, in general, the differences in the referred physical characteristics appear to be bigger in neuronal cultures-derived EVs than in NESCs-derived EVs.

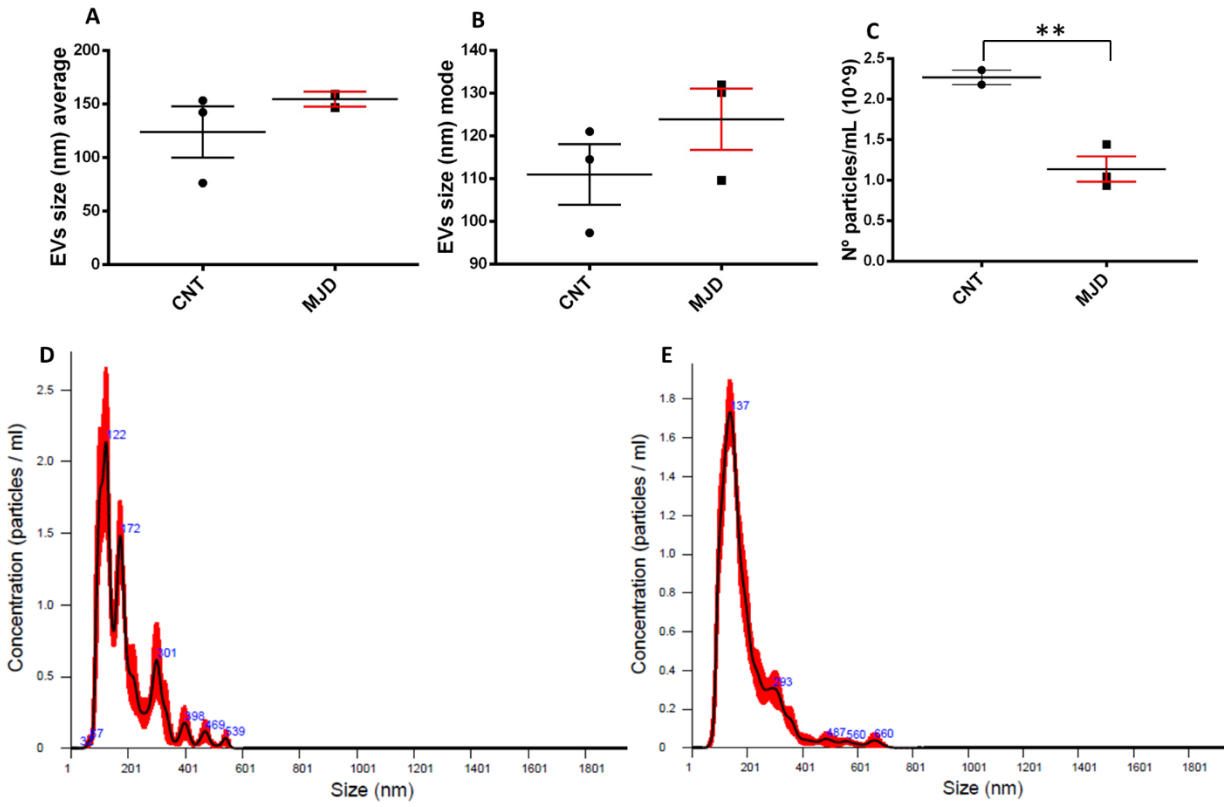


Figure 3.2. Physical characterization of control and MJD neuronal cultures-derived EVs. Comparison of the (A) particle size average, (B) particle size mode, and (C) particle concentration. Size distribution profile of (D) control neuronal cultures-derived EVs and (E) MJD neuronal cultures-derived EVs. Unpaired t test with Welch's correction, **p<0.01; n=3.

3.1.3. Visualization of MJD and Control EVs by Transmisson Electron Mircroscopy

The structure and integrity of MJD and control NESCs and neuronal cultures-derived EVs was further analyzed by Transmission Electron Microscopy (TEM) (Figure 3.3). As expected, round nanometric structures were observed. After measurement, the (Figure 3.3 A) control NESCs-derived EVs present 128.37 nm and (Figure 3.3 B) MJD NESCs-derived EVs exhibited 165.58 nm. (Figure 3.3 C D) Both control and MJD NESCs-derived neuronal cultures present 120.63 nm. This result are consistent with NTA measurements; however demands further confirmation by increasing the number of samples evaluated by TEM.

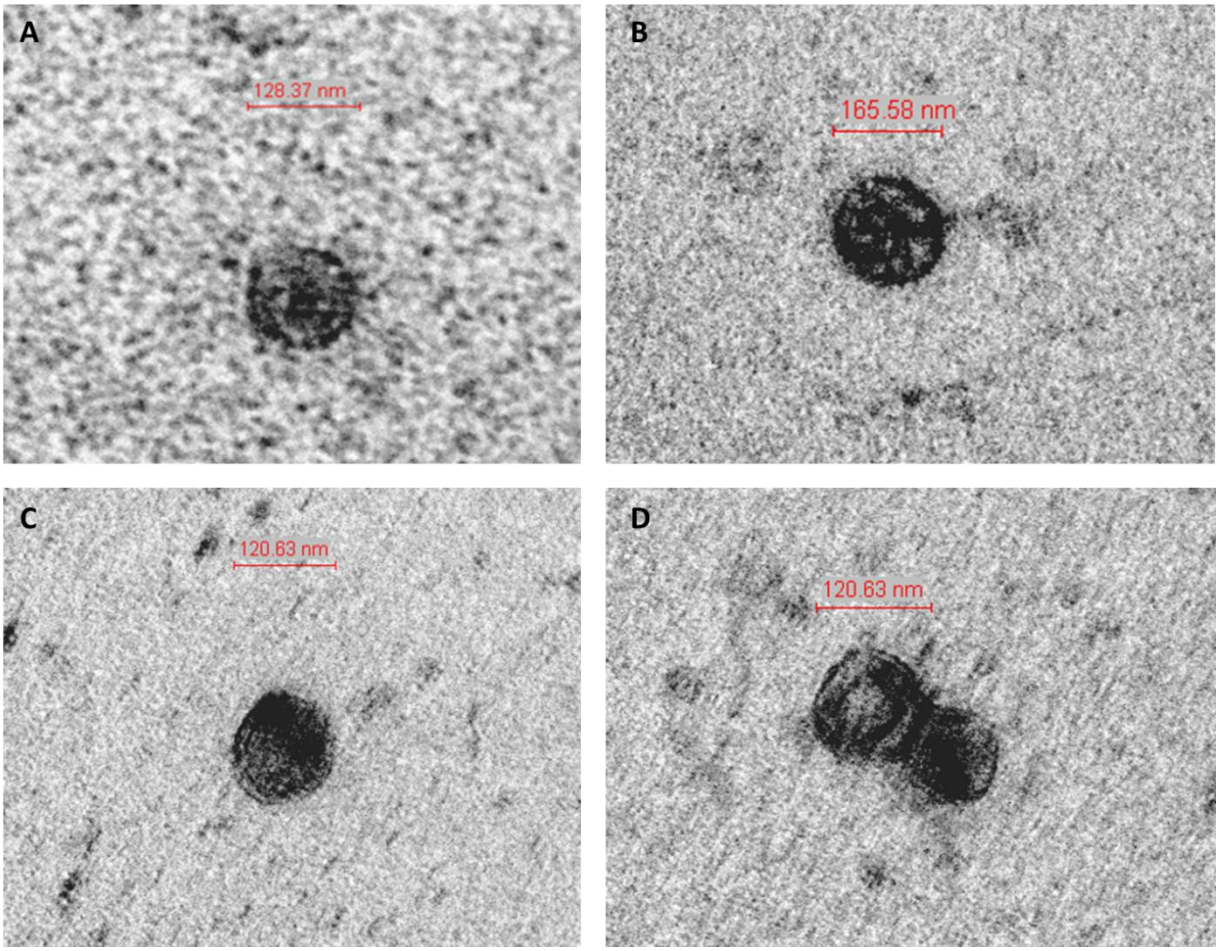


Figure 3.3. Transmission Electron Microscopy (TEM) analysis of control and MJD-derived EVs. (A) Control NESCs-derived EVs. (B) MJD NESCs-derived EVs. (C) Control neuronal cultures-derived EVs. (D) MJD neuronal cultures-derived EVs.

3.1.4. EVs Protein Cargo Characterization

After the physical characterization of the control and MJD NESCs-derived EVs population, an assessment of the protein content of these EVs was performed by Western-Blot.

3.1.4.1. Assessment of Typical EVs Markers

Firstly, using western blot we investigated the presence of protein markers typically associated to EVs, to assure that the EVs isolation protocol used result in EVs populations (Figure 3.4). It was observed that control and MJD NESCs-derived EVs samples are positive for the EVs markers such as ALIX, CD63 and Flotilin-1. We also tested the presence of the negative EVs marker Calnexin, which was not found in our

samples, as comparing with the progenitor cells (MJD NESCs and CNT NESCs). The observed results are in accordance with the previously observations made in NTA and TEM, indicating the isolation of EVs by this method. Thus, it was confirmed the presence of EVs in our samples by the current standards.

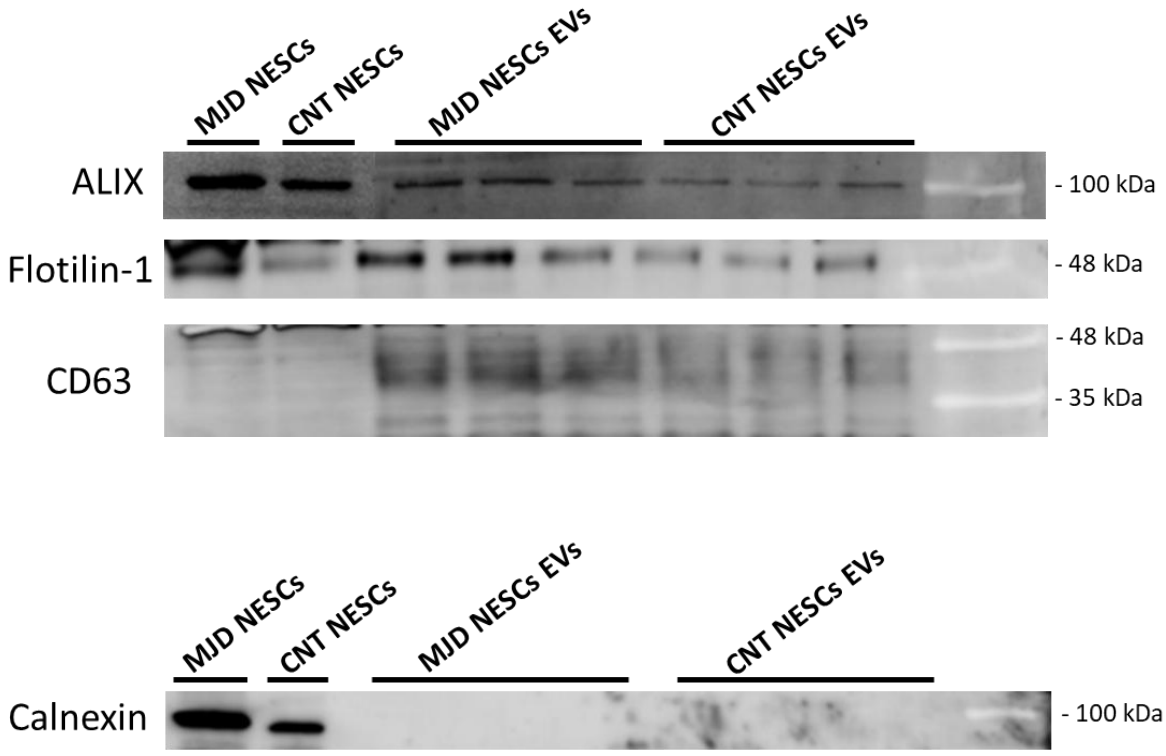


Figure 3.4. Evaluation of EVs positive (ALIX, CD63 and Flotilin-1, upper panel) and negative (Calnexin, down pannel) markers in MJD NESCs-derived EVs (MJD NESCS EVs) and control NESCs-derived EVs (CNT NESCs EVs) through western blot. Control samples of MJD iPSCs-derived NESCs (MJD NESCs) and control iPSCs-derived NESCs (CNT NESCs).

3.1.4.2. Screening of Proteins Related to MJD-associated Neuropathological Pathways

Then, a series of proteins that might be related to pathways deregulated in MJD, as apoptosis and autophagy, and the disease causing mutant Ataxin-3 protein, were screened to assess which ones were present in the NESCs-derived EVs. Several proteins were screened, namely protein associated with (Figure 3.4 A) apoptosis as Akt-1, p-ERK, p-p38 and SOD-1, and (Figure 3.4 B) autophagy, namely Atg7, p62,

Beclin-1, Atg3 and Bcl-2. A control of progenitors iPSCs-derived NESCs (MJD NESCs and CNT NESCs) for each population of EVs was also used.

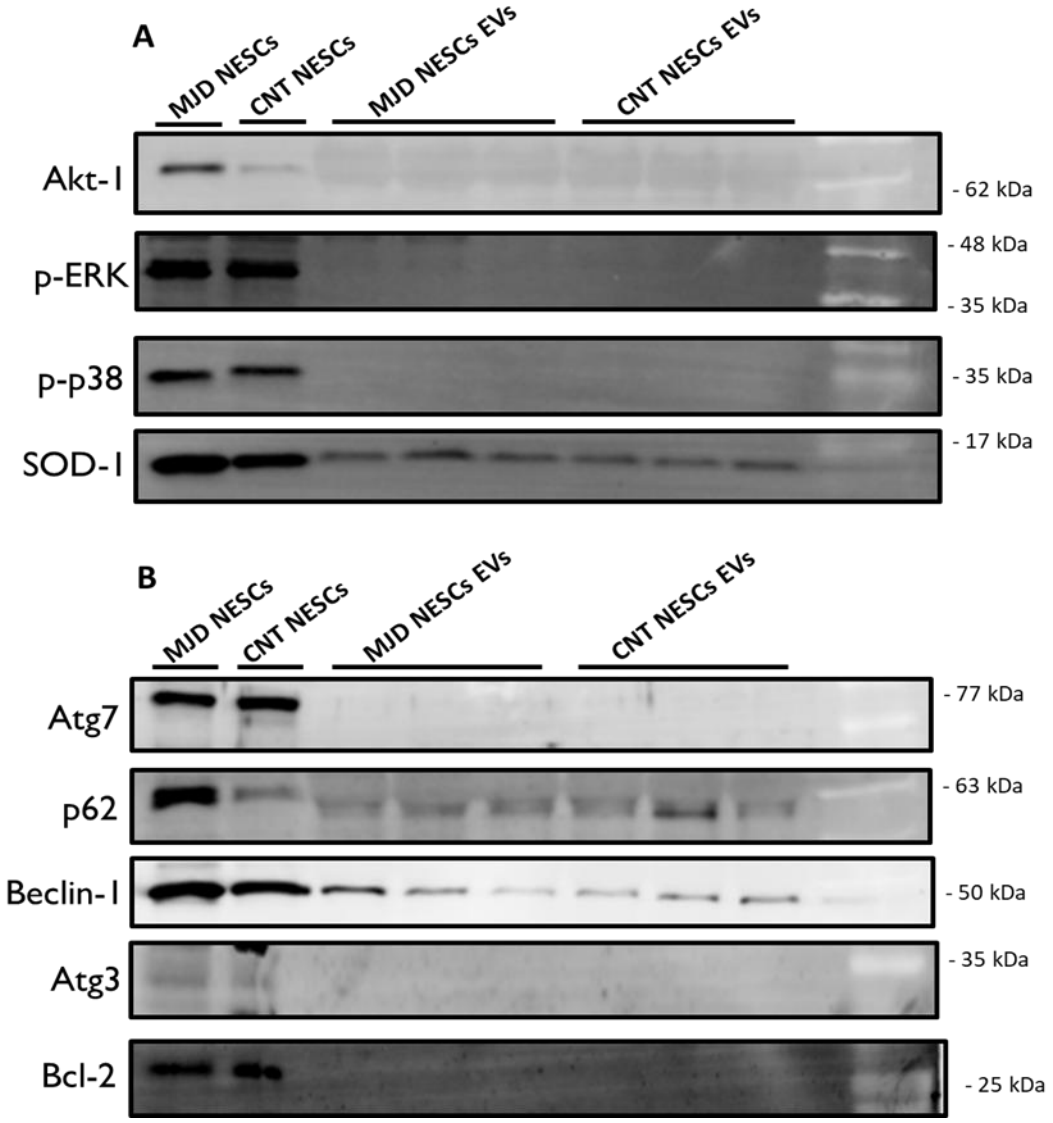


Figure 3.5. Evaluation of (A) proteins associated with apoptosis (Akt-1, p-ERK, p-p38 and SOD-1) and (B) proteins involved in autophagy (Atg7, p62, Beclin-1, Atg3 and Bcl-2) in control NESCs-derived EVs (CNT NESCs EVs) and MJD NESCs-derived EVs (MJD NESCs EVs) through western-blot. Control samples of MJD iPSCs-derived NESCs (MJD NESCs) and control iPSCs-derived NESCs (CNT NESCs).

As can be observed in Figure 3.5, the apoptosis-related protein SOD-1 and the autophagy proteins p62 and Beclin-1 were found to be present in both control and MJD NESCs-derived EVs. These proteins were further quantified, to evaluate possible differences between the two populations of EVs.

Regarding autophagy, the proteins (Figure 3.6 A, D) p62 and (Figure 3.6 B, D) Beclin-1 have a tendency to be less abundant in MJD NESCs-derived EVs. The p62 levels in MJD NESCs-derived EVs were 40% (0.598 ± 0.059) lower as compared to the control NESCs-derived EVs. As for Beclin-1, it was observed a 31% (0.686 ± 0.060) lower expression in MJD NESCs-derived EVs. On the other hand, (Figure 3.6 C, D) SOD-1 protein was found to be three times more expressed (3.021 ± 0.739) in the MJD NESCs-derived EVs than in the control NESCs-derived EVs (1.000 ± 0.190).

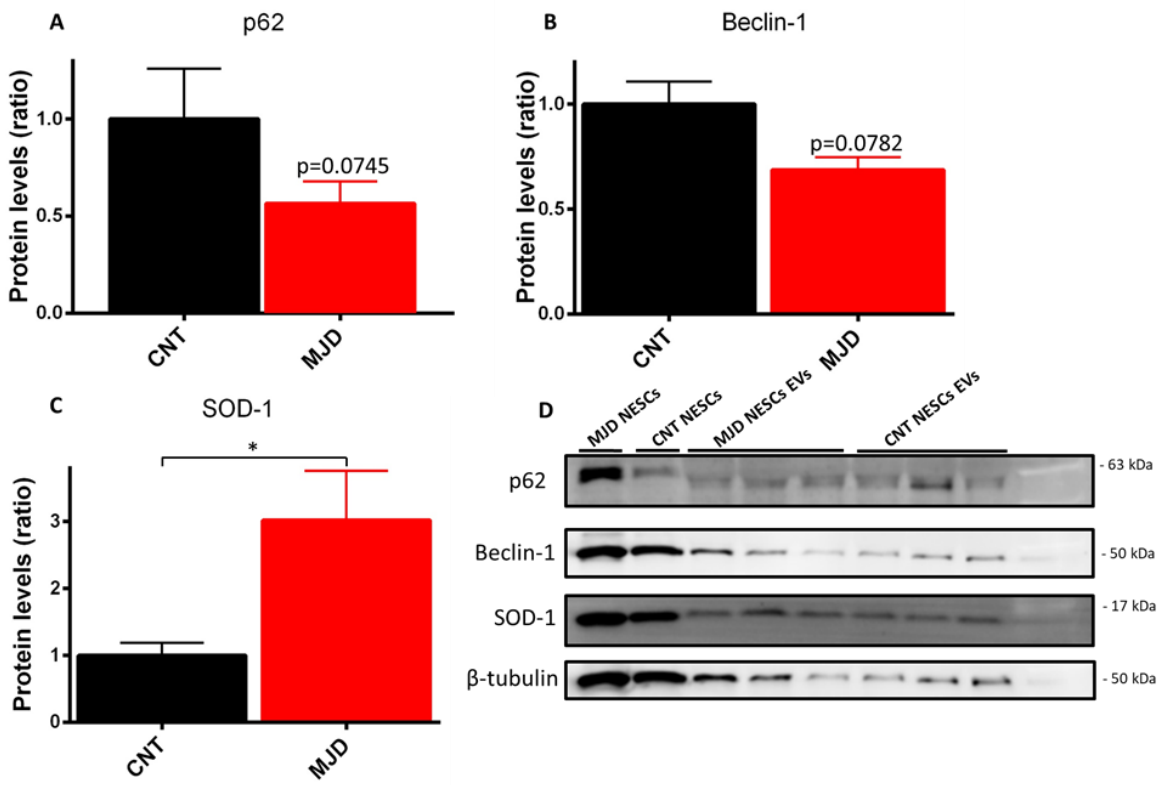


Figure 3.6. Evaluation of autophagy and apoptosis-related proteins in control NESCs-derived EVs (CNT) and MJD NESCs-derived EVs (MJD) through western blot. (A) Quantification of p62, (B) Beclin-1 and (C) SOD-1 levels normalized for β-tubulin and control average. (D) Western-blot representative image. Control samples of MJD iPSCs-derived NESCs (MJD NESCs) and control iPSCs-derived NESCs (CNT NESCs). Data are expressed as mean ± SEM; *p<0.05, unpaired t test with Welch's correction; p62 n=8, Beclin-1 n=3, SOD-1 n=9.

The presence of mutant Ataxin-3 protein in MJD NESCs-derived EVs was also assessed but, as demonstrated in Figure 3.7, this protein was not found in the EVs.

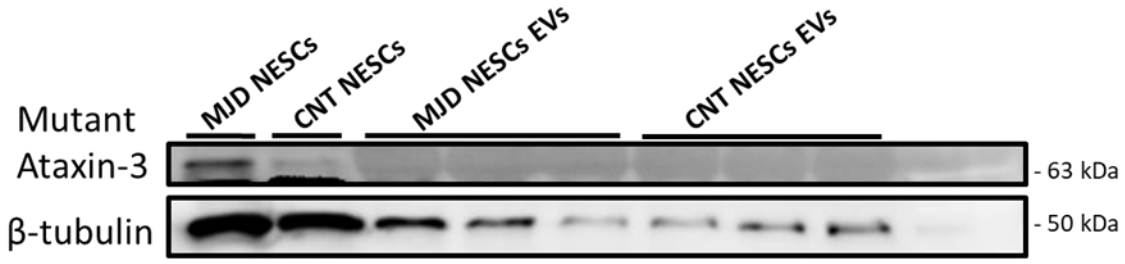


Figure 3.7. Evaluation of mutant Ataxin-3 protein presence in MJD NESCs-derived EVs through western blot analysis. MJD NESCs-derived EVs (MJD NESCs EVs) and control NESCs-derived EVs (CNT NESCs EVs). Control samples of MJD iPSCs-derived NESCs (MJD NESCs) and control iPSCs-derived NESCs (CNT NESCs).

3.1.4.3. Screening of Autophagy and Apoptosis-related Proteins in Neuronal Cultures-derived EVs

Guided by the results obtained above, the autophagy and apoptosis-related markers found in the NESCs-derived EVs were also sought in the neuronal cultures-derived EVs population (Figure 3.8).

The p62 protein (Figure 3.8 A, D) levels were significantly decreased in 34 % (0.664 ± 0.081) in MJD neuronal cultures-derived EVs population (MJD) comparing to the control EVs (CNT). Relatively to Beclin-1 and SOD-1 (Figure 3.7 B, C, D) no significant differences were observed between the EVs populations.

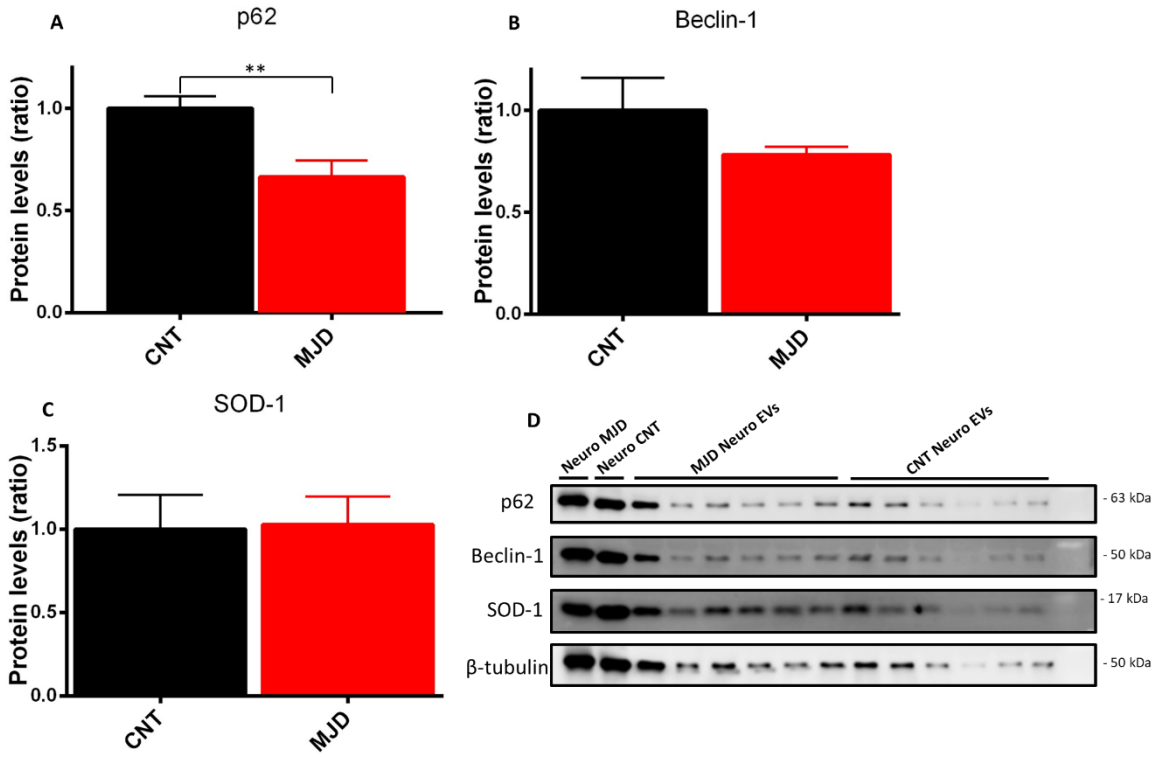


Figure 3.8. Quantification of autophagy and apoptosis-related proteins in neuronal cultures-derived EVs. (A) Quantification of p62, (B) Beclin-1 and (C) SOD-1 normalized with β-tubulin and the average of the controls. (D) Western-blot image of P62, Beclin-1 and SOD-1 expression of control neuronal cultures-derived EVs (CNT Neuro EVs) and MJD neuronal cultures-derived EVs (MJD Neuro EVs). Control samples of control iPSCs-derived NESCs neuronal cultures (Neuro CNT) and MJD iPSCs-derived NESCs neuronal cultures (Neuro MJD). Data are expressed as mean ± SEM; *p<0.01, unpaired t test with Welch's correction; n=6.

Thus, our results indicate differences in terms of protein content between MJD and control EVs.

3.1.5. Screening of Mutant Ataxin-3, Autophagy, Apoptosis and Inflammation-related RNA in NESCs-derived EVs

Furthermore, the RNA content from control and MJD NESCs-derived EVs was analyzed.

For all samples was possible to extract total RNA. The RNA of several proteins related to apoptosis such as CASP3, CASP8, CASP7, CAS9 and Cytochrome C; inflammation, as IL-8 and TNF-α, and autophagy, as LC3-B and Beclin-1 were screened through qRT-PCR. As summarized in Table 3.1, several of the screened RNA were non-detectable by this method, and the ones that were detected exhibited high and

irregular threshold cycles making the quantification not possible. The exception was the gene of the mitochondrial-related apoptosis marker Cytochrome C (*CYCS*) that exhibited regular threshold cycles.

Detected mRNA	Non-detected mRNA
CASP3 (CT=38.24)	IL-8
CASP8 (CT=30.77)	CASP9
CASP7 (CT=33.11)	LC3-B
CYCS (CT=29.10)	TNF-α
	Beclin-1

Table 3.1. Detected and non-detected RNA of genes related to autophagy, apoptosis and inflammation. CASP: Caspase; CYCS: Cytochrome C; IL: Interleukin; LC3-B: Light Chain Protein 3; TNF-α: Tumor Necrosis Factor-α; CT: Threshold Cycle.

The presence of mutant *ATXN3* in EVs derived from NESCs carrying MJD was evaluated through semi-quantitative RT-PCR. As can be observed in Figure 3.9, the band that characterizes wild type Ataxin-3 (182 bp) is present both in control and MJD NESCs-derived EVs; while the mutant Ataxin-3 (353 bp) was only found in 3 of the 5 MJD NESCs-derived EVs tested.

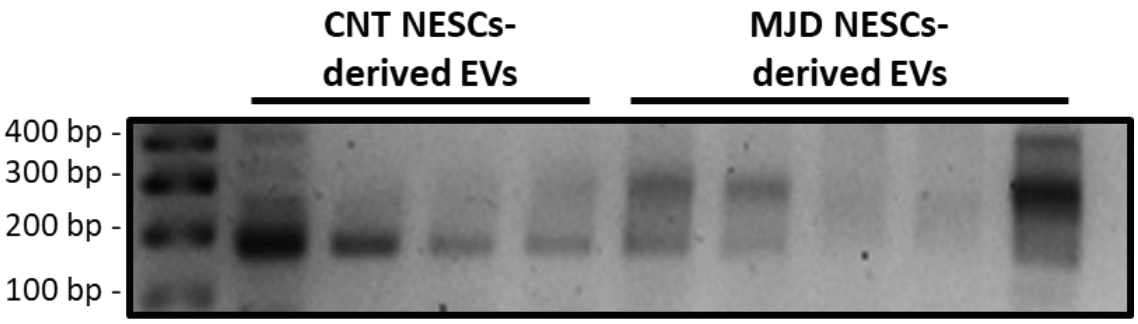


Figure 3.9. Evaluation of mutant Ataxin-3 mRNA presence in MJD NESCs-derived EVs through semi-quantitative RT-PCR.

3.2. *In Vitro* Studies with Extracellular Vesicles

Next, a series of in vitro experiments assessing potential interactions between EVs and neuronal cultures were performed. Additionally, effects of NESCs and neuronal cultures-derived EVs in control NESCs-derived neuronal cultures were also evaluated.

3.2.1 EVs Internalization by NESCs-derived neuronal cultures

Before starting the evaluation of the impact of EVs administration in cellular pathways related to MJD, it was first evaluated if the cells used in this study internalize the obtained EVs. For this, CFSE-labeled EVs were incubated with control NESCs-derived neuronal cultures for 14 hours. As can be observed in Figure 3.10, CFSE-labeled EVs (green puncta) are internalized by the neuronal culture (white arrow).

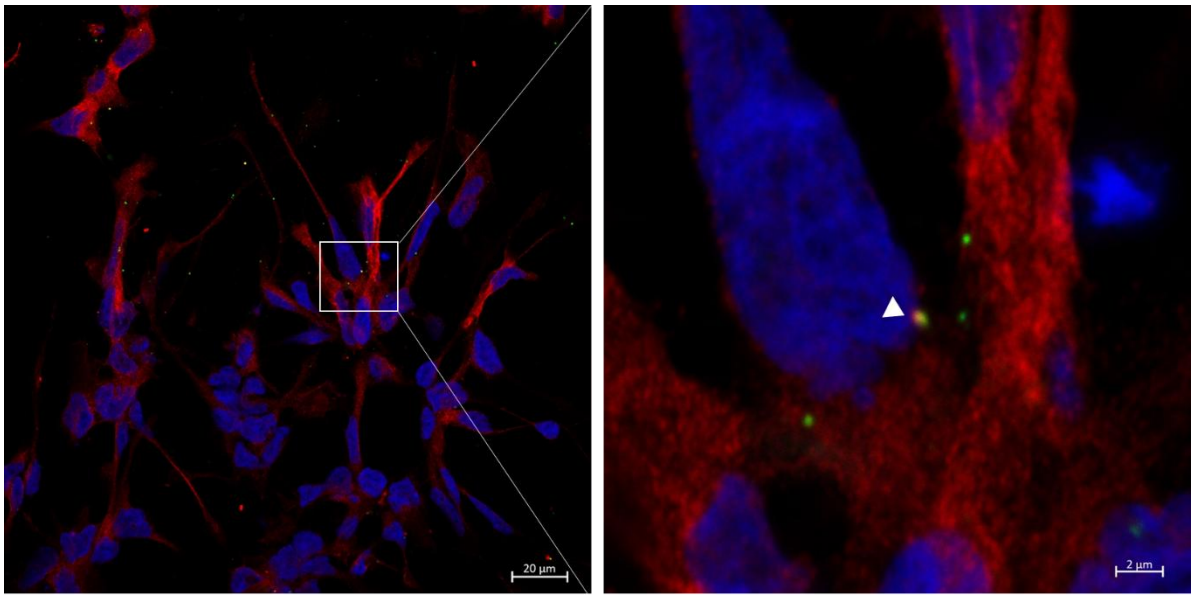


Figure 3.10. EVs internalization by control NESCs-derived neuronal cultures. Neural cultures stained for MAP2 neuronal marker (red), CFSE-labeled EVs (green) and nuclei (DAPI, blue). Representative confocal microscopy images of 3 independent experiments.

3.2.2. Incubation of Neuronal Cultures with Control and MJD NESCs-derived EVs for 2 Weeks

To study the effects of control and MJD NESCs-derived EVs in control NESCs-derived neuronal cultures, with one week of differentiation, these cells were incubated with 50 and 100 µg/ml of control and MJD NESCs-derived EVs for two weeks. Cell viability (Figure 3.11) was performed and as indicated, the tested vesicles triggered no effect on cell viability at any of the tested concentrations.

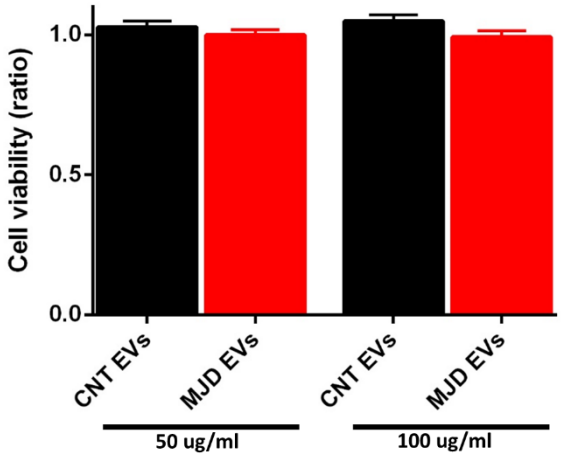
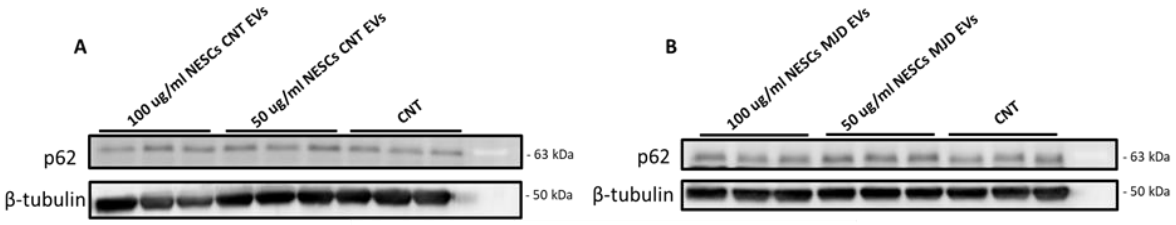


Figure 3.11. Cell viability of control NESCs-derived neuronal cultures incubated for two weeks with 50 and 100 μg/ml of control NESCs-derived EVs (CNT EVs) and MJD NESCs-derived EVs (MJD EVs). The variables were normalized to control neuronal cultures that were not incubated with EVs. Data are expressed as mean ± SEM of 3 independent experiments; One-way ANOVA with Tukey's multiple comparison test.

Then, the proteins found to be differently expressed between control and MJD EVs were also evaluated here. Thus, the autophagy-related p62 and Beclin-1, as well as the apoptosis-related protein SOD-1 levels were quantified (Figure 3.12).

The p62 protein levels were found to be decreased with the incubation of 100 μg/ml of MJD NESCs-derived EVs (0.916 ± 0.041), comparing to the amount of protein in the control NESCs-derived neuronal cultures. On the other hand, this protein shows a tendency to increase 1.74 times when control NESCs-derived EVs are added to the culture.



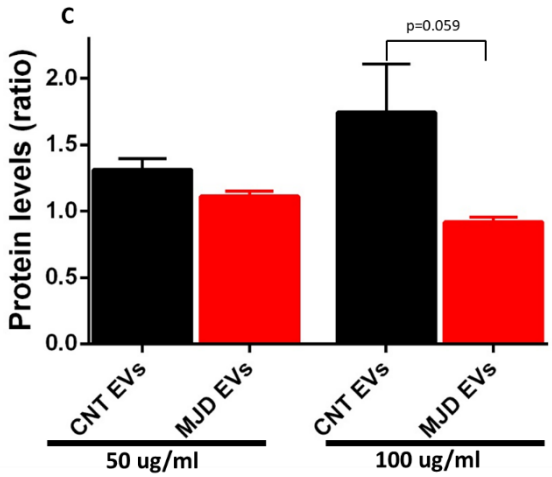
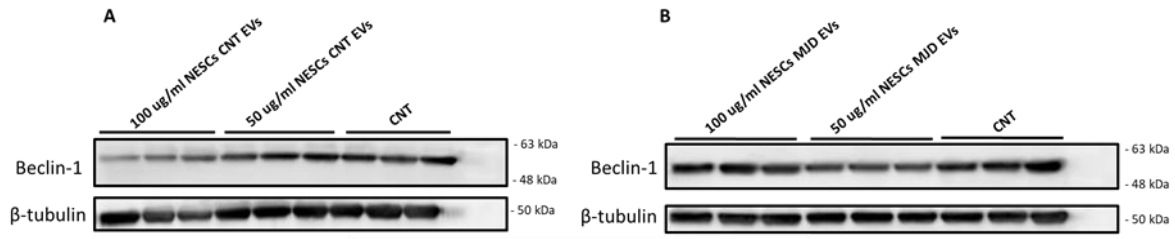


Figure 3.12. Levels of p62 protein in control NESCs-derived neuronal cultures incubated 2 weeks with (CNT EVs) 50 and 100 μg/ml of control NESCs-derived EVs and (MJD EVs) MJD NESCs-derived EVs. (A) Western-blot image of p62 levels in cells incubated with control NESCs-derived EVs or (B) MJD NESCs-derived EVs. (C) Quantification of p62 levels normalized for control NESCs-derived neuronal cultures (CNT) p62 levels. Data are expressed as mean ± SEM of 3 independent experiments. One-way ANOVA with Tukey's multiple comparison test; multiplicity adjusted p values.

Beclin-1 levels of control NESCs-derived neuronal cultures incubated with 50 and 100 μg/ml of control NESCs-derived EVs (Figure 3.13 A, C) or with 50 and 100 μg/ml of MJD NESCs-derived EVs (Figure 3.13 B, C) have a tendency to be decreased, comparing to the amount of protein in the control NESCs-derived neuronal cultures. The higher change was observed with 50 μg/ml of MJD NESCs-derived EVs promoting 43% reduction (0.574 ± 0.011) in the protein levels.



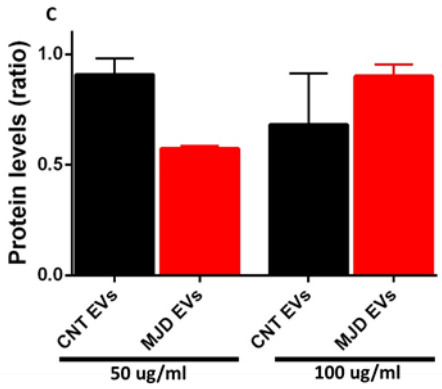
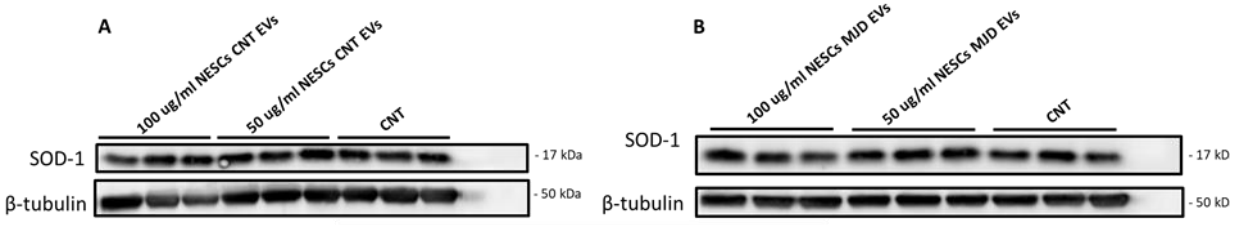


Figure 3.13. Beclin-1 protein levels in control NESCs-derived neuronal cultures incubated 2 weeks with 50 and 100 μg/ml of control and MJD NESCs-derived EVs. Western-blot image of Beclin-1 in cells incubated with (A) control NESCs-derived EVs or (B) MJD NESCs-derived EVs. (C) Quantification of Beclin-1 levels normalized for control NESCs-derived neuronal cultures (CNT) Beclin-1 levels. Data are expressed as mean ± SEM of 3 independent experiments. One-way ANOVA with Tukey's multiple comparison test.

Taken together, our results indicate that autophagy may be affected by the presence of EVs at the time point of 2 weeks. Noteworthy is the fact that the presence of the two different types of EVs appears to have opposite effects in p62 levels.

Furthermore, the apoptosis-related protein SOD-1 was also assessed in control NESCs-derived neuronal cultures incubated with 50 and 100 μg/ml of control NESCs-derived EVs (Figure 3.14 A) or with 50 and 100 μg/ml of of MJD NESCs-derived EVs (Figure 3.14 B). Similar to the results for p62, the biggest observed difference for the variables presented is between the incubation with 100 μg/ml of control NESCs-derived EVs (1.220 ± 0.315) and the incubation with 100 μg/ml of MJD NESCs-derived EVs (0.475 ± 0.090). Therefore, SOD-1 has a tendency to be decreased when 100 μg/ml of MJD NESCs-derived EVs are incubated with the cells.



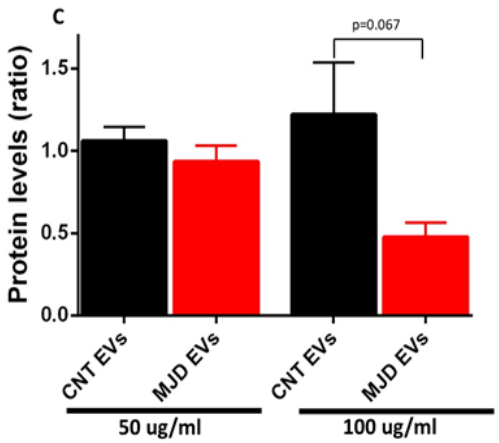


Figure 3.14. SOD-1 protein quantification in control NESCs-derived neuronal cultures incubated 2 weeks with 50 and 100 μg/ml of control NESCs-derived EVs (CNT EVs) and MJD NESCs-derived EVs (MJD EVs). Western-blot image of SOD-1 protein in cells incubated with (A) control NESCs-derived EVs or (B) MJD NESCs-derived EVs. (C) Quantification of SOD-1 levels normalized for control NESCs-derived neuronal cultures (CNT) SOD-1 levels. Data are expressed as mean ± SEM of 3 independent experiments. One-way ANOVA with Tukey's multiple comparison test.

3.2.3. Incubation of Neural Cultures with Control and MJD NESCs-derived EVs for 3 days

In order to evaluate whether shorter incubation times might result in higher changes in the cells, we performed the evaluation of the effect of the incubation of EVs at 3 days after adding the vesicles.

Thus, control NESCs-derived neuronal cultures were incubated with 50 and 100 μg/ml of control NESCs-derived EVs or with 50 and 100 μg/ml of MJD NESCs-derived EVs and 3 days after the impact of the EVs in cell viability and p62, Beclin-1 and SOD-1 protein levels was assessed.

Again no significant changes in cell viability were performed by the studied vesicles (Figure 3.15).

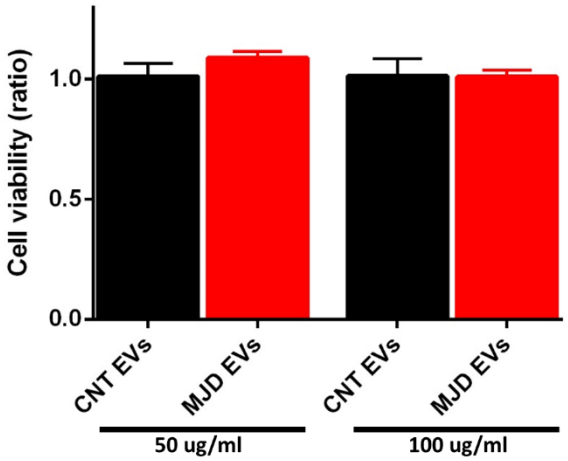


Figure 3.15. Cell viability assay of control NESCs-derived neuronal cultures incubated for 3 days with 50 and 100 μg/ml of control NESCs-derived EVs (CNT EVs) and MJD NESCs-derived EVs (MJD EVs). Data are expressed as mean ± SEM; n=9 for CNT EVs and n=12 for MJD EVs, One-way ANOVA with Tukey's multiple comparison test.

As observed for 2 weeks of incubation with EVs, the p62 protein levels are increased with 100 μg/ml of control NESCs-derived EVs (Figure 3.16 A, C) (1.569 ± 0.142), and this enhancement is dependent of the concentration, being more reduced for 50 μg/ml of control NESCs-derived EVs, 1.099 ± 0.128. Additionally, the MJD NESCs-derived EVs promote no significant changes in the p62 levels (Figure 3.16 B, C).

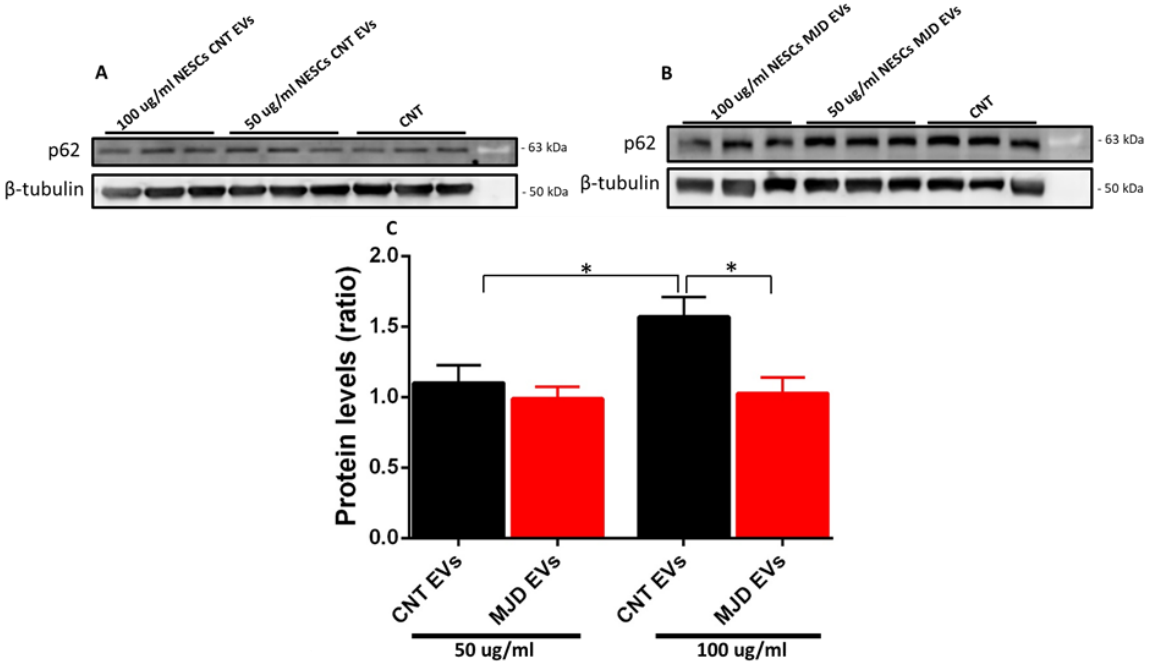


Figure 3.16. Evaluation of p62 protein levels in control NESCs-derived neuronal cultures incubated for 3 days with 50 and 100 μg/ml of control and MJD NESCs-derived EVs. Western-blot image of p62 in cells incubated with (A) control NESCs-derived EVs (CNT EVs)

or (B) MJD NESCs-derived EVs (MJD EVs). (C) Quantification of p62 levels normalized for control NESCs-derived neuronal cultures (CNT) p62 levels. Data are expressed as mean ± SEM; n=9 for CNT EVs and n=11 for MJD EVs; *p<0.05. One-way ANOVA with Tukey's multiple comparison test; multiplicity adjusted p values.

No significant changes were observed for Beclin-1 (Figure 3.17) and SOD-1 (Figure 3.18) protein levels of neuronal cultures incubated with control and MJD NESCs-derived EVs during 3 days.

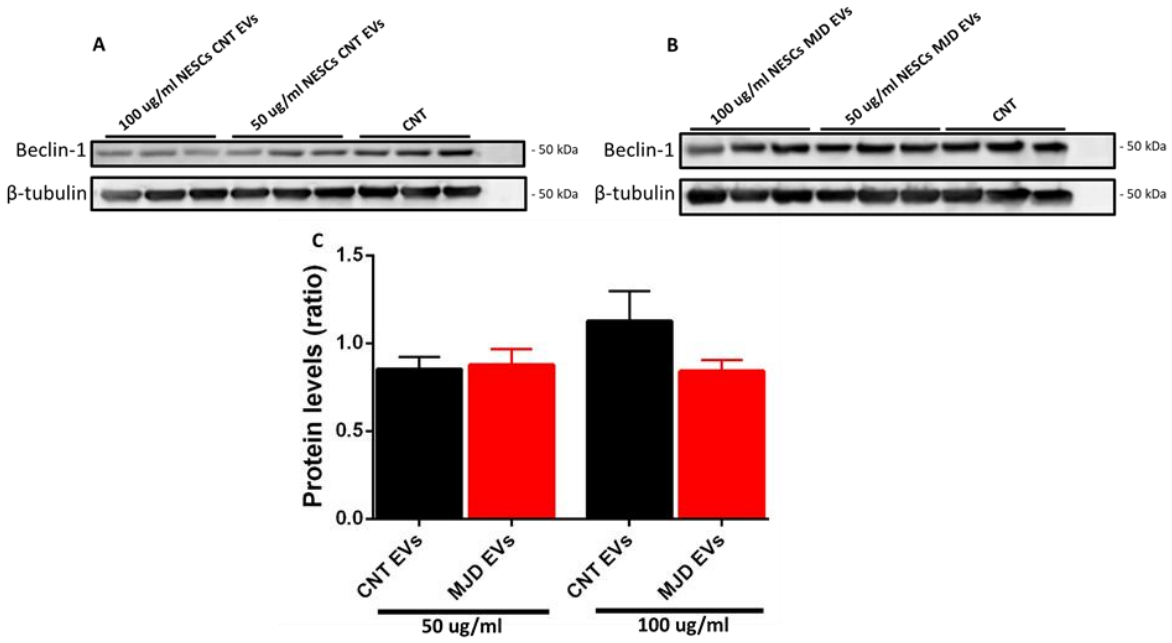
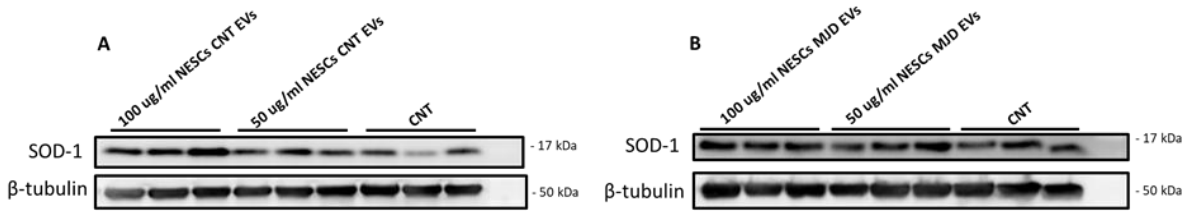


Figure 3.17. Beclin-1 protein levels in control NESCs-derived neuronal cultures incubated for 3 days with 50 and 100 μg/ml of control NESCs-derived EVs (CNT EVs) and MJD NESCs-derived EVs (MJD EVs). Western-blot image of Beclin-1 in cells incubated with (A) CNT EVs and (B) MJD EVs. (C) Quantification of Beclin-1 normalized for control NESCs-derived neuronal cultures (CNT) Beclin-1 levels. Data are expressed as mean ± SEM; n=9 for CNT EVs and n=5 for MJD EVs; One-way ANOVA with Tukey's multiple comparison test; multiplicity adjusted p values.



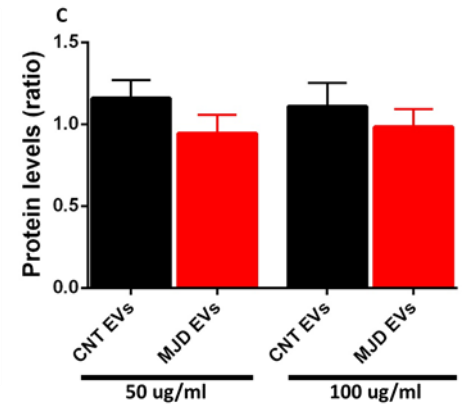


Figure 3.18. SOD-1 protein levels in control NESCs-derived neural cultures incubated for 3 days with 50 and 100 μg/ml of control and MJD NESCs-derived EVs. Western-blot image of SOD-1 in cells incubated with (A) control NESCs-derived EVs (CNT EVs) and (B) MJD NESCs-derived EVs (MJD EVs). (C) Quantification of SOD-1 normalized for control NESCs-derived neuronal cultures (CNT) SOD-1 levels. Data are expressed as mean ± SEM; n=9 for CNT EVs and n=11 for MJD EVs; One-way ANOVA with Tukey's multiple comparison test; multiplicity adjusted p values.

3.2.4. Incubation of Neural Cultures with Control and MJD Neuronal Cultures-derived EVs for 3 days

The effect of control and MJD neuronal-derived EVs on neuronal cultures was also assessed. In these experiments, control NESCs-derived neuronal cultures were incubated with control and MJD neuronal-derived EVs for 3 days and the impact of the vesicles in cell viability and p62, Beclin-1 and SOD-1 protein levels was assessed. Again, no differences were triggered by the tested EVs in the cell viability (Figure 3.19).

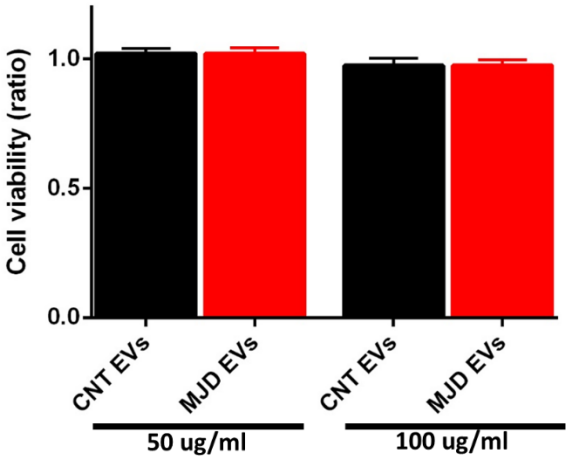


Figure 3.19. Cell viability of control NESCs-derived neural cultures incubated for 3 days with 50 and 100 μg/ml of control neuronal cultures-derived EVs (CNT EVs) and MJD neuronal cultures-derived EVs (MJD EVs). Data are expressed as mean ± SEM of 3 independent experiments. One-way ANOVA with Tukey's multiple comparison test.

Relatively to p62, it was observed that control neuronal cultures incubated with 50 and 100 μg/ml of control neuronal cultures-derived EVs (Figure 3.20 A, C) have a tendency to have p62 levels increased in 1.184 ± 0.092 and 1.133 ± 0.087 times, respectively. As observed for the previously tested particles at 2 weeks, the MJD-derived vesicles promote reduction in the p62 levels (Figure 3.20 B, C), 21 % (0.787 ± 0.027) with 50 μg/ml and 32% (0.684 ± 0.089) with 100 μg/ml of EVs. As previously observed the effect triggered by the control and MJD EVs in the p62 levels are significantly different.

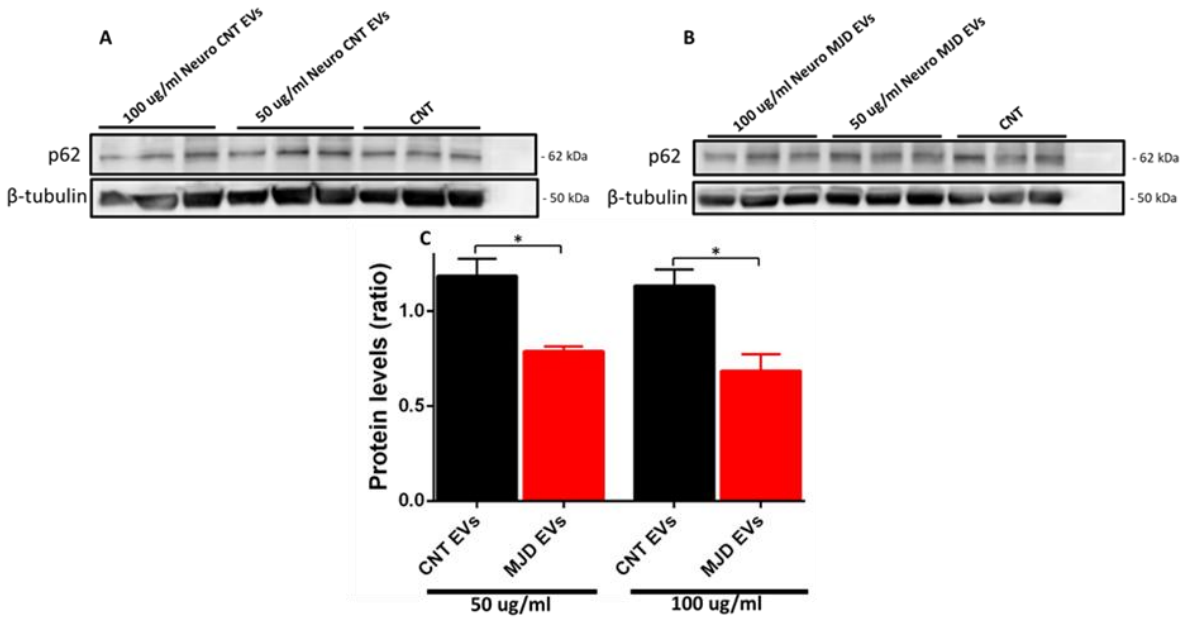


Figure 3.20. Protein p62 levels in control NESCs-derived neural cultures incubated 3 days with 50 and 100 μg/ml of control and MJD neuronal cultures-derived EVs. Western-blot image of p62 in cells incubated with (A) control neuronal cultures-derived EVs (CNT EVs) and (B) MJD neuronal cultures-derived EVs (MJD EVs). (C) Quantification of p62 normalized for control NESCs-derived neuronal cultures (CNT) p62 levels. Data are expressed as mean ± SEM of 3 independent experiments; *$p<0.05$, One-way ANOVA with Tukey's multiple comparison test, multiplicity adjusted p values.

Relatively to SOD-1 protein, likewise the quantifications for p62, for the same quantity of EVs of each of the population, this protein was significantly decreased

(from 1.116 ± 0.074 to 0.750 ± 0.003 with 100 μg/ml) in the cells that were exposed to MJD neuronal cultures-derived EVs as compared to cells exposed to control neuronal cultures derived EVs. Additionally, no differences were found regarding the two different concentrations tested.

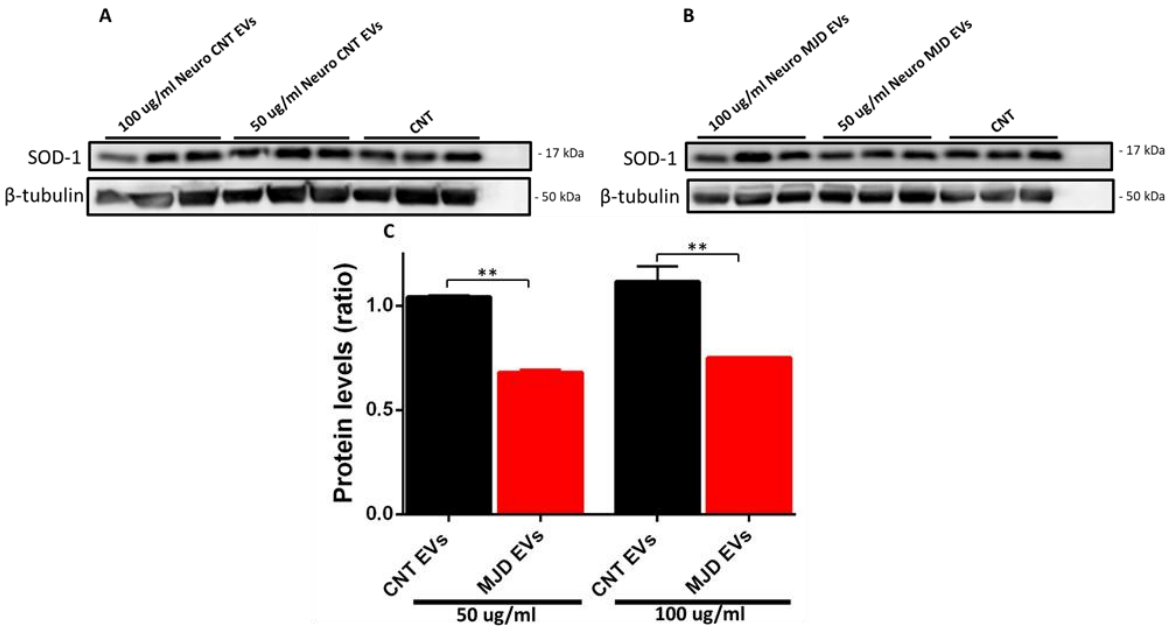


Figure 3.21. SOD-1 quantification in control NESCs-derived neural cultures incubated for 3 days with 50 and 100 μg/ml of control neuronal cultures-derived EVs (CNT EVs) and MJD neuronal cultures-derived EVs (MJD EVs). Western-blot image of SOD-1 in cells incubated with (A) control neuronal cultures-derived EVs and (B) MJD neuronal cultures-derived EVs. (C) Quantification of SOD-1 normalized for control NESCs-derived neuronal cultures (CNT) SOD-1 levels. Data are expressed as mean ± SEM of 3 independent experiments. **p<0.01, One-way ANOVA with Tukey's multiple comparison test; multiplicity adjusted p values.

3.2.5. Reactive Oxygen Species Production Upon Treatment with NESCs-derived EVs

The production and processing of reactive oxygen species (ROS) by cells is an important mechanism and often associated with neuropathological conditions. These ROS are hazardous for the cell and their quick processing is crucial for cell survival.

As so, and since the presence of an important ROS processing enzyme had been found to be present in the EVs, the effect of the administration of EVs in the cellular production of ROS was also assessed with the aid of a probe of ROS (Figure 3.22).

Our results indicate that, as expected control neuronal cultures *per se* exhibit some production of ROS (Figure 3.22 A), since it is a physiological process related energy production. Surprisingly, when 100 µg/ml of control (Figure 3.22 B) and MJD (Figure 3.22 C) NESCs-derived EVs were administrated to these cells a 4 times increase in ROS production was observed (Figure 3. 22 A-F). Additionally, no significant differences were observed between cells treated with control and MJD NESCs-derived EVs.

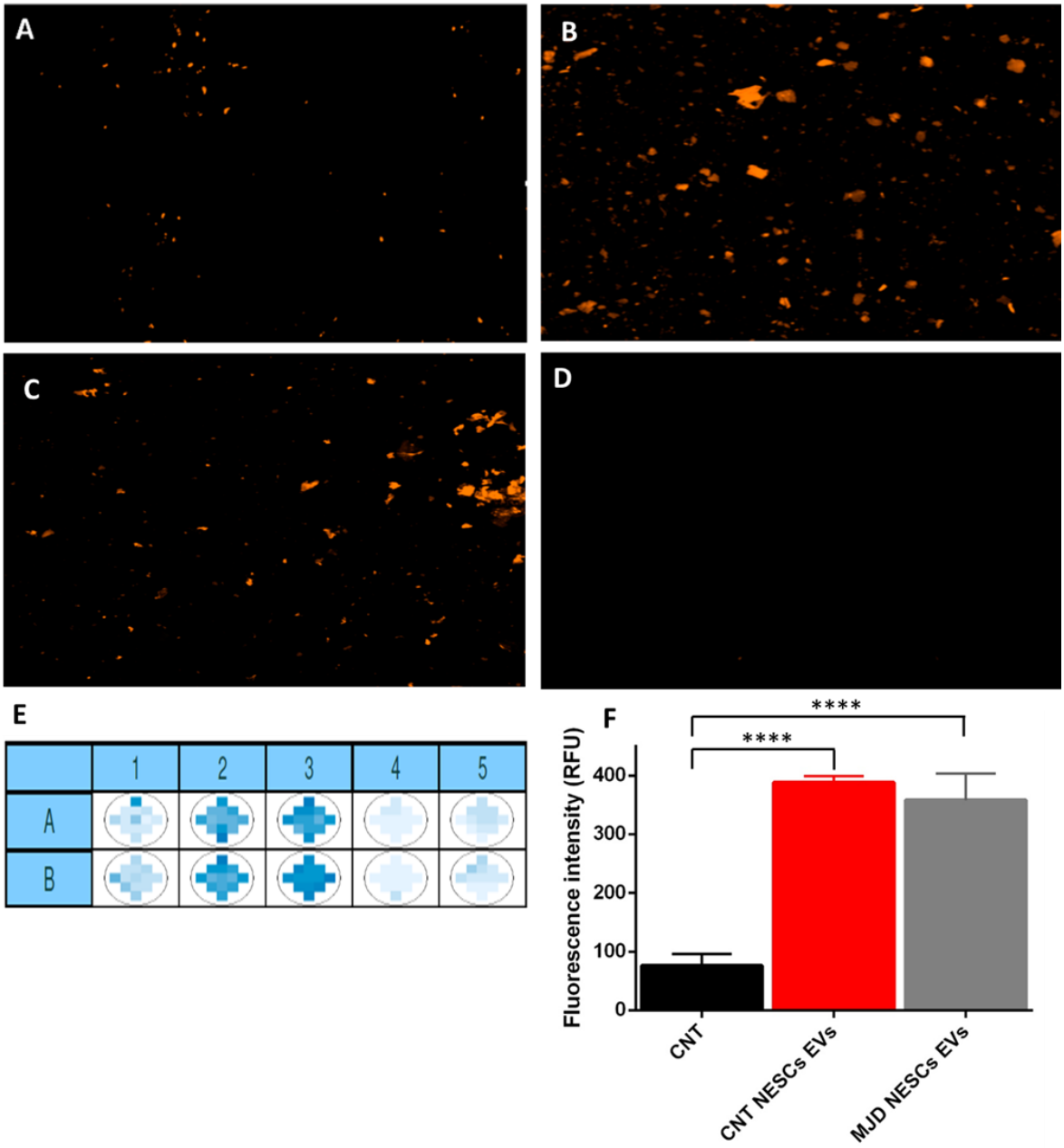


Figure 3.22. Reactive oxygen species production upon treatment with NESCs-derived EVs. (A, E.1, F) Control neuronal cultures without EVs added (CNT), (B, E.2, F) Control neuronal cultures incubated 1 h with 100 µg/ml control NESCs-derived EVs (CNT NESCs EVs), (C, E.3, F) Control neuronal cultures incubated 1 with 100 µg/ml MJD NESCs-derived EVs (MJD NESCs EVs), were incubated 2 h with ROS probe; (D) free probe signal. (E) Fluorescence intensity: (1) neuronal cultures; (2) neuronal cultures with control EVs; (3) neuronal cultures

with MJD EVs; (4) free probe; (5) blank well. (F) Quantification of fluorescence intensity units (RFU). Data are expressed as mean ± SEM of 6 independent experiments. ****p<0.0001 One-way ANOVA with Tukey's multiple comparison test; multiplicity adjusted p values.

3.2.6. Assessment of effects of MJD neuronal cells media in neuropathology

To evaluate whether other vesicles and mediators beside EVs might be interfering in the MJD neuropathological spreading it was submitted control neuronal cells to the effect of MJD neuronal cells media, through contactless media sharing (indirect co-culture). Thus, MJD neuronal cells were plated in inserts (top) and the control neuronal cells were plated in the bottom well, this way both type of cells shared the same culture medium, and this contactless medium sharing was performed during one or three weeks.

The main goals of these experiments were to evaluate the possibility of transmission of mutant Ataxin-3 between cells without the need of cellular contact, as well as possible physiological modifications such as autophagy or typical apoptosis markers deregulations.

3.2.6.1. Mutant Ataxin-3 spreading

As so, with the aim to evaluate the presence of mutant Ataxin-3 aggregates in control neurons, these cells that shared the media with MJD neuronal cells (β3-tubulin positive cells) were immunostained for mutant Ataxin-3 aggregates (Figure 3.23). Control neuronal cells present no mutant Ataxin-3 aggregates (Figure 3.23 A). Nevertheless, after one week of sharing culture medium with MJD neuronal cultures mutant Ataxin-3 aggregates could be observed (Figure 3.23 B); although a very low number of aggregates were detected, indicating that this process might require longer time points.

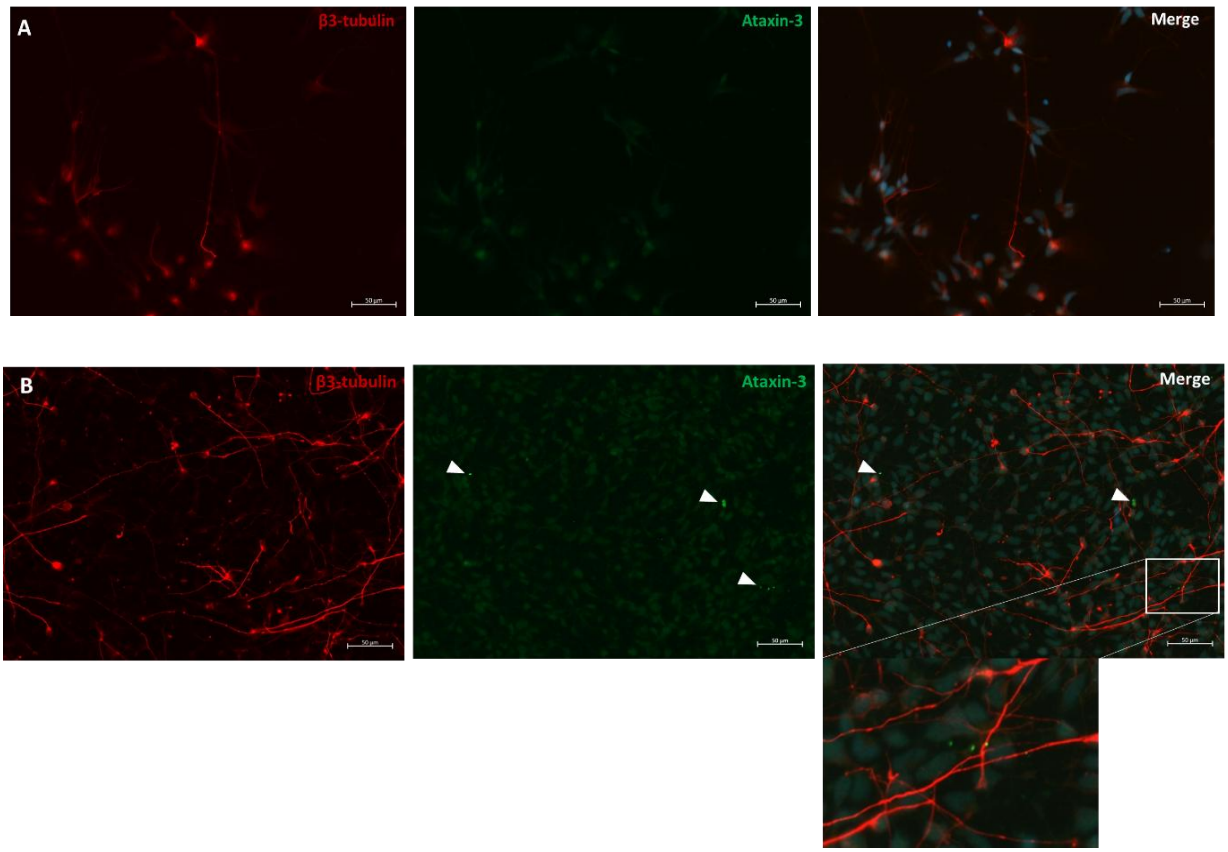


Figure 3.23. Mutant Ataxin-3 spreading assessment with contactless culture medium sharing for 1 week. (A) Control NESCs-derived neuronal cultures not sharing culture medium, (B) Control NESCs-derived neural cultures that shared culture media with MJD NESCs-derived neural cultures for one week, and (C) MJD neuronal cultures, all stained for β3-tubulin neuronal marker (red), mutant Ataxin-3 aggregates (green, white arrows) and nuclei (DAPI, blue). Representative images of 3 independent experiments.

Therefore, a longer time of incubation, 3 weeks, was tested to further assess mutant Ataxin-3 aggregates presence in control neurons (Figure 3.24). Control cells exhibited no presence of mutant Ataxin-3 aggregates (Figure 3.24 A), as for the control NESCs-derived neuronal cultures that shared culture media with MJD NESCs-derived neuronal cultures the mutant Ataxin-3 aggregates were also found (Figure 3.24 B). Surprisingly, apparently it was not observed higher levels of mutant Ataxin-3 aggregation at 3 weeks. Nevertheless mutant Ataxin-3 aggregates number quantification is required to determine whether there is or not differences between the aggregation levels between 1 and 3 weeks.

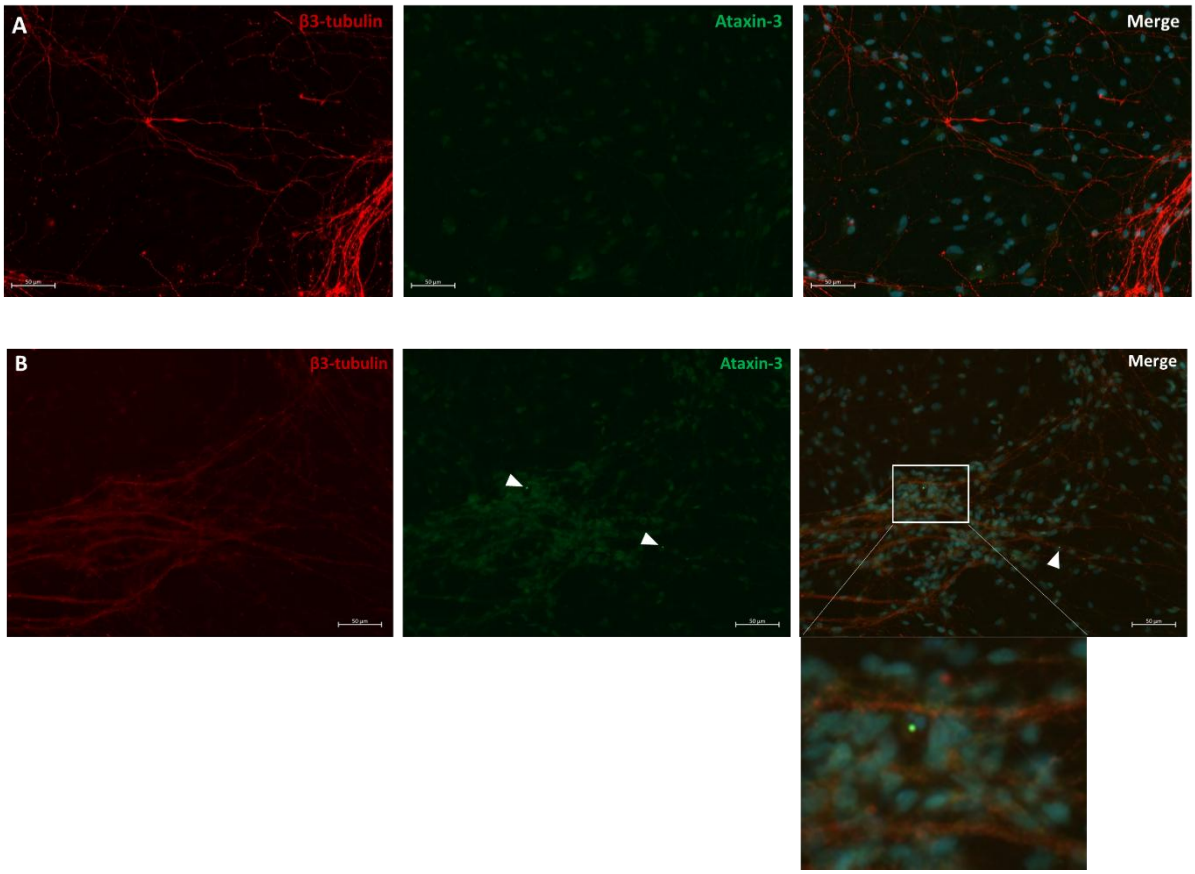


Figure 3.24. Mutant Ataxin-3 spreading assessment with contactless culture medium sharing for 3 weeks. (A) Control NESCs-derived neuronal cultures not sharing culture medium, and (B) control NESCs-derived neural cultures that shared culture media with MJD NESCs-derived neural cultures for 3 weeks. All stained for β3-tubulin neuronal marker (red) and mutant Ataxin-3 aggregates (green, white arrows) through immunocytochemistry; nuclei (DAPI, blue). Representative images of 3 independent experiments.

Mutant Ataxin-3 protein presence in control neurons that shared culture media, without cell contact, with MJD neurons, for 1 and 3 weeks, was also evaluated with Western-blot analysis (Figure 3.25). As can be observed in Figure 3.25, mutant Ataxin-3 (67 KDa) is present in the MJD NESCs-derived neuronal cultures (NESCs MJD), but it was not detected in control neuronal cells that shared culture media with MJD NESCs-derived neural cultures (CNT + MJD) for one week (Figure 3.25 A) or three weeks (Figure 3.25 B). The not detection of mutant Ataxin-3 through this technique might be explained by the very low amount of mutant Ataxin-3 transferred between MJD and control NESCs-derived neuronal cultures, as suggested by immunocytochemistry analysis, which is not enough to be detected in the Western-blot analysis.

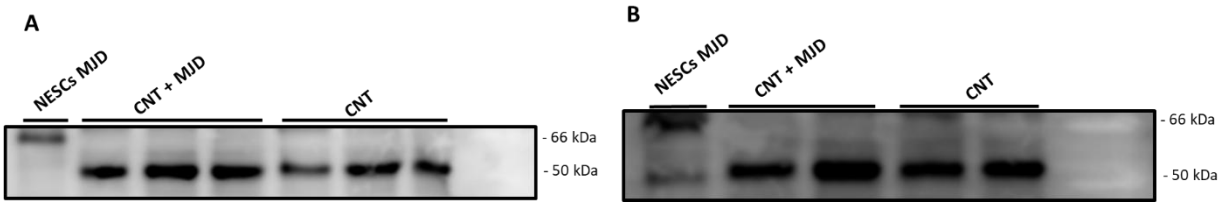


Figure 3.25. Mutant Ataxin-3 protein evaluation in control NESC-derived neuronal cultures sharing culture media, without cell contact, with MJD NESC-derived neuronal cells. Mutant Ataxin-3 evaluation through Western-blot in MJD NESCs-derived neuronal cultures (NESCs MJD), control NESCs-derived neuronal cultures without sharing culture medium (CNT) and control NESCs-derived neuronal cultures sharing culture medium with MJD NESCs-derived neuronal cultures (CNT + MJD) for (A) one week or (B) three weeks.

3.2.6.2. Evaluation of impaired cellular mechanisms

As previously performed, the cellular mechanisms analyzed were autophagy, quantifying changes in p62, LC3-B and Beclin-1 protein levels, and apoptosis, quantifying changes in SOD-1 protein levels.

At the time point of one week (Figure 3.26) the autophagy-related protein p62 presents no significant differences between the control cells (1.000 ± 0.082) and the cells that shared culture media with MJD NESCs-derived neuronal cultures (0.882 ± 0.044) (Figure 3.26 A); while LC3-B has a tendency to be 1.938 ± 0.041 times increased in the cells that shared culture media with MJD NESCs-derived neuronal cultures (Figure 3.26 B). In opposition, the survival signal SOD-1 (Figure 3.26 C) displays a tendency to be 23 % decreased in the control cells that shared media with the diseased neurons (0.766 ± 0.001).

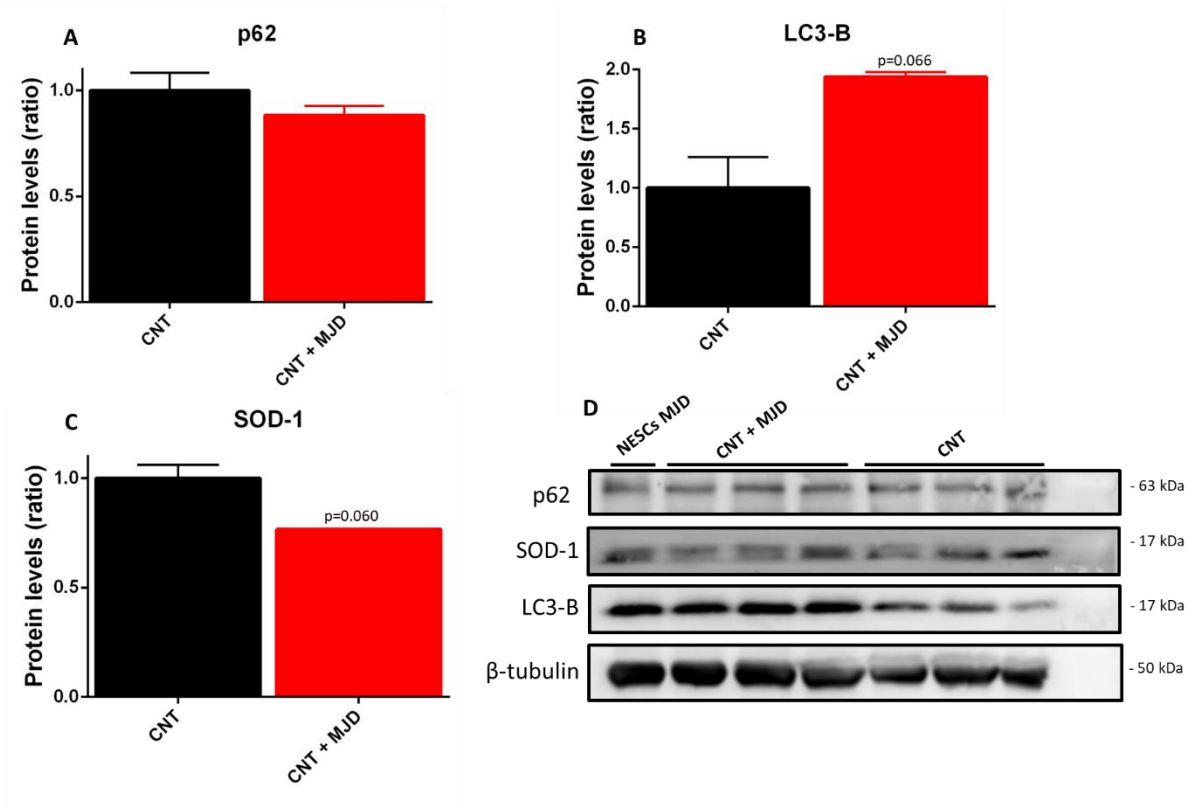


Figure 3.26. Quantification of autophagy and apoptosis-related proteins in control NESCs-derived neuronal cultures sharing culture media, without cell contact, with MJD NESC-derived neuronal cells for one week. Quantification of (A) p62, (B) LC3-B and (C) SOD-1 in MJD NESCs-derived neuronal cultures (NESCs MJD), control NESCs-derived neuronal cultures without sharing culture medium (CNT) and control NESCs-derived neuronal cultures sharing culture medium with MJD NESCs-derived neuronal cultures (CNT + MJD) through Western-blot analysis, normalized for β-tubulin. Data are expressed as mean ± SEM of 3 independent experiments. Unpaired t test with Welch's correction, multiplicity adjusted p values.

To assess if a longer exposure time of the control neuronal cultures to medium of MJD neurons might result in aggravation of autophagy and apoptosis mediators changes, three weeks time point was tested (Figure 3.27). The observed changes in p62, LC3-B and SOD-1 in control neuronal cells that shared culture medium with MJD neurons during 3 weeks are smaller as compared to the observed for 1 week protocol. Nevertheless, these results are from only two independent experiments, thus further N enhancement is required.

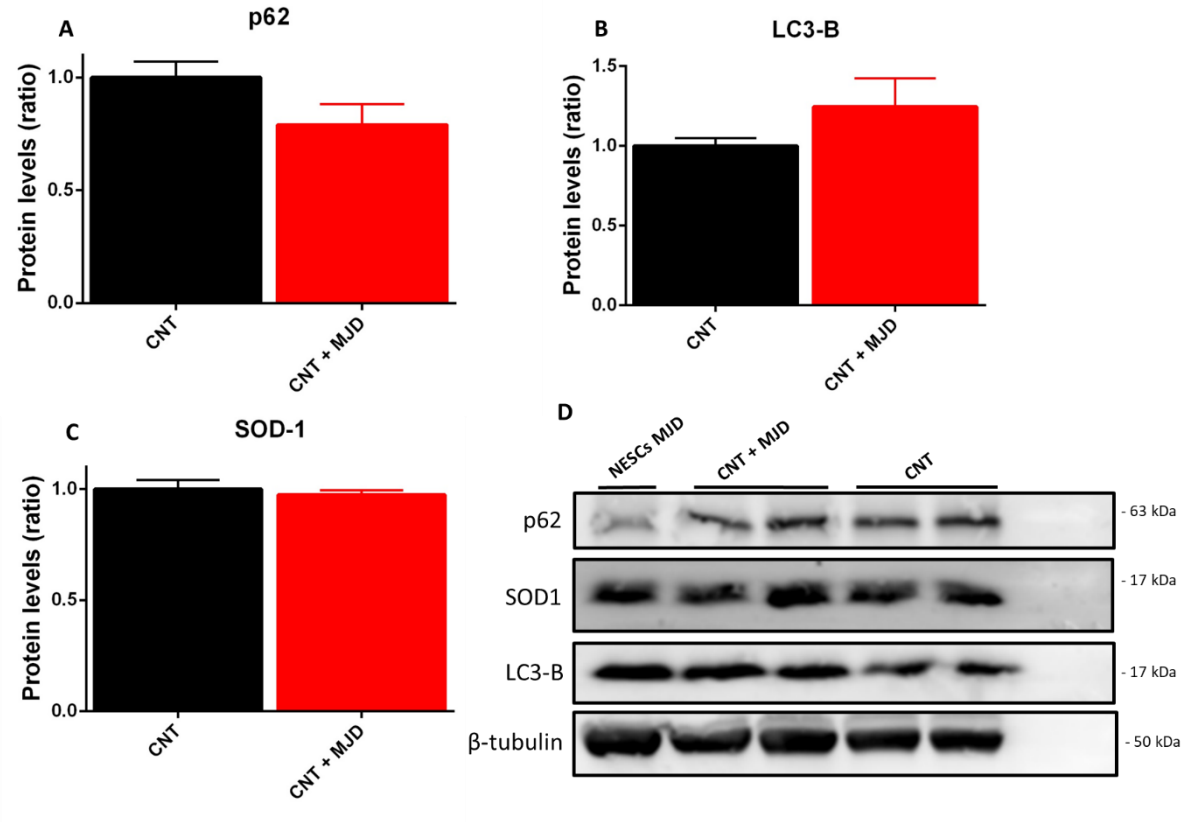


Figure 3.27. Quantification of autophagy and apoptosis-related proteins in control NESCs-derived neuronal cultures sharing culture media, without cell contact, with MJD NESC-derived neuronal cells for three weeks. Quantification of (A) p62, (B) LC3-B and (C) SOD-1 in MJD NESCs-derived neuronal cultures (NESCs MJD), control NESCs-derived neuronal cultures without sharing culture medium (CNT) and control NESCs-derived neuronal cultures sharing culture medium with MJD NESCs-derived neuronal cultures (CNT + MJD) through Western-Blot analysis, normalized for β-tubulin. Data are expressed as mean ± SEM of 2 independent experiments. Unpaired t test with Welch's correction.

3.2.7. Assessment of effects of MJD neuronal cells in neuropathology

Next, the spread of mutant Ataxin-3 from MJD neuronal cells to control ones through direct cellular contact (direct co-culture) was assessed. Having this in mind, a mixed culture of control GFP-expressing and MJD neuronal cells was incubated and the presence of Ataxin-3 inclusion evaluated after two weeks.

In Figure 3.28 the MJD neuronal cells are clearly distinguishable from control ones since they are only stained for MAP2 (red) (Figure 3.28 A), while the control neuronal cells express both GFP (Figure 3.28 B) and MAP2. Mutant Ataxin-3 aggregates (Figure 3.28 C, D, white arrows) were observed in the MJD neuronal cells.

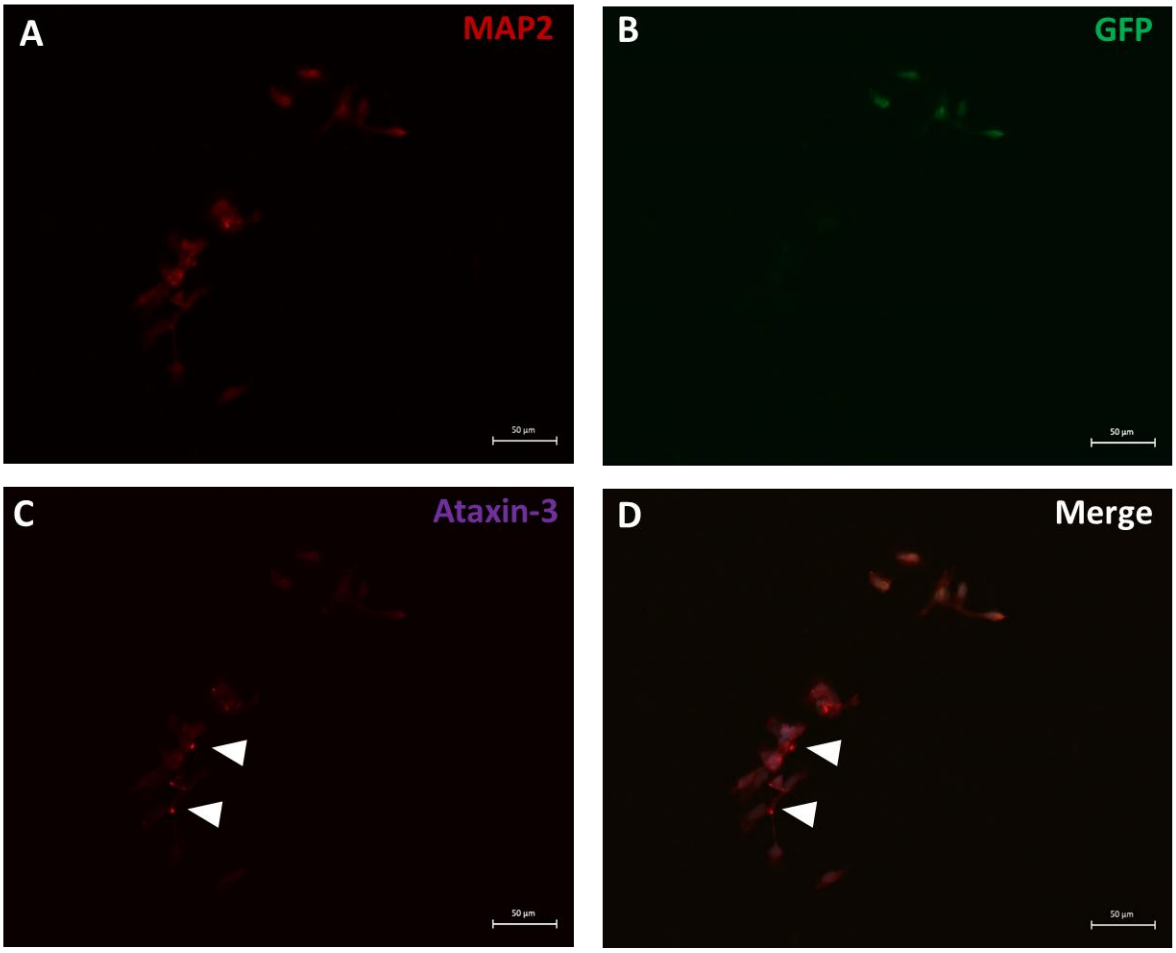


Figure 3.28. Discrimination of MJD and control NESCs-derived neuronal cells incubated for two weeks in a mixed culture. (A) Control and MJD NESCs-derived neuronal cells stained for neuronal marker MAP2 (red), (B) control NESCs-derived neural cells expressing GFP (green), and (C) mutant Ataxin-3 aggregates (purple, white arrows) through immunocytochemistry; nuclei (DAPI, blue). Representative images of 2 independent experiments.

Furthermore, (Figure 3.29 A) in the mixed neuronal cultures, some control GFP-expressing neuronal cells (Figure 3.29 B) were found to have (Figure 3.29 C, white arrows) mutant Ataxin-3 aggregates. This result confirms the diseased-to-control neuronal spread of mutant Ataxin-3 upon direct cellular contact.

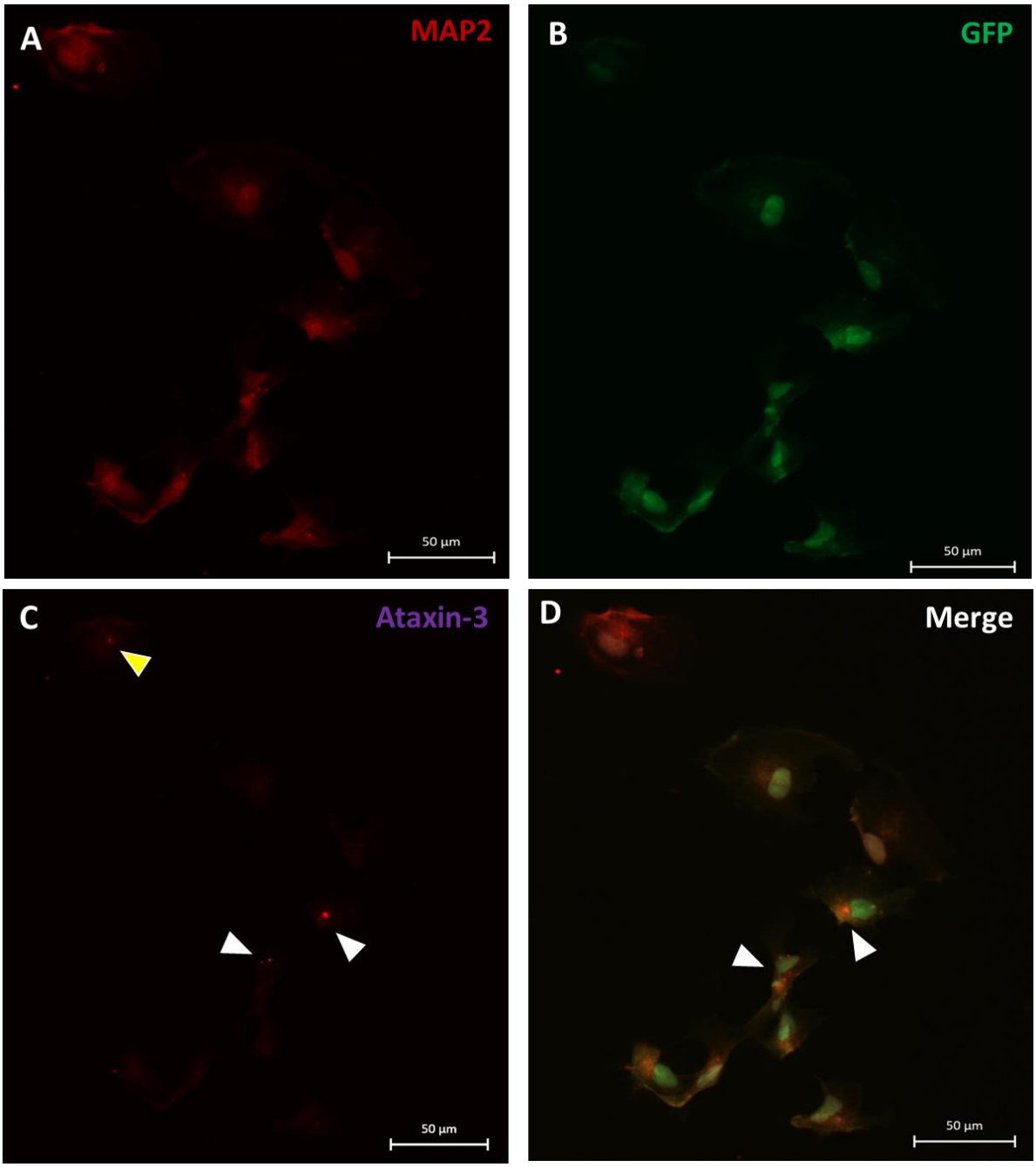


Figure 3.29. Mixed culture of MJD and control NESCs-derived neuronal cells incubated for two weeks. (A) Control and MJD NESCs-derived neuronal cells stained for neuronal marker MAP2 (red), (B) control NESCs-derived neural cells expressing GFP (green), and (C) mutant Ataxin-3 aggregates (purple) in MJD NESCs-derived neurons (yellow arrow) and in control NESCs-derived neurons (white arrows) through immunocytochemistry; nuclei (DAPI, blue). Representative images of 2 independent experiments.

3.3. In vivo assessment of mutant Ataxin-3 spreading

In this task, immunodeficient adult NOD.scid mice were transplanted in the cerebellum with control and MJD iPSC-derived NESCs expressing GFP.

Eight weeks after the transplantation, the potential seeding of mutant Ataxin-3 by MJD NESCs, evaluated by the presence of mutant Ataxin-3 aggregates outside the cells graft, was evaluated (Figure 3.28). As can be observed in Figure 3.28 A, no mutant Ataxin-3 aggregates (red puncta) was observed in control NESCs and in the surrounding tissue. Regarding MJD NESCs (Figure 3.28 B), several mutant Ataxin-3 aggregates were observed in the graft and in the surrounding tissue. Thus, there are aggregates in the GFP-expressing MJD NESCs; as can be observed (white arrows, Figure 3.28 B) some aggregates are present outside the transplanted cells, which indicates that MJD NESCs trigger mutant Ataxin-3 seeding and spreading *in vivo*.

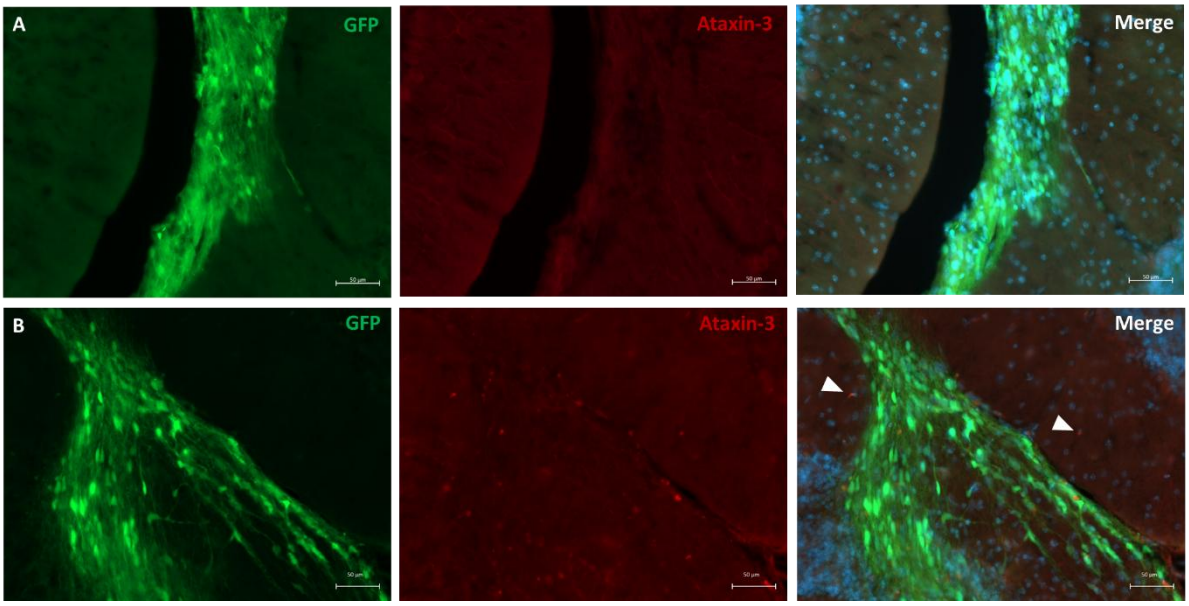


Figure 3.30. Expression of mutant Ataxin-3 protein two months upon the transplantation of iPSC-derived NESC of MJD patients in the cerebellum of adult NOD.scid mice. Brain sections transplanted with (A) control NESCs and (B) MJD NESCs expressing GFP (green) were immunostained for Ataxin-3 (red) and mutant Ataxin-3 aggregates were identified (white arrow); n= 3.

CHAPTER 4

Discussion

Discussion

Machado-Joseph disease (MJD) is caused by an expanded polyQ tract in the Ataxin-3 protein, causing its misfolding and aggregation (Takiyama et al., 1993; Kawaguchi et al., 1994). Evidence exists that several key cellular pathways, such as autophagy, are altered in MJD (Nascimento-Ferreira et al., 2011; Onofre et al., 2016). Extracellular vesicles (EVs) are nanometric structures secreted by all cell types (Raposo and Stoorvogel, 2013) that have been proposed to play an important key role in intercellular communication, namely in the nervous system (van der Pol et al., 2012; Budnik, Ruiz-Cañada, and Wendler, 2016). Furthermore EVs have been associated with the disease progression of several neurodegenerative diseases, such as Creutzfeld-Jacob disease, Alzheimer's disease and Parkinson's disease (Cervenakova et al., 2016; Fevrier et al., 2004; Rajendran et al., 2006; Emmanouilidou et al., 2010). In these works, EVs have been shown to seed aggregation of the disease associated protein, aggravating neuropathology. Regarding polyQ diseases and SCAs, the information regarding the involvement of EVs in the neuropathology is very scarce, and for MJD there are no studies assessing this topic. This fact enhances the relevance of the present study, which is the first work to characterize MJD patients specific neural cells-derived EVs and assess their potential enrolment in MJD neuropathology. Additionally, it was also evaluated for the first time if MJD patients-derived neuronal cells might be involved in *in vivo* spreading of mutant Ataxin-3.

In our samples we observed no significant differences between MJD and control NESCs-derived EVs concerning to size and particles concentration. However, MJD and control neuronal cultures-derived EVs apparently exhibit differences, with the MJD neuronal cultures-derived EVs presenting bigger size and much less concentration than control neuronal cultures-derived EVs. In addition, MJD neuronal cultures-derived EVs appear to be less heterogeneous concerning to the particles size. Thus, a

further enhancement in the number of samples analyzed is required to better clarify these results.

Thus, this data indicates that, beside the possible differences between MJD and control-derived EVs, also the type of cells from which the EVs derive, NESCs or neuronal-cultures, play a role in the observed differences. Nevertheless, this result also requires further confirmation.

Recent data implicate both, autophagy and EVs biogenesis as cellular mechanisms that work in synergy to protect the cell from external insults such as oxidative stress or starvation, maintaining the cell homeostasis, either by lysosomal degradation or cell secretion via EVs (Baixauli, López-Otín, and Mittelbrunn, 2014; Ojha et al., 2017). Autophagy and EVs biogenesis are mechanistically linked by the Endolysosomal pathway, which is implicated in both (Xu, Camfield, and Gorski, 2018).

As so, the finding of key autophagy proteins, such as Beclin-1 or p62 with EVs is not surprising. In our findings, these proteins were present in both, control and MJD neuronal cultures-derived EVs, as well as their respective progenitor cells, with a considerable decrease in the MJD neuronal-cultures and NESCs-derived EVs. This result suggests the presence of differences in autophagy between MJD and control cells. Which is not surprising, since several previous works indicate that autophagy is impaired in MJD (Nascimento-Ferreira et al., 2011; Onofre et al., 2016).

Due to its high energetic demands, the brain and neuronal tissue present particularly high levels of ROS. Moreover, the brain is an organ uniquely sensitive to ROS because of its myriad of peroxidation-susceptible cells and the constant demand of high levels of oxygen. Oxidative stress is commonly associated with the pathophysiology of neurodegenerative diseases, such as AD and PD (Kim et al., 2015). Regarding polyQ diseases, in HD oxidative damage has been detected to be increased in *postmortem* tissue from patients and animal models. In consequence, a role in the neuropathology and disease etiology of HD has been given to oxidative damage (Van Raamsdonk, Vega, and Brundin 2017; Browne and Beal 2006). The accumulation of polyQ proteins also demonstrated direct production of free radicals (Hands et al.,

2011). Concerning MJD, patients exhibit decreased antioxidant capacity and increased production of ROS (de Assis et al., 2017).

SOD-1 is a ROS gathering enzyme, which is linked to cell survival under oxidative stress (Michiels et al., 1994). Our results indicate that this protein is overexpressed in the MJD NESCs-derived EVs population, which can be an attempt of the diseased cells to respond to oxidative stressors by increasing SOD-1 levels, which is reflected in the content of the secreted EVs.

The results obtained when control NESCs-derived neuronal cultures are incubated with (MJD or control) NESCs-derived or neuronal cultures-derived EVs regarding the quantifications of p62, Beclin-1 and SOD-1, indicate that both MJD and control-derived EVs induce cellular alterations in autophagy and cell survival signaling at different time points. Interestingly, MJD-derived EVs, obtained both from NESCs and neuronal cultures, promote reduction in p62 and SOD-1 levels. Regarding p62, as it´s decrease is related to autophagy activation (W. J. Liu et al., 2016), these results might indicate that the MJD-EVs cargo includes other mediators that are impairing this autophagy process, leading to p62 enhancement. Nevertheless, this topic requires further investigation, namely incubating the EVs with autophagy inhibitors (as chloroquine) and in autophagy activation conditions (starvation).

Recently it was hypothesized that stem cells and progenitor cells-derived EVs can promote alleviation effects (Zhang et al., 2016). This might explain the p62 enhancement triggered by control EVs on neuronal cultures. Nevertheless, this hypothesis requires further confirmation.

Surprisingly, no alterations regarding cell viability were observed, possibly due to an agile recovery mechanism from the control NESCs-derived neuronal cultures. However we observed a significant induction of ROS in control neuronal cultures upon incubation over a short period of time with MJD and control NESCs-derived EVs. This result might be a consequence of a too high concentration of EVs used in this experiment that induces an oxidative cell stress, and therefore no differences between control and MJD vesicles were observed. Thus, this experiment has to be repeated with lower vesicles concentrations.

In neurodegenerative diseases like PD, where autophagy is impaired (Karabiyik, Lee, and Rubinsztein, 2017), the cells responds to the insult caused by mutant proteins accumulation by secreting the mutant protein and/or its mRNA via EVs. This interesting mechanism was already shown for several neurodegenerative diseases (Fevrier et al., 2004; Emmanouilidou et al., 2010; Yuyama et al., 2015). This cell mechanism to get relieved from the burden of the disease causing proteins result in its spreading to neighbor cells, aggravating the neuropathology.

This neuropathological spreading was already shown for HD. In a pioneer study, the polyQ tract of mutant hungtintin and its mRNA were found to be present in EVs (X. Zhang et al., 2016). Additionally, they also observed that incubation of striatal neurons with the disease bearing EVs do not induced any toxicity in the neurons, which is in accordance with ours in a way that we proved the presence of mutant Ataxin-3 mRNA in the MJD NESCs-derived EVs, but the incubation of these vesicles carrying the mutant RNA with control neuronal cultures result in no cellular toxicity. Concerning to seeding of mutant Ataxin-3 aggregates promoted by these vesicles in control neuronal cultures, western blot assay was performed (data not shown) and no mutant protein was detected. Nevertheless, the amount of spread protein might not be enough to be detected by this assay. Therefore, immunocytochemistry should also be performed to assess this topic.

However, our results demonstrate that spreading of mutant Ataxin-3 occurs when MJD NESCs-derived neuronal cultures are co-cultured with control NESCs-derived neuronal cultures in conditions without cellular contact. In this conditions control NESCs-derived neuronal cultures exhibit the presence of mutant Ataxin-3 aggregates after one and three weeks. These results suggest that MJD NESCs-derived neuronal cultures release in the media factors that induce mutant Ataxin-3 protein aggregation in the control NESCs-derived neuronal cultures.

Additionally, our *in vivo* results of MJD patient iPSCs-derived NESCs transplanted into NOD.scid mice also indicate that the transplanted cells induce mutant Ataxin-3 seeding and aggregation *in vivo*, given that some of the aggregates were found outside the transplanted cells. Although no work with iPSCs-derived NESCs in MJD have been done before. There is a recent work in PD that demonstrated an

upregulation in α-synuclein levels in mouse brain after transplantation of PD patient iPSCs-derived neurons (obtained from NESCs) bearing the LRRK2-G2019S mutation. However, these elevated levels of α-synuclein were not able to induce protein seeding, aggregation or spreading in the mouse brain (Hemmer et al., 2017). Thus, this work is in accordance with our findings, in the aspect that there is also a neuropathological brain spreading mediated by the diseased cells.

Concluding Remarks and Future Perspectives

In this work, we aimed to assess the potential of mutant Ataxin-3 spreading in MJD as well as other mediators and players of neuropathology spreading using control and MJD-patient specific iPSCs-derived NESCs and neural cultures as source of EVs to investigate whether vesicles from diseased human neural cells might promote this process. Additionally, it was also evaluated the potential disease spreading not mediated by EVs.

In general, the EVs from MJD and control NESCs and their derived neuronal cultures appear to have some differences in terms of the physical characteristics evaluated, mainly the EVs derived from neuronal cultures. Regarding their content, both types of vesicles carry proteins important for key cellular processes, such as autophagy and apoptosis. Additionally, these proteins are differently expressed in MJD and control EVs. Moreover, MJD NESCs-derived EVs were shown to carry the mutant Ataxin-3 mRNA.

The incubation of control NESCs-derived neuronal cultures with MJD and control EVs (derived from NESCs and neuronal cultures) also resulted in differences in the expression of autophagy and apoptosis-related proteins, demonstrating that vesicles from MJD cells promote deregulation of important cellular pathways known to interfere with MJD neuropathology.

Cellular experiments of MJD and control NESCs-derived neuronal cultures co-cultured without contact proved that mutant Ataxin-3 aggregates spread to control cells without the need of cellular contact. Finally, *in vivo* spreading of mutant Ataxin-3 from MJD iPSCs-derived NESCs into the cerebellum of transplanted mice was observed through the presence of Ataxin-3 aggregation outside the graft in mice transplanted with MJD iPSCs-derived NESCs, which was not observed for mice transplanted with control iPSCs-derived NESCs.

In conclusion, this work provides evidences of the pathological spread and seeding of mutant Ataxin-3 and neuropathological mediators in MJD.

This work paved the way for further exploration of the relationship of EVs with MJD neuropathology. As so, it would be interesting to explore:

- Some of the presented experiments that indicated tendencies, namely the physical characterization of MJD vs control EVs;
- Further characterize the presence of non-coding RNA in the MJD vesicles;
- Explore beneficial effects of control EVs in MJD NESCs-derived neuronal cultures;
- Directly inject EVs into the cerebellum of mice and evaluate their capacity to induce mutant Ataxin-3 aggregation or neuroinflammation.

References

Abounit, Saïda et al. 2016b. "Tunneling Nanotubes Spread Fibrillar A-synuclein by Intercellular Trafficking of Lysosomes." *The EMBO Journal* 35(19):2120–38.

Abounit, Saida, Jessica W. Wu, Karen Duff, Guiliana Soraya Victoria, and Chiara Zurzolo. 2016a. "Tunneling Nanotubes: A Possible Highway in the Spreading of Tau and Other Prion-like Proteins in Neurodegenerative Diseases." *Prion* 10(5):344–51.

Abrahams, Vikki M. et al. 2003. "Epithelial Ovarian Cancer Cells Secrete Functional Fas Ligand." *Cancer Research* 63(17):5573–81.

Aguzzi, Adriano, Fabio Montrasio, and Pascal S. Kaeser. 2001. "Prions: Health Scare and Biological Challenge." *Nature Reviews Molecular Cell Biology* 2(2):118–26.

Ahmed, Zeshan et al. 2014. "A Novel in Vivo Model of Tau Propagation with Rapid and Progressive Neurofibrillary Tangle Pathology: The Pattern of Spread Is Determined by Connectivity, Not Proximity." *Acta Neuropathologica* 127(5):667–83.

Akers, Johnny C., David Gonda, Ryan Kim, Bob S. Carter, and Clark C. Chen. 2013. "Biogenesis of Extracellular Vesicles (EV): Exosomes, Microvesicles, Retrovirus-like Vesicles, and Apoptotic Bodies." *Journal of Neuro-Oncology* 113(1):1–11.

de Almeida, L. P., D. Zala, P. Aebischer, and N. Deglon. 2001. "Neuroprotective Effect of a CNTF-Expressing Lentiviral Vector in the Quinolinic Acid Rat Model of Huntington's Disease." *Neurobiology of Disease* 8(3):433–46.

Angot, Elodie et al. 2012. "Alpha-Synuclein Cell-to-Cell Transfer and Seeding in Grafted Dopaminergic Neurons in Vivo." *PLoS ONE* 7(6).

Antonucci, Flavia et al. 2012. "Microvesicles Released from Microglia Stimulate Synaptic Activity via Enhanced Sphingolipid Metabolism." *EMBO Journal* 31(5):1231–40.

Arrasate, Montserrat, Siddhartha Mitra, Erik S. Schweitzer, Mark R. Segal, and Steven Finkbeiner. 2004. "Inclusion Body Formation Reduces Levels of Mutant Huntingtin and the Risk of Neuronal Death." *Nature* 431(7010):805–10.

Asai, Hirohide et al. 2015. "Depletion of Microglia and Inhibition of Exosome Synthesis Halt Tau Propagation." *Nature Neuroscience* 18(11):1584–93.

de Assis, Adriano M. et al. 2017. "Peripheral Oxidative Stress Biomarkers in Spinocerebellar Ataxia Type 3/Machado-Joseph Disease." *Frontiers in Neurology* 8(SEP).

Atkin-Smith, Georgia K. et al. 2015. "A Novel Mechanism of Generating Extracellular Vesicles during Apoptosis via a Beads-on-a-String Membrane Structure." *Nature Communications* 6.

Atkin-Smith, Georgia K. and Ivan K. H. Poon. 2017. "Disassembly of the Dying: Mechanisms and Functions." *Trends in Cell Biology* 27(2):151–62.

Aubertin, Kelly et al. 2016. "Massive Release of Extracellular Vesicles from Cancer Cells after Photodynamic Treatment or Chemotherapy." *Scientific Reports* 6.

Babcock, Daniel T. and Barry Ganetzky. 2015. "Transcellular Spreading of Huntingtin Aggregates in the Drosophila Brain." *Proceedings of the National Academy of Sciences* 112(39):E5427–33.

Bahmad, Hisham et al. 2017. "Modeling Human Neurological and Neurodegenerative Diseases: From Induced Pluripotent Stem Cells to Neuronal Differentiation and Its Applications in Neurotrauma." *Frontiers in Molecular Neuroscience* 10.

Baixauli, Francesc, Carlos López-Otín, and Maria Mittelbrunn. 2014. "Exosomes and Autophagy: Coordinated Mechanisms for the Maintenance of Cellular Fitness." *Frontiers in Immunology* 5(AUG).

Baker, H. F., R. M. Ridley, L. W. Duchen, T. J. Crow, and C. J. Bruton. 1993. "Evidence for the Experimental Transmission of Cerebral β-Amyloidosis to Primates." *International Journal of Experimental Pathology* 74(5).

Bart van der Worp, H. et al. 2010. "Can Animal Models of Disease Reliably Inform Human Studies?" *PLoS Medicine* 7(3):1–8.

Bartscherer, Kerstin, Nadège Pelte, Dierk Ingelfinger, and Michael Boutros. 2006. "Secretion of Wnt Ligands Requires Evi, a Conserved Transmembrane Protein." *Cell* 125(3):523–33.

Bassett, Andrew R. 2017. "Editing the Genome of hiPSC with CRISPR/Cas9: Disease Models." *Mammalian Genome* 28(7–8):348–64.

Basso, Manuela and Valentina Bonetto. 2016. "Extracellular Vesicles and a Novel Form of Communication in the Brain." *Frontiers in Neuroscience* 10(MAR).

Bence, N. F., R. M. Sampat, and R. R. Kopito. 2001. "Impairment of the Ubiquitin-Proteasome System by Protein Aggregation." *Science* 292(5521):1552–55.

Berchem, Guy et al. 2016. "Hypoxic Tumor-Derived Microvesicles Negatively Regulate NK Cell Function by a Mechanism Involving TGF-β and miR23a Transfer." *OncoImmunology* 5(4).

Bettencourt, Conceição and Manuela Lima. 2011. "Machado-Joseph Disease: From First Descriptions to New Perspectives." *Orphanet Journal of Rare Diseases* 6(1).

Bettencourt, Conceição, Cristina Santos, Teresa Kay, João Vasconcelos, and Manuela Lima. 2008. "Analysis of Segregation Patterns in Machado-Joseph Disease Pedigrees." *Journal of Human Genetics* 53(10):920–23.

Bezprozvanny, Ilya. 2009. "Calcium Signaling and Neurodegenerative Diseases." *Trends in Molecular Medicine* 15(3):89–100.

Bhatnagar, Sanchita, Kazuhiko Shinagawa, Francis J. Castellino, and Jeffrey S. Schorey. 2007. "Exosomes Released from Macrophages Infected with Intracellular Pathogens Stimulate a Proinflammatory Response in Vitro and in Vivo." *Blood* 110(9):3234–44.

Blennow, K. et al. 1995. "Tau Protein in Cerebrospinal Fluid: A Biochemical Marker for Axonal Degeneration in Alzheimer Disease?" *Mol Chem Neuropathol* 26(3):231–45.

Bobrie, Angélique, Marina Colombo, Graça Raposo, and Clotilde Théry. 2011. "Exosome Secretion: Molecular Mechanisms and Roles in Immune Responses." *Traffic* 12(12):1659–68.

Böing, A. N. et al. 2013. "Active Caspase-3 Is Removed from Cells by Release of Caspase-3-Enriched Vesicles." *Biochimica et Biophysica Acta - Molecular Cell Research* 1833(8):1844–52.

Braga-Neto, Pedro et al. 2012. "Cerebellar Cognitive Affective Syndrome in Machado Joseph Disease: Core Clinical Features." Pp. 549–56 in *Cerebellum*, vol. 11.

Breuer, Peter, Annette Haacke, Bernd O. Evert, and Ullrich Wüllner. 2010. "Nuclear Aggregation of Polyglutamine-Expanded Ataxin-3: Fragments Escape the Cytoplasmic Quality Control." *Journal of Biological Chemistry* 285(9):6532–37.

Brini, Marisa, Tito Calì, Denis Ottolini, and Ernesto Carafoli. 2014. "Neuronal Calcium Signaling: Function and Dysfunction." *Cellular and Molecular Life Sciences* 71(15):2787–2814.

Browne, Susan E. and M.Flint Beal. 2006. "Oxidative Damage in Huntington's Disease Pathogenesis." *Antioxidants & Redox Signaling* 8(11–12):2061–73. Retrieved (http://www.ncbi.nlm.nih.gov/pubmed/17034350).

Budnik, Vivian, Catalina Ruiz-Cañada, and Franz Wendler. 2016. "Extracellular Vesicles Round off Communication in the Nervous System." *Nature Reviews Neuroscience* 17(3):160–72.

Burnett, Barrington, Fusheng Li, and Randall N. Pittman. 2003. "The Polyglutamine Neurodegenerative Protein Ataxin-3 Binds Polyubiquitylated Proteins and Has Ubiquitin Protease Activity." *Human Molecular Genetics* 12(23):3195–3205.

De Calignon, Alix et al. 2012. "Propagation of Tau Pathology in a Model of Early Alzheimer's Disease." *Neuron* 73(4):685–97.

Carmona, Vitor et al. 2017. "Unravelling Endogenous MicroRNA System Dysfunction as a New Pathophysiological Mechanism in Machado-Joseph Disease." *Molecular Therapy* 25(4):1038–55.

Carvalho, Daniel R., Alessandra La Rocque-Ferreira, Isabela M. Rizzo, Erica U. Imamura, and Carlos E. Speck-Martins. 2008. "Homozygosity Enhances Severity in Spinocerebellar Ataxia Type 3." *Pediatric Neurology* 38(4):296–99.

Cecchin, C. R. et al. 2006. "Depressive Symptoms in Machado-Joseph Disease (SCA3) Patients and Their Relatives." *Community Genetics* 10(1):19–26.

Cervenakova, Larisa et al. 2016. "Are Prions Transported by Plasma Exosomes?" *Transfusion and Apheresis Science* 55(1):70–83.

Chalmin, Fanny et al. 2010. "Membrane-Associated Hsp72 from Tumor-Derived Exosomes Mediates STAT3-Dependent Immunosuppressive Function of Mouse and Human Myeloid-Derived Suppressor Cells." *Journal of Clinical Investigation* 120(2):457–71.

Chang, Chongwang et al. 2013. "Exosomes of BV-2 Cells Induced by Alpha-Synuclein: Important Mediator of Neurodegeneration in PD." *Neuroscience Letters* 548:190–95.

Chen, Xi et al. 2008. "Deranged Calcium Signaling and Neurodegeneration in Spinocerebellar Ataxia Type 3." *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 28(48):12713–24.

Chivet, Mathilde et al. 2013. "Exosomes as a Novel Way of Interneuronal Communication." *Biochemical Society Transactions* 41(1):241–44.

Chou, An-Hsun et al. 2008. "Polyglutamine-Expanded Ataxin-3 Causes Cerebellar Dysfunction of SCA3 Transgenic Mice by Inducing Transcriptional Dysregulation." *Neurobiology of Disease* 31(1):89–101.

Chou, An Hsun et al. 2006. "Polyglutamine-Expanded Ataxin-3 Activates Mitochondrial Apoptotic Pathway by Upregulating Bax and Downregulating Bcl-xL." *Neurobiology of Disease* 21(2):333–45.

Christianson, H. C., K. J. Svensson, T. H. van Kuppevelt, J. P. Li, and M. Belting. 2013. "Cancer Cell Exosomes Depend on Cell-Surface Heparan Sulfate Proteoglycans for Their Internalization and Functional Activity." *Proceedings of the National Academy of Sciences* 110(43):17380–85.

Cicchetti, F. et al. 2009. "Neural Transplants in Patients with Huntington's Disease Undergo Disease-like Neuronal Degeneration." *Proceedings of the National Academy of Sciences* 106(30):12483–88.

Clavaguera, Florence et al. 2013. "Brain Homogenates from Human Tauopathies Induce Tau Inclusions in Mouse Brain." *Proceedings of the National Academy of Sciences* 110(23):9535–40.

Clavaguera, Florence et al. 2014. "Peripheral Administration of Tau Aggregates Triggers Intracerebral Tauopathy in Transgenic Mice." *Acta Neuropathologica* 127(2):299–301.

Clavaguera, Florence et al. 2009. "Transmission and Spreading of Tauopathy in Transgenic Mouse Brain." *Nature Cell Biology* 11(7):909–13.

Cocucci, Emanuele and Jacopo Meldolesi. 2015. "Ectosomes and Exosomes: Shedding the Confusion between Extracellular Vesicles." *Trends in Cell Biology* 25(6):364–72.

Colombo, Marina, Graça Raposo, and Clotilde Théry. 2014. "Biogenesis, Secretion, and Intercellular Interactions of Exosomes and Other Extracellular Vesicles." *Annual Review of*

Cell and Developmental Biology 30(1):255–89.
Conti, Luciano and Elena Cattaneo. 2010. "Neural Stem Cell Systems: Physiological Players or in Vitro Entities?" *Nature Reviews Neuroscience* 11(3):176–87.
Costanzo, M. et al. 2013. "Transfer of Polyglutamine Aggregates in Neuronal Cells Occurs in Tunneling Nanotubes." *Journal of Cell Science* 126(16):3678–85.
Coutinho, P. 1992. "Doença de Machado-Joseph: Tentativa de Definição." *Porto: Universidade Do Porto* 247.
Croese, Tommaso and Roberto Furlan. 2017. "Extracellular Vesicles in Neurodegenerative Diseases." *Molecular Aspects of Medicine*.
D'Abreu, a et al. 2009. "Sleep Symptoms and Their Clinical Correlates in Machado-Joseph Disease." *Acta Neurologica Scandinavica* 119(4):277–80.
D'Abreu, Anelyssa, Marcondes C. França, Henry L. Paulson, and Iscia Lopes-Cendes. 2010. "Caring for Machado-Joseph Disease: Current Understanding and How to Help Patients." *Parkinsonism & Related Disorders* 16(1):2–7.
D'Souza-Schorey Crislyn, C. and James W. Clancy. 2012. "Tumor-Derived Microvesicles: Shedding Light on Novel Microenvironment Modulators and Prospective Cancer Biomarkers." *Genes and Development* 26(12):1287–99.
Danzer, Karin M. et al. 2012. "Exosomal Cell-to-Cell Transmission of Alpha Synuclein Oligomers." *Molecular Neurodegeneration* 7(1).
Deatheragea, Brooke L. and Brad T. Cooksona. 2012. "Membrane Vesicle Release in Bacteria, Eukaryotes, and Archaea: A Conserved yet Underappreciated Aspect of Microbial Life." *Infection and Immunity* 80(6):1948–57.
Desplats, P. et al. 2009. "Inclusion Formation and Neuronal Cell Death through Neuron-to-Neuron Transmission of -Synuclein." *Proceedings of the National Academy of Sciences* 106(31):13010–15.
Van Deun, Jan et al. 2014. "The Impact of Disparate Isolation Methods for Extracellular Vesicles on Downstream RNA Profiling." *Journal of Extracellular Vesicles* 3(1).
Dieriks, Birger Victor et al. 2017. "α-Synuclein Transfer through Tunneling Nanotubes Occurs in SH-SY5Y Cells and Primary Brain Pericytes from Parkinson's Disease Patients." *Scientific Reports* 7.
Van Dijk, K. D. et al. 2014. "Reduced α-Synuclein Levels in Cerebrospinal Fluid in Parkinson's Disease Are Unrelated to Clinical and Imaging Measures of Disease Severity." *European Journal of Neurology* 21(3):388–94.
Dimos, John T. et al. 2008. "Induced Pluripotent Stem Cells Generated from Patients with ALS Can Be Differentiated into Motor Neurons." *Science (New York, N.Y.)* 321(5893):1218–21.
Donaldson, K. M. et al. 2003. "Ubiquitin-Mediated Sequestration of Normal Cellular Proteins into Polyglutamine Aggregates." *Proceedings of the National Academy of Sciences* 100(15):8892–97.
Doss-Pepe, E. W., E. S. Stenroos, W. G. Johnson, and K. Madura. 2003. "Ataxin-3 Interactions with Rad23 and Valosin-Containing Protein and Its Associations with Ubiquitin Chains and the Proteasome Are Consistent with a Role in Ubiquitin-Mediated Proteolysis." *Molecular and Cellular Biology* 23(18):6469–83.
Ebert, Antje D., Ping Liang, and Joseph C. Wu. 2012. "Induced Pluripotent Stem Cells as a Disease Modeling and Drug Screening Platform." *Journal of Cardiovascular Pharmacology* 60(4):408–16.
Efthymiou, A. et al. 2014. "Functional Screening Assays with Neurons Generated from Pluripotent Stem Cell-Derived Neural Stem Cells." *J Biomol Screen* 19(1):32–43.
Eisele, Yvonne S. et al. 2010. "Peripherally Applied Aβ-Containing Inoculates Induce Cerebral β-Amyloidosis." *Science* 330(6006):980–82.

Eitan, Erez et al. 2016. "Extracellular Vesicle-Associated Aβ Mediates Trans-Neuronal Bioenergetic and Ca 2+ -Handling Deficits in Alzheimer's Disease Models." *Nature Publishing Group* 2(10).

Elmore, Susan. 2007. "Apoptosis: A Review of Programmed Cell Death." *Toxicologic Pathology* 35(4):495–516.

Emmanouilidou, E. et al. 2010. "Cell-Produced -Synuclein Is Secreted in a Calcium-Dependent Manner by Exosomes and Impacts Neuronal Survival." *Journal of Neuroscience* 30(20):6838–51.

Esser, Julia et al. 2010. "Exosomes from Human Macrophages and Dendritic Cells Contain Enzymes for Leukotriene Biosynthesis and Promote Granulocyte Migration." *Journal of Allergy and Clinical Immunology* 126(5).

Evers, Melvin M., Lodewijk J. A. Toonen, and Willeke M. C. van Roon-Mom. 2014. "Ataxin-3 Protein and RNA Toxicity in Spinocerebellar Ataxia Type 3: Current Insights and Emerging Therapeutic Strategies." *Molecular Neurobiology* 49(3):1513–31.

Evert, B. O. et al. 2006. "Ataxin-3 Represses Transcription via Chromatin Binding, Interaction with Histone Deacetylase 3, and Histone Deacetylation." *Journal of Neuroscience* 26(44):11474–86.

Evert, Bernd O. et al. 2003. "Gene Expression Profiling in Ataxin-3 Expressing Cell Lines Reveals Distinct Effects of Normal and Mutant Ataxin-3." *Journal of Neuropathology and Experimental Neurology* 62(10):1006–18.

Evert, Bernd O. et al. 2001. "Inflammatory Genes Are Upregulated in Expanded Ataxin-3-Expressing Cell Lines and Spinocerebellar Ataxia Type 3 Brains." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 21(15):5389–96.

Fabbri, M. et al. 2012. "MicroRNAs Bind to Toll-like Receptors to Induce Prometastatic Inflammatory Response." *Proceedings of the National Academy of Sciences* 109(31):E2110–16.

Fairchild, Paul J. 2010. "The Challenge of Immunogenicity in the Quest for Induced Pluripotency." *Nature Reviews Immunology* 10(12):868–75.

Falk, Anna et al. 2012. "Capture of Neuroepithelial-like Stem Cells from Pluripotent Stem Cells Provides a Versatile System for in Vitro Production of Human Neurons." *PLoS ONE* 7(1).

Fauré, J. et al. 2006. "Exosomes Are Released by Cultured Cortical Neurones." *Molecular and Cellular Neuroscience* 31(4):642–48.

Feng, Du et al. 2010. "Cellular Internalization of Exosomes Occurs through Phagocytosis." *Traffic* 11(5):675–87.

Fernandes, Hugo J. R. et al. 2016. "ER Stress and Autophagic Perturbations Lead to Elevated Extracellular α-Synuclein in GBA-N370S Parkinson's iPSC-Derived Dopamine Neurons." *Stem Cell Reports* 6(3):342–56.

Fevrier, B. et al. 2004. "Cells Release Prions in Association with Exosomes." *Proceedings of the National Academy of Sciences* 101(26):9683–88.

Fitzner, D. et al. 2011. "Selective Transfer of Exosomes from Oligodendrocytes to Microglia by Macropinocytosis." *Journal of Cell Science* 124(3):447–58.

Franca, Jr M. C., Anelyssa D'Abreu, Joseph H. Friedman, Anamarli Nucci, and Iscia Lopes-Cendes. 2007. "Chronic Pain in Machado-Joseph Disease: A Frequent and Disabling Symptom." *Archives of Neurology* 64(12):1767–70.

Frängsmyr, L. et al. 2005. "Cytoplasmic Microvesicular Form of Fas Ligand in Human Early Placenta: Switching the Tissue Immune Privilege Hypothesis from Cellular to Vesicular Level." *Molecular Human Reproduction* 11(1):35–41.

Fraser, Kyle B. et al. 2016. "Ser(P)-1292 LRRK2 in Urinary Exosomes Is Elevated in Idiopathic Parkinson's Disease." *Movement Disorders* 31(10):1543–50.

French, Kinsley C., Marc A. Antonyak, and Richard A. Cerione. 2017. "Extracellular Vesicle Docking at the Cellular Port: Extracellular Vesicle Binding and Uptake." *Seminars in Cell and Developmental Biology* 67:48–55.

Friedman, Joseph H. and Melissa M. Amick. 2008. "Fatigue and Daytime Somnolence in Machado Joseph Disease (Spinocerebellar Ataxia Type 3)." *Movement Disorders* 23(9):1323–24.

Frost, Bess, Rachel L. Jacks, and Marc I. Diamond. 2009. "Propagation of Tau Misfolding from the Outside to the inside of a Cell." *Journal of Biological Chemistry* 284(19):12845–52.

Frühbeis, Carsten, Dominik Fröhlich, Wen Ping Kuo, Jesa Amphornrat, et al. 2013. "Neurotransmitter-Triggered Transfer of Exosomes Mediates Oligodendrocyte-Neuron Communication." *PLoS Biology* 11(7).

Frühbeis, Carsten, Dominik Fröhlich, Wen Ping Kuo, and Eva-Maria Krämer-Albers. 2013. "Extracellular Vesicles as Mediators of Neuron-Glia Communication." *Frontiers in Cellular Neuroscience* 7.

Ghidoni, Roberta et al. 2011. "Cystatin C Is Released in Association with Exosomes: A New Tool of Neuronal Communication Which Is Unbalanced in Alzheimer's Disease." *Neurobiology of Aging* 32(8):1435–42.

Gilligan, Katie E. and Róisín M. Dwyer. 2017. "Engineering Exosomes for Cancer Therapy." *International Journal of Molecular Sciences* 18(6).

Goedert, Michel. 2015. "Alzheimer's and Parkinson's Diseases: The Prion Concept in Relation to Assembled Aβ, Tau, and α-Synuclein." *Science* 349(6248).

Goti, D. 2004. "A Mutant Ataxin-3 Putative-Cleavage Fragment in Brains of Machado-Joseph Disease Patients and Transgenic Mice Is Cytotoxic above a Critical Concentration." *Journal of Neuroscience* 24(45):10266–79.

Gould, Stephen J. and Graça Raposo. 2013. "As We Wait: Coping with an Imperfect Nomenclature for Extracellular Vesicles." *Journal of Extracellular Vesicles* 2(1).

Gousset, Karine et al. 2009. "Prions Hijack Tunnelling Nanotubes for Intercellular Spread." *Nature Cell Biology* 11(3):328–36.

Grskovic, Marica, Ashkan Javaherian, Berta Strulovici, and George Q. Daley. 2011. "Induced Pluripotent Stem Cells--Opportunities for Disease Modelling and Drug Discovery." *Nature Reviews. Drug Discovery* 10(12):915–29.

Guest, Will C. et al. 2011. "Generalization of the Prion Hypothesis to Other Neurodegenerative Diseases: An Imperfect Fit." *Journal of Toxicology and Environmental Health - Part A: Current Issues* 74(22–24):1433–59.

Guo, Jing L. and Virginia M. Y. Lee. 2014. "Cell-to-Cell Transmission of Pathogenic Proteins in Neurodegenerative Diseases." *Nature Medicine* 20(2):130–38.

Haacke, Annette, F.Ulrich Hartl, and Peter Breuer. 2007. "Calpain Inhibition Is Sufficient to Suppress Aggregation of Polyglutamine-Expanded Ataxin-3." *Journal of Biological Chemistry* 282(26):18851–56.

Hands, Sarah, Mohammad U. Sajjad, Michael J. Newton, and Andreas Wyttenbach. 2011. "In Vitro and in Vivo Aggregation of a Fragment of Huntingtin Protein Directly Causes Free Radical Production." *Journal of Biological Chemistry* 286(52):44512–20.

Hansen, Christian et al. 2011. "α-Synuclein Propagates from Mouse Brain to Grafted Dopaminergic Neurons and Seeds Aggregation in Cultured Human Cells." *Journal of Clinical Investigation* 121(2):715–25.

Hansen, Susanne K. et al. 2016. "Induced Pluripotent Stem Cell - Derived Neurons for the Study of Spinocerebellar Ataxia Type 3." *Stem Cell Research* 17(2):306–17.

Hanson, Phyllis I. and Anil Cashikar. 2012. "Multivesicular Body Morphogenesis." *Annual Review of Cell and Developmental Biology* 28(1):337–62.

Harding, C., J. Heuser, and P. Stahl. 1983. "Receptor-Mediated Endocytosis of Transferrin and Recycling of the Transferrin Receptor in Rat Reticulocytes." *Journal of Cell Biology* 97(2):329–39.

Hawari, F. I. et al. 2004. "Release of Full-Length 55-kDa TNF Receptor 1 in Exosome-like Vesicles: A Mechanism for Generation of Soluble Cytokine Receptors." *Proceedings of the National Academy of Sciences* 101(5):1297–1302.

Holmgren, L. et al. 1999. "Horizontal Transfer of DNA by the Uptake of Apoptotic Bodies." *Blood* 93(11):3956–63.

Hsu, Chieh et al. 2010. "Regulation of Exosome Secretion by Rab35 and Its GTPase-Activating Proteins TBC1D10A-C." *Journal of Cell Biology* 189(2):223–32.

Hübener, Jeannette et al. 2013. "Calpain-Mediated Ataxin-3 Cleavage in the Molecular Pathogenesis of Spinocerebellar Ataxia Type 3 (SCA3)." *Human Molecular Genetics* 22(3):508–18.

Hurley, James H. and Phyllis I. Hanson. 2010. "Membrane Budding and Scission by the ESCRT Machinery: It's All in the Neck." *Nature Reviews Molecular Cell Biology* 11(8):556–66.

Ichikawa, Y. et al. 2001. "The Genomic Structure and Expression of MJD, the Machado-Joseph Disease Gene." *J Hum Genet* 46(7):413–22.

Jacobi, Heike et al. 2015. "Long-Term Disease Progression in Spinocerebellar Ataxia Types 1, 2, 3, and 6: A Longitudinal Cohort Study." *The Lancet Neurology* 14(11):1101–8.

Jeong, Whun Kim et al. 2005. "Fas Ligand-Positive Membranous Vesicles Isolated from Sera of Patients with Oral Cancer Induce Apoptosis of Activated T Lymphocytes." *Clinical Cancer Research* 11(3):1010–20.

Jiang, Lanzhou et al. 2017. "Determining the Contents and Cell Origins of Apoptotic Bodies by Flow Cytometry." *Scientific Reports* 7(1).

Johri, Ashu and M.Flint Beal. 2012. "Mitochondrial Dysfunction in Neurodegenerative Diseases." *The Journal of Pharmacology and Experimental Therapeutics* 342(3):619–30.

Jung, Joonil, Kexiang Xu, Derek Lessing, and Nancy M. Bonini. 2009. "Preventing Ataxin-3 Protein Cleavage Mitigates Degeneration in a Drosophila Model of SCA3." *Human Molecular Genetics* 18(24):4843–52.

Kanai, Kazuaki et al. 2003. "Muscle Cramp in Machado-Joseph Disease - Altered Motor Axonal Excitability Properties and Mexiletine Treatment." *Brain* 126(4):965–73.

Kane, M. D. et al. 2000. "Evidence for Seeding of Beta -Amyloid by Intracerebral Infusion of Alzheimer Brain Extracts in Beta -Amyloid Precursor Protein-Transgenic Mice." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 20(10):3606–11.

Karabiyik, Cansu, Min Jae Lee, and David C. Rubinsztein. 2017. "Autophagy Impairment in Parkinson's Disease." *Essays In Biochemistry* 61(6):711–20.

Kawaguchi, Yoshiya et al. 1994. "CAG Expansions in a Novel Gene for Machado-Joseph Disease at Chromosome 14q32.1." *Nature Genetics* 8(3):221–28.

Kawai, Yoshinari et al. 2004. "Cognitive Impairments in Machado-Joseph Disease." *Archives of Neurology* 61(11):1757.

Kazachkova, Nadiya et al. 2013. "Patterns of Mitochondrial DNA Damage in Blood and Brain Tissues of a Transgenic Mouse Model of Machado-Joseph Disease." *Neurodegenerative Diseases* 11(4):206–14.

Keerthikumar, Shivakumar et al. 2016. "ExoCarta: A Web-Based Compendium of Exosomal Cargo." *Journal of Molecular Biology* 428(4):688–92.

Keller, Sascha, Johannes Ridinger, Anne Kathleen Rupp, Johannes W. G. Janssen, and Peter Altevogt. 2011. "Body Fluid Derived Exosomes as a Novel Template for Clinical Diagnostics." *Journal of Translational Medicine* 9.

Kieling, C., P. R. Prestes, M. L. Saraiva-Pereira, and Laura Bannach Jardim. 2007. "Survival Estimates for Patients with Machado-Joseph Disease (SCA3)." *Clinical Genetics* 72(6):543–45.

Kim, Geon Ha, Jieun E. Kim, Sandy Jeong Rhie, and Sujung Yoon. 2015. "The Role of Oxidative Stress in Neurodegenerative Diseases." *Experimental Neurobiology* 24(4):325.

Klockgether, T. et al. 1999. "Age Related Axonal Neuropathy in Spinocerebellar Ataxia Type 3/Machado-Joseph Disease (SCA3/MJD)." *Journal of Neurology, Neurosurgery, and Psychiatry* 66(2):222–24.

Koch, Philipp et al. 2011. "Excitation-Induced Ataxin-3 Aggregation in Neurons from Patients with Machado-Joseph Disease." *Nature* 480(7378):543–46.

Koch, Philipp et al. 2012. "Presenilin-1 L166P Mutant Human Pluripotent Stem Cell-Derived Neurons Exhibit Partial Loss of γ-Secretase Activity in Endogenous Amyloid-β Generation." *American Journal of Pathology* 180(6):2404–16.

Koch, Philipp, Thoralf Opitz, Julius A. Steinbeck, Julia Ladewig, and Oliver Brüstle. 2009. "A Rosette-Type, Self-Renewing Human ES Cell-Derived Neural Stem Cell with Potential for in Vitro Instruction and Synaptic Integration." *Proceedings of the National Academy of Sciences of the United States of America* 106(9):3225–30.

Konoshenko, Maria Yu, Evgeniy A. Lekchnov, Alexander V. Vlassov, and Pavel P. Laktionov. 2018. "Isolation of Extracellular Vesicles: General Methodologies and Latest Trends." *BioMed Research International* 2018.

Kordower, Jeffrey H., Yaping Chu, Robert A. Hauser, Thomas B. Freeman, and C.Warren Olanow. 2008a. "Lewy Body-like Pathology in Long-Term Embryonic Nigral Transplants in Parkinson's Disease." *Nature Medicine* 14(5):504–6.

Kordower, Jeffrey H., Yaping Chu, Robert A. Hauser, C.Warren Olanow, and Thomas B. Freeman. 2008b. "Transplanted Dopaminergic Neurons Develop PD Pathologic Changes: A Second Case Report." *Movement Disorders* 23(16):2303–6.

Korkut, Ceren et al. 2009. "Trans-Synaptic Transmission of Vesicular Wnt Signals through Evi/Wntless." *Cell* 139(2):393–404.

Kowal, Joanna et al. 2016. "Proteomic Comparison Defines Novel Markers to Characterize Heterogeneous Populations of Extracellular Vesicle Subtypes." *Proceedings of the National Academy of Sciences* 113(8):E968–77.

Krämer-Albers, Eva Maria et al. 2007. "Oligodendrocytes Secrete Exosomes Containing Major Myelin and Stress-Protective Proteins: Trophic Support for Axons?" *Proteomics - Clinical Applications* 1(11):1446–61.

Langer, F. et al. 2011. "Soluble A Seeds Are Potent Inducers of Cerebral -Amyloid Deposition." *Journal of Neuroscience* 31(41):14488–95.

Li, B., M. A. Antonyak, J. Zhang, and R. A. Cerione. 2012. "RhoA Triggers a Specific Signaling Pathway That Generates Transforming Microvesicles in Cancer Cells." *Oncogene* 31(45):4740–49.

Li, Fusheng, Todd Macfarlan, Randall N. Pittman, and Debabrata Chakravarti. 2002. "Ataxin-3 Is a Histone-Binding Protein with Two Independent Transcriptional Corepressor Activities." *Journal of Biological Chemistry* 277(47):45004–12.

Li, Jia Yi et al. 2008. "Lewy Bodies in Grafted Neurons in Subjects with Parkinson's Disease Suggest Host-to-Graft Disease Propagation." *Nature Medicine* 14(5):501–3.

Lindenbergh, Marthe F. S. and Willem Stoorvogel. 2018. "Antigen Presentation by Extracellular Vesicles from Professional Antigen-Presenting Cells." *Annual Review of Immunology* 36(1):annurev-immunol-041015-055700.

Liu, C. et al. 2006. "Murine Mammary Carcinoma Exosomes Promote Tumor Growth by Suppression of NK Cell Function." *The Journal of Immunology* 176(3):1375–85.

Liu, Chen Geng, Jing Song, Yue Qi Zhang, and Pei Chang Wang. 2014. "MicroRNA-193b Is a Regulator of Amyloid Precursor Protein in the Blood and Cerebrospinal Fluid Derived Exosomal microRNA-193b Is a Biomarker of Alzheimer's Disease." *Molecular Medicine Reports* 10(5):2395–2400.

Liu, Chun, Angelos Oikonomopoulos, Nazish Sayed, and Joseph C. Wu. 2018. "Modeling Human Diseases with Induced Pluripotent Stem Cells: From 2D to 3D and beyond." *Development* 145(5):dev156166.

Liu, Hongmei et al. 2016. "The Machado–Joseph Disease Deubiquitinase Ataxin-3 Regulates the Stability and Apoptotic Function of p53." *PLoS Biology* 14(11).

Liu, Li et al. 2012. "Trans-Synaptic Spread of Tau Pathology in Vivo." *PLoS ONE* 7(2).

Liu, Wei Jing et al. 2016. "p62 Links the Autophagy Pathway and the Ubiqutin-Proteasome System upon Ubiquitinated Protein Degradation." *Cellular and Molecular Biology Letters* 21(1).

Liu, Xin, Wenjuan Li, Xuemei Fu, and Yang Xu. 2017. "The Immunogenicity and Immune Tolerance of Pluripotent Stem Cell Derivatives." *Frontiers in Immunology* 8(JUN).

Lo, Raymond Y. et al. 2016. "Depression and Clinical Progression in Spinocerebellar Ataxias." *Parkinsonism and Related Disorders* 22:87–92.

Lötvall, Jan et al. 2014. "Minimal Experimental Requirements for Definition of Extracellular Vesicles and Their Functions: A Position Statement from the International Society for Extracellular Vesicles." *Journal of Extracellular Vesicles*.

Luk, K. C. et al. 2009. "Exogenous -Synuclein Fibrils Seed the Formation of Lewy Body-like Intracellular Inclusions in Cultured Cells." *Proceedings of the National Academy of Sciences* 106(47):20051–56.

Luk, Kelvin C., Victoria M. Kehm, et al. 2012. "Intracerebral Inoculation of Pathological α-Synuclein Initiates a Rapidly Progressive Neurodegenerative α-Synucleinopathy in Mice." *The Journal of Experimental Medicine* 209(5):975–86.

Luk, Kelvin C., Victoria Kehm, et al. 2012. "Pathological α-Synuclein Transmission Initiates Parkinson-like Neurodegeneration in Nontransgenic Mice." *Science* 338(6109):949–53.

Maciel, P. et al. 1995. "Correlation between CAG Repeat Length and Clinical Features in Machado-Joseph Disease." *American Journal of Human Genetics* 57(1):54–61.

MacKenzie, Amanda et al. 2001. "Rapid Secretion of Interleukin-1β by Microvesicle Shedding." *Immunity* 15(5):825–35.

Manèek-Keber, Mateja et al. 2015. "Toll-like Receptor 4 Senses Oxidative Stress Mediated by the Oxidation of Phospholipids in Extracellular Vesicles." *Science Signaling* 8(381):ra60.

Marie, Grey et al. 2015. "Acceleration of α-Synuclein Aggregation by Exosomes." *Journal of Biological Chemistry* 290(5):2969–82.

Maury, Yves, Morgane Gauthier, Marc Peschanski, and Cécile Martinat. 2011. "Human Pluripotent Stem Cells for Disease Modelling and Drug Screening." *BioEssays : News and Reviews in Molecular, Cellular and Developmental Biology* 1–11.

Melentijevic, Ilija et al. 2017. "C. Elegans Neurons Jettison Protein Aggregates and Mitochondria under Neurotoxic Stress." *Nature* 542(7641):367–71.

Mendonca, Liliana S., Clevio Nobrega, Hirokazu Hirai, Brian K. Kaspar, and Luis Pereira de Almeida. 2015. "Transplantation of Cerebellar Neural Stem Cells Improves Motor Coordination and Neuropathology in Machado-Joseph Disease Mice." *Brain : A Journal of Neurology* 138(2014):320–35.

Mendonca, Nuno, Marcondes C.Jr. Franca, Antonio Freire Goncalves, and Cristina Januario. 2018. "Clinical Features of Machado-Joseph Disease." *Advances in Experimental Medicine and Biology* 1049:255–73.

Menzies, Fiona M. et al. 2010. "Autophagy Induction Reduces Mutant Ataxin-3 Levels and

Toxicity in a Mouse Model of Spinocerebellar Ataxia Type 3." *Brain* 133(1):93–104.
van der Merwe, Yolandi and Michael B. Steketee. 2017. "Extracellular Vesicles: Biomarkers, Therapeutics, and Vehicles in the Visual System." *Current Ophthalmology Reports* 5(4):276–82.
Meyer-Luehmann, Melanie et al. 2006. "Exogenous Induction of Cerebral β-Amyloidogenesis Is Governed Bf Agent and Host." *Science* 313(5794):1781–84.
Michiels, Carine, Martine Raes, Olivier Toussaint, and José Remacle. 1994. "Importance of SE-Glutathione Peroxidase, Catalase, and CU/ZN-SOD for Cell Survival against Oxidative Stress." *Free Radical Biology and Medicine* 17(3):235–48.
Miller, Justine D. et al. 2013. "Human iPSC-Based Modeling of Late-Onset Disease via Progerin-Induced Aging." *Cell Stem Cell* 13(6):691–705.
Miller, Justine and Lorenz Studer. 2014. "Aging in iPS Cells." *Aging*, 246–47.
Mo, Jin-A. et al. 2015. "Cerebrospinal Fluid β-Amyloid1–42 Levels in the Differential Diagnosis of Alzheimer's Disease—Systematic Review and Meta-Analysis." *PLOS ONE* 10(2):e0116802.
Möbius, W. et al. 2003. "Recycling Compartments and the Internal Vesicles of Multivesicular Bodies Harbor Most of the Cholesterol Found in the Endocytic Pathway." *Traffic* 4(4):222–31.
Mollenhauer, Brit et al. 2013. "Total CSF α-Synuclein Is Lower in de Novo Parkinson Patients than in Healthy Subjects." *Neuroscience Letters* 532(1):44–48.
Monleon, I. et al. 2001. "Differential Secretion of Fas Ligand- or APO2 Ligand/TNF-Related Apoptosis-Inducing Ligand-Carrying Microvesicles During Activation-Induced Death of Human T Cells." *The Journal of Immunology* 167(12):6736–44.
Montecalvo, Angela et al. 2012. "Mechanism of Transfer of Functional microRNAs between Mouse Dendritic Cells via Exosomes." *Blood* 119(3):756–66.
Morales, R., C. Duran-Aniotz, J. Castilla, L. D. Estrada, and C. Soto. 2012. "De Novo Induction of Amyloid-β Deposition in Vivo." *Molecular Psychiatry* 17(12):1347–53.
Mougenot, Anne Laure et al. 2012. "Prion-like Acceleration of a Synucleinopathy in a Transgenic Mouse Model." *Neurobiology of Aging* 33(9):2225–28.
Mulcahy, Laura Ann, Ryan Charles Pink, and David Raul Francisco Carter. 2014. "Routes and Mechanisms of Extracellular Vesicle Uptake." *Journal of Extracellular Vesicles* 3(1).
Mulligan, Kimberly A. and Benjamin N. R. Cheyette. 2012. "Wnt Signaling in Vertebrate Neural Development and Function." *Journal of Neuroimmune Pharmacology* 7(4):774–87.
Munich, Stephan, Andrea Sobo-Vujanovic, William J. Buchser, Donna Beer-Stolz, and Nikola L. Vujanovic. 2012. "Dendritic Cell Exosomes Directly Kill Tumor Cells and Activate Natural Killer Cells via TNF Superfamily Ligands." *OncoImmunology* 1(7):1074–83.
Nakano, KK., Dawson, DM., Spence, A. 1972. "Machado Disease. A Hereditary Ataxia in Portuguese Emigrants to Massachusetts." *Neurology* 22(1):49–55.
Nascimento-Ferreira, Isabel et al. 2011. "Overexpression of the Autophagic Beclin-1 Protein Clears Mutant Ataxin-3 and Alleviates Machado-Joseph Disease." *Brain* 134(5):1400–1415.
Nath, S. et al. 2012. "Spreading of Neurodegenerative Pathology via Neuron-to-Neuron Transmission of -Amyloid." *Journal of Neuroscience* 32(26):8767–77.
van Niel, Guillaume, Gisela D'Angelo, and Graça Raposo. 2018. "Shedding Light on the Cell Biology of Extracellular Vesicles." *Nature Reviews Molecular Cell Biology*.
Nóbrega, C. Pereira de Almeida, L. 2012. "Machado-Joseph Disease/Spinocerebellar Ataxia Type 3. 'SPINOCEREBELLAR ATAXIA'."
Nóbrega, C. et al. 2018. *Molecular Mechanisms and Cellular Pathways Implicated in Machado-Joseph Disease Pathogenesis*.

Nolte'T Hoen, Esther N. M. et al. 2012. "Deep Sequencing of RNA from Immune Cell-Derived Vesicles Uncovers the Selective Incorporation of Small Non-Coding RNA Biotypes with Potential Regulatory Functions." *Nucleic Acids Research* 40(18):9272–85.

O'Brien, John, Ian Wilson, Terry Orton, and Fran??ois Pognan. 2000. "Investigation of the Alamar Blue (Resazurin) Fluorescent Dye for the Assessment of Mammalian Cell Cytotoxicity." *European Journal of Biochemistry* 267(17):5421–26.

Ojha, Chet et al. 2017. "Interplay between Autophagy, Exosomes and HIV-1 Associated Neurological Disorders: New Insights for Diagnosis and Therapeutic Applications." *Viruses* 9(7):176. Retrieved (http://www.mdpi.com/1999-4915/9/7/176).

Okabe, Shigeo, Karin Forsberg-Nilsson, A.Cyril Spiro, Menahem Segal, and Ronald D. G. McKay. 1996. "Development of Neuronal Precursor Cells and Functional Postmitotic Neurons from Embryonic Stem Cells in Vitro." *Mechanisms of Development* 59(1):89–102.

Onder, Tamer T. and George Q. Daley. 2012. "New Lessons Learned from Disease Modeling with Induced Pluripotent Stem Cells." *Current Opinion in Genetics & Development* 22(5):500–508.

Onofre, Isabel et al. 2016. "Fibroblasts of Machado Joseph Disease Patients Reveal Autophagy Impairment." *Scientific Reports* 6.

Osterberg, Valerie R. et al. 2015. "Progressive Aggregation of Alpha-Synuclein and Selective Degeneration of Lewy Inclusion-Bearing Neurons in a Mouse Model of Parkinsonism." *Cell Reports* 10(8):1252–60.

Ostrowski, Matias et al. 2010. "Rab27a and Rab27b Control Different Steps of the Exosome Secretion Pathway." *Nature Cell Biology* 12(1):19–30.

Ouyang, S., Xie, Y., Xiong, Z., Yang, Y., Xian, Y., Ou, Z., Song, B., Chen, Y., Xie, Y., Li, H., Sun, X. 2018. "CRISPR/Cas9-Targeted Deletion of Polyglutamine in Spinocerebellar Ataxia Type 3-Derived Induced Pluripotent Stem Cells." *Stem Cells Development* 27(11):756–70.

Pan, Bin Tao and Rose M. Johnstone. 1983. "Fate of the Transferrin Receptor during Maturation of Sheep Reticulocytes in Vitro: Selective Externalization of the Receptor." *Cell* 33(3):967–78.

Paolicelli, Rosa C., Giorgio Bergamini, and Lawrence Rajendran. 2018. "Cell-to-Cell Communication by Extracellular Vesicles: Focus on Microglia." *Neuroscience*.

Paulson, H. L. et al. 1997. "Intranuclear Inclusions of Expanded Polyglutamine Protein in Spinocerebellar Ataxia Type 3." *Neuron* 19(2):333–44.

Paulson, Henry. 2012. "Machado-Joseph Disease/spinocerebellar Ataxia Type 3." Pp. 437–49 in *Handbook of Clinical Neurology*.

Pecho-Vrieseling, Eline et al. 2014. "Transneuronal Propagation of Mutant Huntingtin Contributes to Non-Cell Autonomous Pathology in Neurons." *Nature Neuroscience* 17(8):1064–72.

Pedroso, José Luiz et al. 2011. "Sleep Disorders in Machado-Joseph Disease: Frequency, Discriminative Thresholds, Predictive Values, and Correlation with Ataxia-Related Motor and Non-Motor Features." *Cerebellum* 10(2):291–95.

van der Pol, E., A. N. Boing, P. Harrison, A. Sturk, and R. Nieuwland. 2012. "Classification, Functions, and Clinical Relevance of Extracellular Vesicles." *Pharmacological Reviews* 64(3):676–705.

Poon, Anna et al. 2017. "Modeling Neurodegenerative Diseases with Patient-Derived Induced Pluripotent Cells: Possibilities and Challenges." *New Biotechnology* 39:190–98.

Poon, Ivan K. H., Christopher D. Lucas, Adriano G. Rossi, and Kodi S. Ravichandran. 2014. "Apoptotic Cell Clearance: Basic Biology and Therapeutic Potential." *Nature Reviews Immunology* 14(3):166–80.

Poppe, Daniel et al. 2018. "Genome Editing in Neuroepithelial Stem Cells to Generate Human

Neurons with High Adenosine-Releasing Capacity." *Stem Cells Translational Medicine*.
Pozzi, Chiara et al. 2008. "Study of Subcellular Localization and Proteolysis of Ataxin-3." *Neurobiology of Disease* 30(2):190–200.
Prada, Ilaria et al. 2016. "A New Approach to Follow a Single Extracellular Vesicle–cell Interaction Using Optical Tweezers." *BioTechniques* 60(1):35–41.
Prusiner, S. B. 1998. "Prions." *Proceedings of the National Academy of Sciences of the United States of America* 95(23):13363.
Van Raamsdonk, Jeremy M., Irving E. Vega, and Patrik Brundin. 2017. "Oxidative Stress in Neurodegenerative Disease: Causation or Association?" *Oncotarget* 8(7).
Rajendran, L. et al. 2006. "Alzheimer's Disease Beta-Amyloid Peptides Are Released in Association with Exosomes." *Proceedings of the National Academy of Sciences* 103(30):11172–77.
Ramos, Amanda et al. 2014. "Differential mtDNA Damage Patterns in a Transgenic Mouse Model of Machado–Joseph Disease (MJD/SCA3)." *Journal of Molecular Neuroscience* 55(2):449–53.
Raposo, G. and HW Nijman. 1996. "B Lymphocytes Secrete Antigen Presenting Vesicles." *J. Exp. Med* 183(March):1161–72.
Raposo, Graça and Willem Stoorvogel. 2013. "Extracellular Vesicles: Exosomes, Microvesicles, and Friends." *Journal of Cell Biology* 200(4):373–83.
Ratajczak, J. et al. 2006. "Embryonic Stem Cell-Derived Microvesicles Reprogram Hematopoietic Progenitors: Evidence for Horizontal Transfer of mRNA and Protein Delivery." *Leukemia* 20(5):847–56.
Ravikumar, B. 2002. "Aggregate-Prone Proteins with Polyglutamine and Polyalanine Expansions Are Degraded by Autophagy." *Human Molecular Genetics* 11(9):1107–17.
Reinhardt, Peter et al. 2013. "Derivation and Expansion Using Only Small Molecules of Human Neural Progenitors for Neurodegenerative Disease Modeling." *PLoS ONE* 8(3).
Ren, Pei Hsien et al. 2009. "Cytoplasmic Penetration and Persistent Infection of Mammalian Cells by Polyglutamine Aggregates." *Nature Cell Biology* 11(2):219–25.
Robbins, Paul D., Akaitz Dorronsoro, and Cori N. Booker. 2016. "Regulation of Chronic Inflammatory and Immune Processes by Extracellular Vesicles." *Journal of Clinical Investigation* 126(4):1173–80.
Robbins, Paul D. and Adrian E. Morelli. 2014. "Regulation of Immune Responses by Extracellular Vesicles." *Nature Reviews Immunology* 14(3):195–208.
Robinton, Daisy. A. a. and Gq George Q. Daley. 2012. "The Promise of Induced Pluripotent Stem Cells in Research and Therapy." *Nature* 481(7381):295–305.
Rüb, Udo, Ewout R. Brunt, and Thomas Deller. 2008. "New Insights into the Pathoanatomy of Spinocerebellar Ataxia Type 3 (Machado-Joseph Disease)." *Current Opinion in Neurology* 21(2):111–16.
Rufino-Ramos, David et al. 2017. "Extracellular Vesicles: Novel Promising Delivery Systems for Therapy of Brain Diseases." *Journal of Controlled Release* 262:247–58.
Rusten, Tor Erik, Thomas Vaccari, and Harald Stenmark. 2012. "Shaping Development with ESCRTs." *Nature Cell Biology* 14(1):38–45.
Sacino, Amanda N. et al. 2013. "Conformational Templating of α-Synuclein Aggregates in Neuronal-Glial Cultures." *Molecular Neurodegeneration* 8(1).
Saman, Sudad et al. 2012. "Exosome-Associated Tau Is Secreted in Tauopathy Models and Is Selectively Phosphorylated in Cerebrospinal Fluid in Early Alzheimer Disease." *Journal of Biological Chemistry* 287(6):3842–49.
Sarko, Diana K. and Cindy E. McKinney. 2017. "Exosomes: Origins and Therapeutic Potential for Neurodegenerative Disease." *Frontiers in Neuroscience* 11(FEB).

Sayed, Nazish, Chun Liu, and Joseph C. Wu. 2016. "Translation of Human-Induced Pluripotent Stem Cells from Clinical Trial in a Dish to Precision Medicine." *Journal of the American College of Cardiology* 67(18):2161–76.

Scherzed, W. et al. 2012. "Pathoanatomy of Cerebellar Degeneration in Spinocerebellar Ataxia Type 2 (SCA2) and Type 3 (SCA3)." *Cerebellum* 11(3):749–60.

Schmitz-Hübsch, Tanja et al. 2010. "Self-Rated Health Status in Spinocerebellar Ataxia - Results from a European Multicenter Study." *Movement Disorders* 25(5):587–95.

Schöls, Ludger, Peter Bauer, Thorsten Schmidt, Thorsten Schulte, and Olaf Riess. 2004. "Autosomal Dominant Cerebellar Ataxias: Clinical Features, Genetics, and Pathogenesis." *Lancet Neurology* 3(5):291–304.

Schulz, Jörg B. et al. 2010. "Visualization, Quantification and Correlation of Brain Atrophy with Clinical Symptoms in Spinocerebellar Ataxia Types 1, 3 and 6." *NeuroImage* 49(1):158–68.

Segura, Elodie, Sebastian Amigorena, and Clotilde Théry. 2005. "Mature Dendritic Cells Secrete Exosomes with Strong Ability to Induce Antigen-Specific Effector Immune Responses." *Blood Cells, Molecules, and Diseases* 35(2):89–93.

Sharples, R. A. et al. 2007. "Inhibition of -Secretase Causes Increased Secretion of Amyloid Precursor Protein C-Terminal Fragments in Association with Exosomes." *The FASEB Journal* 22(5):1469–78.

Shoesmith Berke, Sarah J., Francisca A. Flores Schmied, Ewout R. Brunt, Lisa M. Ellerby, and Henry L. Paulson. 2004. "Caspase-Mediated Proteolysis of the Polyglutamine Disease Protein Ataxin-3." *Journal of Neurochemistry* 89(4):908–18.

Simões, Ana T. et al. 2012. "Calpastatin-Mediated Inhibition of Calpains in the Mouse Brain Prevents Mutant Ataxin 3 Proteolysis, Nuclear Localization and Aggregation, Relieving Machado-Joseph Disease." *Brain* 135(8):2428–39.

Simões, Ana Teresa, Nélio Gonçalves, Rui Jorge Nobre, Carlos Bandeira Duarte, and Luís Pereira de Almeida. 2014. "Calpain Inhibition Reduces Ataxin-3 Cleavage Alleviating Neuropathology and Motor Impairments in Mouse Models of Machado-Joseph Disease." *Human Molecular Genetics* 23(18):4932–44.

Singh, Vimal K., Manisha Kalsan, Neeraj Kumar, Abhishek Saini, and Ramesh Chandra. 2015. "Induced Pluripotent Stem Cells: Applications in Regenerative Medicine, Disease Modeling, and Drug Discovery." *Frontiers in Cell and Developmental Biology* 3.

Soong, Bing-Wen et al. 2017. "Generation of Induced Pluripotent Stem Cells from a Patient with Spinocerebellar Ataxia Type 3." *Stem Cell Research* 18:29–32.

Stuendl, A. et al. 2016. "Induction of α-Synuclein Aggregate Formation by CSF Exosomes from Patients with Parkinson's Disease and Dementia with Lewy Bodies." *Brain* 139(2).

Szatanek, Rafal, Jarek Baran, Maciej Siedlar, and Monika Baj-Krzyworzeka. 2015. "Isolation of Extracellular Vesicles: Determining the Correct Approach (Review)." *International Journal of Molecular Medicine* 36(1):11–17.

Szebenyi, Györgyi et al. 2003. "Neuropathogenic Forms of Huntingtin and Androgen Receptor Inhibit Fast Axonal Transport." *Neuron* 40(1):41–52.

Takahashi, K. and S. Yamanaka. 2006. "Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors." *Cell* 126(4):663–76.

Takahashi, Kazutoshi et al. 2007. "Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors." *Cell* 131(5):861–72.

Takiyama, Y. et al. 1993. "The Gene for Machado-Joseph Disease Maps to Human Chromosome 14q." *Nature Genetics* 4(3):300–304.

Tapiola, Tero et al. 2009. "Cerebrospinal Fluid β-Amyloid 42 and Tau Proteins as Biomarkers of Alzheimer-Type Pathologic Changes in the Brain." *Archives of Neurology* 66(3):382–89.

Tardivel, Meryem et al. 2016. "Tunneling Nanotube (TNT)-Mediated Neuron-to Neuron

Transfer of Pathological Tau Protein Assemblies." *Acta Neuropathologica Communications* 4(1):117.

Taroni, Franco and Stefano DiDonato. 2004. "Pathways to Motor Incoordination: The Inherited Ataxias." *Nature Reviews Neuroscience* 5(8):641–55.

Taylor, Rebecca C., Sean P. Cullen, and Seamus J. Martin. 2008. "Apoptosis: Controlled Demolition at the Cellular Level." *Nature Reviews Molecular Cell Biology* 9(3):231–41.

Théry, Clotilde, Sebastian Amigorena, Graça Raposo, and Aled Clayton. 2006. "Isolation and Characterization of Exosomes from Cell Culture Supernatants and Biological Fluids." *Current Protocols in Cell Biology / Editorial Board, Juan S. Bonifacino ... [et Al.]* Chapter 3:Unit 3.22.

Thompson, Alexander G. et al. 2016. "Extracellular Vesicles in Neurodegenerative Disease — Pathogenesis to Biomarkers." *Nature Reviews Neurology* 12(6):346–57.

Tian, Tian et al. 2013. "Dynamics of Exosome Internalization and Trafficking." *Journal of Cellular Physiology* 228(7):1487–95.

Trajkovic, Katarina et al. 2008. "Ceramide Triggers Budding of Exosome Vesicles into Multivesicular Endosomes." *Science* 319(5867):1244–47.

Valadi, Hadi et al. 2007. "Exosome-Mediated Transfer of mRNAs and microRNAs Is a Novel Mechanism of Genetic Exchange between Cells." *Nature Cell Biology* 9(6):654–59.

Vella, L. J. et al. 2007. "Packaging of Prions into Exosomes Is Associated with a Novel Pathway of PrP Processing." *Journal of Pathology* 211(5):582–90.

Vilette, Didier et al. 2015. "Efficient Inhibition of Infectious Prions Multiplication and Release by Targeting the Exosomal Pathway." *Cellular and Molecular Life Sciences* 72(22):4409–27.

Volpicelli-Daley, Laura A. et al. 2011. "Exogenous α-Synuclein Fibrils Induce Lewy Body Pathology Leading to Synaptic Dysfunction and Neuron Death." *Neuron* 72(1):57–71.

Wang, S. et al. 2011. "Synapsin I Is an Oligomannose-Carrying Glycoprotein, Acts As an Oligomannose-Binding Lectin, and Promotes Neurite Outgrowth and Neuronal Survival When Released via Glia-Derived Exosomes." *Journal of Neuroscience* 31(20):7275–90.

Wang, Y., J. Cui, X. Sun, and Y. Zhang. 2011. "Tunneling-Nanotube Development in Astrocytes Depends on p53 Activation." *Cell Death and Differentiation* 18(4):732–42.

Van De Warrenburg, B. P. C., R. J. Sinke, C. C.Verschuuren Bemelmans, and H. Scheffer. 2002. "Spinocerebellar Ataxias in the Netherlands." *Neurology* 58(97252):702–8.

Wellington, Cheryl L. et al. 1998. "Caspase Cleavage of Gene Products Associated with Triplet Expansion Disorders Generates Truncated Fragments Containing the Polyglutamine Tract." *Journal of Biological Chemistry* 273(15):9158–67.

Williams, Roger L. and Sylvie Urbé. 2007. "The Emerging Shape of the ESCRT Machinery." *Nature Reviews Molecular Cell Biology* 8(5):355–68.

Winborn, Brett J. et al. 2008. "The Deubiquitinating Enzyme Ataxin-3, a Polyglutamine Disease Protein, Edits Lys63 Linkages in Mixed Linkage Ubiquitin Chains." *Journal of Biological Chemistry* 283(39):26436–43.

Wolf, Peter. 1967. "The Nature and Significance of Platelet Products in Human Plasma." *British Journal of Haematology* 13(3):269–88.

Xie, Y. Z. and R. X. Zhang. 2014. "Neurodegenerative Diseases in a Dish: The Promise of iPSC Technology in Disease Modeling and Therapeutic Discovery." *Neurological Sciences* 36(1):21–27.

Xin, Hongqi et al. 2012. "Exosome-Mediated Transfer of miR-133b from Multipotent Mesenchymal Stromal Cells to Neural Cells Contributes to Neurite Outgrowth." *Stem Cells* 30(7):1556–64.

Xu, Jing, Robert Camfield, and Sharon M. Gorski. 2018. "The Interplay between Exosomes and

Autophagy-Partners in Crime." *Journal of Cell Science*. Retrieved (http://jcs.biologists.org.gate2.inist.fr/content/joces/131/15/jcs215210.full.pdf).

Xu, Xiao-hong and Zhong Zhong. 2013. "Disease Modeling and Drug Screening for Neurological Diseases Using Human Induced Pluripotent Stem Cells." *Acta Pharmacologica Sinica* 34(6):755–64.

Yamada, Mitsunori, C. F. Tan, C. Inenaga, S. Tsuji, and H. Takahashi. 2004. "Sharing of Polyglutamine Localization by the Neuronal Nucleus and Cytoplasm in CAG-Repeat Diseases." *Neuropathology and Applied Neurobiology* 30(6):665–75.

Yáñez-Mó, María et al. 2015. "Biological Properties of Extracellular Vesicles and Their Physiological Functions." *Journal of Extracellular Vesicles* 4(2015):1–60.

Yang, W. 2002. "Aggregated Polyglutamine Peptides Delivered to Nuclei Are Toxic to Mammalian Cells." *Human Molecular Genetics* 11(23):2905–17.

Yoshihara, Masahito, Yoshihide Hayashizaki, and Yasuhiro Murakawa. 2017. "Genomic Instability of iPSCs: Challenges Towards Their Clinical Applications." *Stem Cell Reviews and Reports* 13(1):7–16.

Younesi, Erfan and Martin Hofmann-Apitius. 2013. "From Integrative Disease Modeling to Predictive, Preventive, Personalized and Participatory (P4) Medicine." *EPMA Journal* 4(1).

Yu, Ya-Chun, Chen-Ling Kuo, Wen-Ling Cheng, Chin-San Liu, and Mingli Hsieh. 2009. "Decreased Antioxidant Enzyme Activity and Increased Mitochondrial DNA Damage in Cellular Models of Machado-Joseph Disease." *Journal of Neuroscience Research* 87(8):1884–91.

Yuyama, Kohei et al. 2015. "A Potential Function for Neuronal Exosomes: Sequestering Intracerebral Amyloid-β Peptide." *FEBS Letters* 589(1):84–88.

Zhang, Bin, Ronne Wee Yeh Yeo, Kok Hian Tan, and Sai Kiang Lim. 2016. "Focus on Extracellular Vesicles: Therapeutic Potential of Stem Cell-Derived Extracellular Vesicles." *International Journal of Molecular Sciences* 17(2).

Zhang, H. G. et al. 2006. "A Membrane Form of TNF- Presented by Exosomes Delays T Cell Activation-Induced Cell Death." *The Journal of Immunology* 176(12):7385–93.

Zhang, Su-Chun, Marius Wernig, Ian D. Duncan, Oliver Brüstle, and James A. Thomson. 2001. "In Vitro Differentiation of Transplantable Neural Precursors from Human Embryonic Stem Cells." *Nature Biotechnology* 19(12):1129–33.

Zhang, Xuan et al. 2016. "Potential Transfer of Polyglutamine and CAG-Repeat RNA in Extracellular Vesicles in Huntington's Disease: Background and Evaluation in Cell Culture." *Cellular and Molecular Neurobiology* 36(3):459–70.

Zhuang, Xiaoying et al. 2011. "Treatment of Brain Inflammatory Diseases by Delivering Exosome Encapsulated Anti-Inflammatory Drugs from the Nasal Region to the Brain." *Molecular Therapy* 19(10):1769–79.

Zitvogel, Laurence et al. 1998. "Eradication of Established Murine Tumors Using a Novel Cell-Free Vaccine: Dendritic Cell-Derived Exosomes." *Nature Medicine* 4(5):594–600.