João Carlos Versos Varanda Barata

Unravelling the players of Mesenchymal Stromal Cells' Neuroprotective Effect in Machado-Joseph Disease

Setembro 2017

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UNIVERSIDADE DE COIMBRA

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Dissertação de Mestrado em Biologia Celular e Molecular,
realizada sob a orientação científica da Doutora Catarina Oliveira Miranda
e orientação interina da Professora Doutora Emília Duarte,
apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra

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Front cover:

Fluorescence microphotograph of MSCs differentiated into chondrocytes (collagen II in red, DAPI in blue).

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Abbreviations

AD – Alzheimer's disease

ALS – amyotrophic lateral sclerosis

BDNF – brain-derived neurotrophic factor

CBP – CREB-binding protein

CREB – cAMP response element-binding protein

DC – dendritic cell

DRPLA – dentatorubral pallidoluysian atrophy

DUB – deubiquitinating activity

ECM – extracellular matrix

EGF – epidermal growth factor

ER – endoplasmic reticulum

ERAD – endoplasmic reticulum ER-associated protein degradation

ERK – extracellular signal-regulated kinase

FAAH – fatty acid amide hydrolase

FGF – fibroblast growth factor

GDNF – glial cell line-derived neurotrophic factor

GFAP – glial fibrillary acidic protein

HD – Huntington's disease

HGF – hepatocyte growth factor

HSC – hematopoietic stem cell

Hsp – heat-shock protein

IDO – indoleamine 2,3-dioxygenase

IFN – interferon

IGF-1 – insulin-like growth factor-1

IL – interleukin

ISCT – International Society for Cellular Therapy

MAP – microtubule-associated protein

MAPK – mitogen-activated protein kinase

MHC – major histocompatibility complex

MJD – Machado-Joseph disease

MSC – multipotent mesenchymal stromal cell

mTOR – mammalian target of rapamycin

NES – nuclear export signal

NGF – nerve growth factor

NIN – neuronal intranuclear inclusion

NK – natural killer

NLS – nuclear localization signal

NO – nitric oxide

PCAF – P300/CBP-associated factor

PD – Parkinson's disease

PD-1 - programmed death-1

PDGF – platelet-derived growth factor

PI3K – phosphatidylinositol-4,5-bisphosphate 3-kinase

PolyQ – polyglutamine

SBMA – spinal and bulbar muscular atrophy

SCA – spinocerebellar ataxia

SCA3 – spinocerebellar ataxia type 3

SIRT1 – sirtuin 1

STAT – signal transducer and activator of transcription

Tg – transgenic

TLR – toll-like receptor

TNF-α – tumor necrosis factor alpha

TSG-6 – tumor necrosis factor-alpha stimulated gene/protein 6

UPS – ubiquitin-proteasome system

UTR – untranslated region

VCP – valosin-containing protein

VEGF – vascular endothelial growth factor

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Abstract

Machado-Joseph Disease (MJD), also known as spinocerebellar ataxia type 3 (SCA3), is a neurodegenerative disorder caused by an abnormally expanded number of CAG repeats in the gene codifying the protein ataxin-3. This mutation leads to dysfunction of several cellular mechanisms, such as autophagy, and ends with neuronal death, mainly in the cerebellum and brain stem. Currently, there is no cure or therapy for this fatal disorder. Multipotent mesenchymal stromal cells (MSCs) have been widely used as a therapeutic strategy in neurodegenerative disorders but few studies have been done using them in SCAs.

In this study, we intended to evaluate the capacity of MSCs in enhancing autophagy, and leading to functional benefits in MJD. In order to unravel this, we created a co-culture *in vitro* system using a cellular MJD model and MSCs and evaluated the expression of autophagy-related proteins. Moreover, we assessed for phenotypic improvements and autophagy-related proteins after performing repeated systemic treatments in a transgenic mouse model of MJD. Proteins involved in the autophagic pathway were found to be normalized in the presence of MSCs both *in vitro* and *in vivo.* Furthermore, we observed a decrease in mammalian target of rapamycin (mTOR) activity, an inhibitor of autophagy. Concomitantly, we found that the levels of mutant ataxin-3 were decreased in MJD mice treated with MSCs. These findings suggest that MSCs' beneficial effects in MJD might be mediated through paracrine signaling by decreasing mutant ataxin-3-derived toxicity through an increment of the autophagic pathway.

In conclusion, we demonstrated that MSCs can induce functional benefits in MJD models through a stimulation of the autophagic flux in an mTOR-dependent way, thus showing MSCs are a viable therapeutic approach for MJD patients.

Keywords: Machado-Joseph disease; multipotent mesenchymal stromal cells; autophagy; paracrine signaling; ataxin-3.

Resumo

A doença de Machado-Joseph (MJD), também conhecida como ataxia espinocerebelosa do tipo 3, é uma doença neurodegenerativa causada por um aumento anormal do número de repetições CAG no gene que codifica a proteína ataxina-3. Esta mutação leva à perturbação de diversos mecanismos celulares, como a autofagia, e acaba por levar a morte neuronal, maioritariamente no cerebelo e tronco cerebral. Actualmente, não existe uma cura ou terapia que atrase ou pare a progressão desta doença fatal. As células multipotentes mesenquimatosas do estroma (MSCs) têm sido bastante utilizadas como estratégia terapêutica em doenças neurodegenerativas, mas poucos estudos foram realizados em ataxias espinocerebelosas..

Neste estudo, avaliámos a capacidade das MSCs em promover a autofagia e assim induzir benefícios funcionais em MJD. Para desvendar esta questão, críamos um sistema de co-culturas *in vitro* usando um modelo celular de MJD e MSCs e avaliámos a expressão de proteínas relacionadas com a autofagia. Além disso, investigámos a existência de melhorias fenotípicas e proteínas envolvidas na autofagia depois de realizarmos um múltiplo tratamento sistémico com MSCs num modelo transgénico de murganhos. Na presença de MSCs, observámos que proteínas envolvidas na autofagia se encontravam normalizadas, quer *in vitro* quer *in vivo*. Adicionalmente, foi observada uma diminuição da actividade da proteína mTOR, um inibidor da autofagia. Os níveis de ataxina-3 mutante também se encontravam diminuídos em murganhos MJD tratados com MSCs. Estas descobertas sugerem que o efeito benéfico das MSCs em MJD poderá ser, pelo menos em parte, mediado por sinalização parácrina através da diminuição da toxicidade derivada da ataxina-3 mutante ao haver indução do fluxo autofágico.

Com este projecto, demonstrámos que as MSCs têm a capacidade de induzir benefícios em modelos de MJD através de um aumento da autofagia, mostrando assim que são uma promissora terapia para pacientes de MJD.

Palavras-chave: Doença de Machado-Joseph; células multipotentes mesenquimatosas do estroma; autofagia, sinalização parácrina, ataxina-3

1 – Introduction

1.1 Polyglutamine diseases

Polyglutamine (polyQ) diseases are a group of neurodegenerative disorders that includes Huntington's disease (HD), dentatorubral-pallidoluysian atrophy (DRPLA), X-linked spinal and bulbar muscular atrophy (SBMA, also known as Kennedy's disease) and several types of spinocerebellar ataxias (SCAs).

Their name derives from the fact that they originate from the expansion of an unstable CAG repeat in an associated mutated gene (Fu et al. 1991; La Spada et al. 1991), with the number of repeats from which a pathological phenotype can start being specific of each disorder (Gatchel and Zoghbi 2005). The affected genes code proteins with almost no homology and with different functions (Zoghbi and Orr 2000; Gatchel and Zoghbi 2005), suggesting that the excessive repeats might induce mechanisms of pathogenesis that are also defined by the other regions of the protein and its cellular function.

The biochemical mechanisms that elicit neuronal death in polyQ diseases have not been clearly unraveled yet. However, as age of onset can almost always be correlated with the number of repeats (Rolfs et al. 2003; Netravathi et al. 2009; Durr et al. 2010) it is thought that the extended polyQ tract is essential for abnormal function of affected protein. Some clinical features also appear to correlate with the number of repeats but other unidentified factors are suggested to also influence the phenotype (Kremer et al. 1993; Ranum et al. 1994). Most patients with polyQ disorders are heterozygous for the respective mutant allele but some homozygotes have been found. The symptoms in these cases can be similar (Ross et al. 2002) or the patients might display a worse profile (Lerer et al. 1996), depending on the disorder.

Symptomatic features also differ widely between disorders but they typically start in midlife (usually between 10 and 20 years), with neuronal dysfunction thus increasing and ending in selective neuronal death (Zoghbi and Orr 2000). This selective neuronal loss occurs in different areas of the nervous system for each disorder but there is some overlap in areas such as the cerebellum, basal ganglia, brainstem nuclei and spinal motor nuclei (Ross 1995; Zoghbi and Orr 2000). Neuronal progressive loss finally culminates in patients' death (Zoghbi and Orr 2000).

1.1.1 Spinocerebellar ataxias

SCAs are a heterogeneous group of hereditary autosomal dominant neurodegenerative disorders characterized by progressive neuronal loss in the cerebellum, brain stem and spinocerebellar tracts (Dohlinger et al. 2008; Jacobi et al. 2011). More than 20 SCAs have been recognized until now but only a few (1, 2, 3, 6, 7 and 17) belong to the

group of polyglutamine diseases, meaning that they originate from an expansion of a CAG repeat (Holmes et al. 1999; Koob et al. 1999; Matsuura et al. 2000; Zoghbi and Orr, 2000), with others being linked with dysfunctions in proteins such as ion channels or kinases (van Swieten et al. 2003; Chen et al. 2003; Sasaki et al. 2003; Waters et al. 2006).

Different SCAs share common clinical symptoms which include gait and limb ataxia, oculomotor disturbances and dysarthria, but specific features can help distinguish certain ataxias from the others (Schöls et al. 2004). Length of the repeated polyQ tract correlates with age of onset of the disease in 50 to 80% of the cases, similarly to HD (Maciel et al. 1995; Durr et al. 1996; Riess et al. 2008). An increase of its length may occur during transmission, leading to an earlier disease onset in successive generations, mostly due to paternal transmission, in some SCAs (Lebre et al. 2003; Paulson et al. 2009).
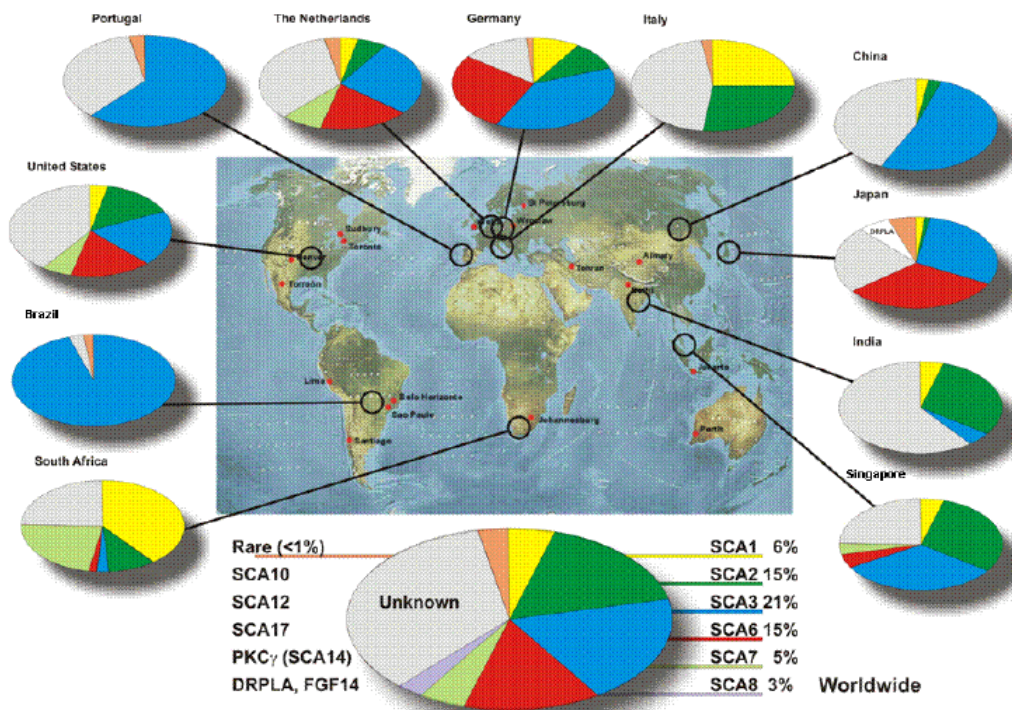
Figure 1 - Distribution of SCA subtypes worldwide. Adapted from Bird et al. 1993

These inherited ataxias are considered rare diseases as their prevalence is thought to be around 0,3 to 2 per 100,000 inhabitants (van de Warrenburg et al. 2002), though most existing surveys are not recent and might not present the prevalence of all the identified SCAs or otherwise only show data from minor regions which are known to have high prevalence of a particular SCA, such as the Azores archipelago from Portugal for Machado-Joseph disease (Bettencourt et al. 2008).

1.1.2 Machado-Joseph disease

Machado-Joseph disease (MJD), also known as spinocerebellar ataxia type 3 (SCA3), is a polyQ disease that originates in an extended polyQ tract in the protein ataxin-3 and is the most common type of autosomal dominant ataxia worldwide (Cagnoli et al. 2005). Its geographic distribution pattern shows that some countries such as Portugal, Brazil, Singapore, China and the Netherlands have the highest frequencies of the pathology, but inside them there is high heterogeneity in their distribution (Jardim et al. 2001; van de Warrenburg et al. 2002; Zhao et al. 2002; Jiang et al. 2005; Vale et al. 2010). Portugal's example clearly illustrates this as MJD is quite rare in the mainland (1:100,000 (Coutinho 1992)) but a very high prevalence exists in one of the Azores Islands, with around 1 case for each 239 habitants in Flores Island (Bettencourt et al. 2008).

MJD was first described in families descending from this archipelago, where it constitutes a major public health problem. In 1972 this disorder was named as a "nigro-spino-dentatal degeneration with nuclear ophthalmoplegia" (Woods and Schaumburg 1972) and later as "Azorean disease of the nervous system" (Romanul et al. 1977), "autosomal dominant striatonigral degeneration" (Rosenberg et al. 1976) and "Machado disease" (Nakano et al. 1972) but later, they were all classified as being the same autosomal dominant genetic disorder (Coutinho et al. 1978). These set of studies showcased well the diverse symptoms of this specific SCA, with some cases only being correctly diagnosed after the mutation responsible for it was discovered.

1.1.2.1 ATXN3 gene

The locus responsible for MJD was only mapped in 1993 (Takiyama et al. 1993) in the gene of ATXN3 (also known as MJD1 or MJD), which is located in 14q32.1, and the gene was cloned in the following year (Kawaguchi et al. 1994). The cause of the pathology was attributed to an expansion of a CAG repeat that was found to be present in all MJD patients (Takiyama et al. 1993).

Its genomic structure was afterwards described in 2001 (Ichikawa et al. 2001) and 13 exons have been described, with the extended CAG repeats being present in exon 10 (Ichikawa et al. 2001; Bettencourt et al. 2009). Presently, about 50 different transcripts can be found due to combined splicing events, but it is not known if they differ in terms of biological functions. It is thought that they are found in similar levels in all the neuronal and non-neuronal tissues where the gene is expressed (Bettencourt et al. 2010). However, only neuronal cells appear to be affected in MJD.

Ataxin-3 is the protein encoded by this gene, with the polyQ tract codified by the expanded CAG repeats being in its C-terminal (Ichikawa et al. 2001). The number of CAG repeats in the ATXN3 gene is between 12 and 44 in normal persons, and 60 or more in MJD patients (Cancel et al. 1995; Maciel et al. 1995, Paulson et al. 2015), but these numbers are still widely discussed as some cases have been reported in which the extended tract has an intermediate number of repeats, and there is doubt whether the disease is even present or not because the symptoms presented by these patients are usually not as severe as those with a higher number of CAG repeats (Takiyama et al. 1995; Van Alfen et al. 2001). Even though heterozygotes constitute the vast majority of cases, some homozygotes for the mutated allele have been described and usually display a more severe phenotype of the disease (Lerer et al. 1996). In this disorder, an increase in the number of CAG repeats is known to occur in future generations, mainly due to paternal transmission in which there is great expansion of the polyQ tract (Takiyama et al. 1995; Maciel et al. 1995; Durr et al. 1996).

1.1.2.2 Ataxin-3 protein

The protein ataxin-3 has a molecular weight of 42kDa (Kawaguchi et al. 1994) and it is most known has having deubiquitinating activity (DUB), and is widely expressed in the brain and other parts of the body (Paulson et al. 1997). It is predominantly present in the cytoplasm but also in the nucleus and mitochondria (Trottier et al. 1998).

The human form of the protein has 339 amino acid residues, followed by a variable number of glutamine repeats. The N-terminal is more compact and has a conserved motif, named Josephin, with a globular domain (Goto et al. 1997; Masino et al. 2003). It also possesses papain-like cysteine protease activity, which originates from this domain (Albrecht et al. 2004; Nicastro et al. 2005). The polyQ tract, which is extended in MJD patients, is found at the C-terminal. This region is more flexible and can suffer proteolytic degradation (Masino et al. 2003). Its capacity to bind ubiquitin residues comes from 2 or 3 ubiquitin-interaction motifs that are also found at the C-terminal (Masino et al. 2003; Burnett et al. 2003).
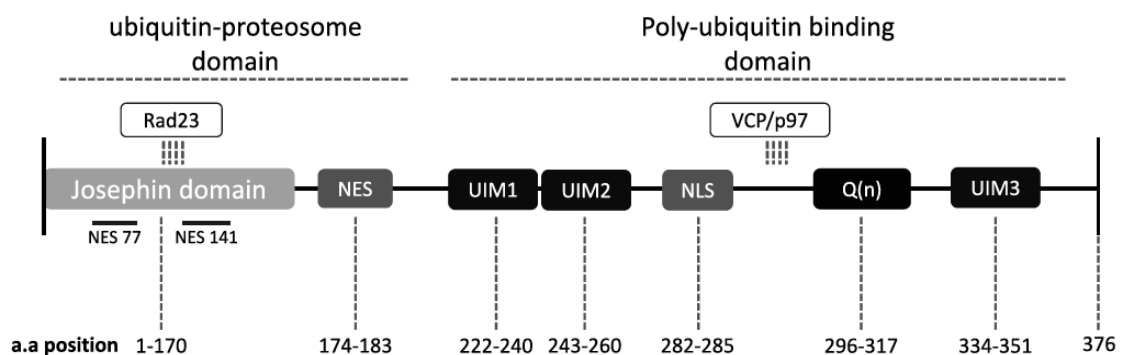

Figure 2 - Structure of the ataxin-3 protein. Adapted from Nóbrega and de Almeida (2012)

Different ataxin-3 isoforms have long been known to exist (Trottier et al. 1998; Schmidt et al. 1998). It was first thought that specific isoforms originating from the mutated protein could go to the nucleus and would induce pathogenesis once present there (Schmidt et al. 1998). However, this view was quickly proven to be wrong as even normal ataxin-3 was found in the nucleus of cells of healthy individuals due to the presence of a nuclear localization signal (NLS), usually found associated with the inner nuclear matrix (Tait et al. 1998). Two nuclear export signals (NESs) are also known to exist in ataxin-3, NES 77 and NES141. These have a greater activity than the NLS, thus leading to the low levels of ataxin-3 usually found in the nucleus of healthy individuals (Albrecht et al. 2004).

Ataxin-3 seems to be involved in several cellular pathways, though its most known role is the participation in the ubiquitin-proteasome system (UPS), where it can direct proteins for degradation by binding polyubiquitinated proteins and other proteins that help in the delivery process to the proteasome (Doss-Pepe et al. 2003), or trim polyubiquitin chains to regulate their presentation to the proteasome (Burnett et al. 2003). Impairment of the UPS system can lead to aggresome formation. Proteins can also be degraded through lysosomes (García-Mata et al. 2002), with ataxin-3 being able to regulate this process by interacting with microtubules through tubulin (Mazzuchelli et al. 2009) and dynein (Burnett et al. 2005). Knowledge on what proteins are regulated by ataxin-3 is fundamental to understand the pathogenesis process in MJD. Recently, p53 was identified as one of ataxin-3 substrates both in its native or polyubiquitinated form, repressing its degradation. Indeed, it was shown that the mutated form of ataxin-3 has an enhanced ability to promote the stabilization of p53, which then promotes the expression of several pro-apoptotic genes in an abnormally increased way (Liu et al. 2016).

Another way by which ataxin-3 promotes the maintenance of cellular protein quality is through the endoplasmic reticulum (ER)-associated protein degradation (ERAD) system: by binding to valosin-containing protein (VCP, also known as p97), which is able to extract substrates of this mechanism from the ER to the proteasome, proteins can be degraded after being ubiquitinated (Meusser et al. 2005; Zhong et al. 2006; Blount et al. 2014).

Ataxin-3 is also able to control transcription through binding and inhibition of certain activators, such as CREB-binding protein (CBP), p300 and P300/CBP-associated factor (PCAF), or through direct binding to histones, both mechanisms leading to the blockage of histone acetylation, thus reducing transcription of the affected genes (Li et al. 2002).

Finally, a role in myogenesis has also been attributed to ataxin-3 as it was shown that, if interference RNA was used, an abnormal cellular phenotype was produced in mouse embryos, which was attributed to a decreased expression of integrin α-5 and -7 because ataxin-3 is thought to be able to stabilize them and repress their degradation (Costa et al. 2010). Ataxin-3 might also have a role in cytoskeleton organization as microtubules are found

to be in a random disorganized fashion in its absence (Rodrigues et al. 2010). Cell adhesion is also decreased, probably due to decreased expression of cell-extracellular matrix (ECM) and cell-cell communication proteins, such as talin or α-1 subunit of integrin. This does not lead to changes in terms of mitosis even if more cells appear to be in the G0 or G1 phase, which can occur due to apoptosis induced by the abnormal cell shape and adhesion (Rodrigues et al. 2010).

1.1.2.3 Clinical features

The main common symptoms observed in MJD patients are ataxia, dysarthria and dysphasia (Coutinho et al. 1978; Paulson et al. 2007; Costa et al. 2012) but, due to the heterogeneity of features that can occur, clinicians usually use a classification that divides the pathology in five clinical types, based mainly on the age of onset and symptoms observed (Sequeiros and Coutinho 1993; Matsumura et al. 1996). Type I is known to start earlier (at around 25 years of age) and is characterized by bradykinesia, spasticity, rigidity and almost no ataxia. The most common type observed is type II, which begins in mid adult years and with progressive ataxia and upper motor neuron deficiencies being its hallmarks. Type III has a late onset (around 50 years of age) and is characterized by ataxia but while also displaying severe peripheral nerve abnormalities that lead to amyotrophy and areflexia. A rare type IV is also found in some cases that show parkinsonian features (Cancel et al. 1995). A type V has been proposed where patients present pure spastic paraplegia (Wang et al. 2009) and for a homozygote male patient (Lysenko et al. 2010).

Other nonmotor and extracerebellar features that are also commonly observed in MJD patients include sleep disturbances (derived from restless legs syndrome), olfactory dysfunction, pseudoexophthalmos (bulging eyes) and impaired temperature discrimination (mostly in the limbs, face and trunk) (Durr et al. 1996; Paulson et al. 2007; Costa et al. 2012; Pedroso et al. 2013).

The mean age of onset is around 40.2 years of age but some premature cases have been reported (7 years of age), with others only appearing very late in life (70 years of age) (Sequeiros and Coutinho 1993; Carvalho et al. 2008; Bettencourt and Lima 2011). These differences in the age of onset can mostly be explained by the number of CAG repeats in the ATXN3 gene, with an increased number leading to earlier age of onset (Durr et al. 1996). The mean survival time, after the first symptoms are reported, is 20 years (Klockgether et al., 1998).

1.1.2.4 Neuropathology

The main brain areas affected in MJD are the pontine nuclei and caudal region of the brainstem, pontocerebellar and spinocerebellar fibers in the cerebellum (with loss of Purkinje cells) and anterior horn and Clarke's column in the spinal cord (Coutinho et al. 1978; Paulson et al. 2007; Yamada et al. 2007). The substantia nigra is found to be depigmented due to degeneration of neuromelanin-containing neurons. The dentate nucleus, subthalamic nucleus, putamen, globus pallidus and striatum are also found to be atrophied whereas the thalamic intralaminar nucleus, lateral geniculate body, inferior olive and dorsal root are not (Yamada et al. 2007; Schulz et al. 2010). In terms of brain weight, there's a significant decrease in MJD patients in comparison with healthy individuals (Iwabuchi et al. 1999).
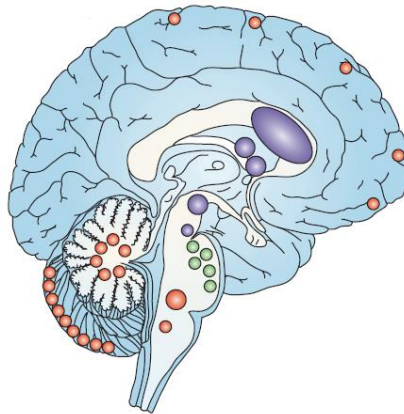
Figure 3 - Main brain areas affected in MJD. Severe neuronal loss is indicated in red, involvement of extrapyramidal nuclei in blue and cranial nerve involvement in green. Adapted from Taroni and DiDonato (2004)

Glucose utilization is known to be impaired in the cortical areas and also in the cerebellum and brainstem, even before symptoms of the disease start appearing (Soong and Liu 1998). Neurotransmission is also thought to be dysfunctional as some genes correlated with dopaminergic and glutamatergic transmission are differentially expressed in MJD (Taniwaki et al. 1997; Wullner et al. 2005), together with some voltage-gated potassium channels that can explain some of the motor symptoms observed, and which precede neuronal death (Shakkottai et al. 2011).

Intranuclear inclusions have long been known to occur in MJD (Paulson et al. 1997; Trottier et al. 1998), both in affected and spared areas (Paulson et al. 1997; Schmidt et al. 1998, Rub et al. 2006), with their role in the pathogenesis process still being controversial. They are eosinophilic spheres with a size of more or less 0.7 to 3.7 μm and do not only include aggregates of mutated ataxin-3 but also ubiquitin, transcription factors, chaperones,

other proteins that possess polyQ stretches and components of the UPS (Perez et al. 1998; Chai et al. 1999a; Chai et al. 1999b; Hayashi et al. 2003; Seidel et al. 2010). Cytoplasmic inclusions can also be found (Hayashi et al. 2003), bearing polyQ stretches from the mutated form of the protein, associated with lysosomes. Axonal inclusions have also been identified, usually in fiber tracts that are known to degenerate in MJD (Seidel et al. 2010).

1.1.2.5 Pathogenesis and potential therapeutic targets

Since several cellular processes appear to be affected by mutant ataxin-3, there certainly isn't a single mechanism responsible for the pathophysiology of this protein. Therapies that target one (or more) of these mechanisms have shown some promising results, though most of them have only been performed in animal models of the disease. Still, future clinical trials that might use these approaches will previously need to better evaluate their safety.

Neuronal intranuclear inclusions (NINs) were once thought to induce pathogenesis by themselves (Paulson et al. 1997; Schmidt et al. 1998) but as they are also found in brain areas not affected in MJD, they are now seen as a failed attempt of the cell to properly fold or degrade the mutated ataxin-3 (Paulson et al. 1997; Schmidt et al. 1998, Rub et al. 2006). This incapacity to clear the mutant form of the protein might arise from post-translational modifications, such as phosphorylation and ubiquitination on specific sites, which differ between the normal and mutant form of ataxin-3 (Kristensen et al. 2017). These altered post-translational modifications can not only alter its cellular fate but also modify its cellular function, which might end up explaining other disruptions observed in the pathology. NINs are SDS-soluble in healthy individuals, while the ones found in MJD patients are SDS-insoluble due to the presence of the mutated form of the protein (Ellisdon et al. 2006; Mueller et al. 2009; Reina et al. 2010). A transition in the conformation of mutant ataxin-3 is also known to occur (Bevivino et al. 2001), which might precede its aggregation. Targeting the extended polyQ tract is then a possible therapeutical approach, as shown through the use of polyglutamine binding peptide 1, which prevents the formation of a toxic conformational transition (Nagai et al. 2007). The selective silencing of the mutated form of the protein, using RNAi, also showed promising results (Nóbrega et al. 2013, Conceição et al. 2015). Mice lacking ataxin-3 appear to be phenotypically normal and strategies that involve the suppression of the mutated gene might thus be viable, as indicated by a study in which antisense oligonucleotides specific for human ataxin-3 were able to reduce the protein levels of ataxin-3 without significant inflammation occurring in a mouse model of MJD, but no behavioral or neuropathological analysis was reported (Moore et al. 2017).

Supporting the 'toxic fragment hypothesis', in which aggregation is started by a fragment (containing the extended polyQ tract) that is cleaved from the full length form of mutant ataxin-3, it was found that aggregation requires the removal of the N-terminal of the mutated protein (Haacke et al. 2006). As a result of the aggregation process, more components important for the cellular homeostasis mechanisms also start being aggregated, and an increase in calpain and caspase proteases as well as a decrease in chaperone levels can occur (as seen in some human patients) (Haacke et al. 2006), inducing an increment of the proteolytic cleavage and a reduction on the clearance of misfolded proteins. Identification of calpain cleavage sites in ataxin-3 is essential to understand how its proteolysis is changed in a pathological state and for possible pharmacological treatments. Indeed, the inhibition of calpain proteases by calpastatin blocks aggregation of mutant ataxin-3, thus suppressing formation of NINs (Haacke et al. 2007; Simões et al. 2012; Weber et al. 2017). Use of n-butilydenephthalide has been shown to decrease the levels of active calpains on cell and animal models of MJD by acting on tryptophan metabolism, more specifically through downregulation of 2,3-dyoxygenase (TDO2), and thus showing it can be a potential therapeutic strategy, with motor deficits also being reduced in mice (Rajamani et al. 2017). Increase of chaperone levels by use of heat-shock protein (Hsp) 104 was also successful in halting disease progression in a *Drosophila* model by reducing protein misfolding (Cushman-Nick et al. 2013). Another interesting finding was that the addition of an extra NES was enough to suppress aggregate formation in the nucleus (Antony et al. 2009).
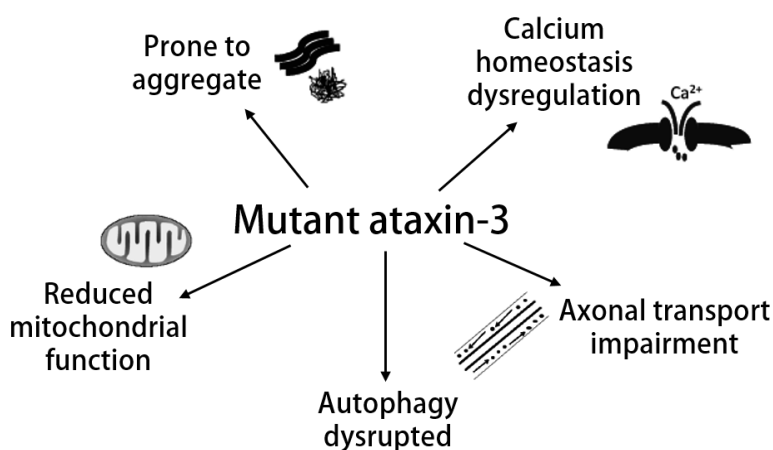

Figure 4 - Examples of mechanisms of pathogenesis in MJD. Adapted from Nóbrega and de Almeida (2012)

NINs do indeed include UPS components and Hsps (Chai et al. 1999; Chai et al. 2 1999), which might impair the normal function of both systems (Rub et al. 2006). Interaction of ataxin-3 with certain proteins can also be altered in its mutated form, as seen in its reduced

binding to VCP/p97 and Rad23, which leads to less protein degradation through the ERAD system as both proteins act in the selection of proteins to be transported, and degraded, in the proteasome (Burnett et al. 2003; Meusser et al. 2005; Zhong et al. 2006; Blount et al. 2015). Aggresome formation is also reduced in MJD as it requires dynein binding to ataxin-3 but the extended polyQ tract impairs it (Burnett et al. 2005).

Transcription factors, such as cAMP response element-binding protein (CREB) or CBP, have also been found in NINs (Yamada et al. 2000, McCampbell et al. 2000), which might also lead to abnormal expression of some genes. Indeed, genes involved in inflammatory processes, nuclear transcription and cell surface-associated components are known to be differentially expressed in MJD (Evert et al. 2001; Evert et al. 2003), leading to an increase in inflammation by microglia and astrocyte activation, reduced neurotrophic support and decreased cell adhesion. The endocannabinoid signaling system is known to be able to modulate inflammation and analysis of cerebellar post-mortem samples of patients showed it was perturbed. Further studies using transgenic (Tg) mice revealed increased levels of CB1 receptors in the Purkinje cell layer and a reduction in the dentate nucleus. Decreased levels of endocannabinoid lipids, such as anandamide and oleoylethanolamide, were also found in the brainstem of the animals, together with increased levels of fatty acid amide hydrolase (FAAH), an endocannabinoid-inactivating enzyme (Rodríguez-Cueto et al. 2016). Cystathionine γ-lyase and neuropeptide Y are found to be decreased in MJD patients, and overexpression of both led to significant improvements in animal models (Snijder et al. 2015; Duarte-Neves et al. 2015). In Tg MJD mice, caloric restriction led to improvements of both motor and neuropathological deficits, due to a rescue of the levels of sirtuin 1 (SIRT1). The same results were also obtained if there was only increased expression of SIRT1 by a gene delivery approach (Cunha-Santos et al. 2016). Utilizing the same model, activation of A_{2A} receptors through chronic caffeine consumption was also shown to attenuate neuronal loss and motor function impairment (Gonçalves et al. 2016).

Studies characterizing microRNA expression and function in MJD are still few but proteins involved in miRNA biogenesis, such as DGCR8, Dicer and FMR1, have recently been shown to be decreased in MJD, together with ones that act in miRNA-mediated gene silencing, including Ago2, TARBP2 and DDX6. In terms of miRNA expression profile, miR9, miR181a and miR494 appear to be downregulated in MJD, with the 3' untranslated region (UTR) of the ATXN3 gene being essential for their role in downregulating the expression of ataxin-3 (Carmona et al. 2017). Other miRNAs which are also abnormally expressed in MJD include miR25b, miR29a and miR34B (Shi et al. 2013).

Finally, DNA damage is increased due to reduced mammalian polynucleotide kinase 3'-phosphatase activity (Chatterjee et al. 2015) as mutant ataxin-3 is thought to be able to inactivate it, which ultimately leads to activation of apoptotic DNA damage-response

pathways (Gao et al. 2015). Mitochondrial damage due to decreased antioxidant activity has also been shown to occur in MJD cellular models (Yu et al. 2009), with activation of apoptotic pathways by cytochrome c being a possible explanation for neuronal dysfunction in certain brain areas (Laço et al. 2012). Calcium homeostasis impairment through activation of an intracellular calcium channel is thought to occur in MJD (Chen et al. 2008), with the use of a Ca^{2+} stabilizer (dantrolene) restoring it and reducing neuronal loss at the pontine nuclei and substantia nigra (Chen et al. 2008).

1.1.2.5.1 Autophagy

Autophagy is an intracellular clearance pathway known to be essential in the degradation of proteins and organelles **(Figure V)**. Several proteins, such as ataxin-3, when mutated are more prone to aggregate and create insoluble-species. In normal conditions, these proteins are degraded through the autophagic pathway, but an impairment of this system is now known to occur in these disorders, thus making it an interesting therapeutic target (Nascimento-Ferreira et al. 2011; Shin et al. 2014).
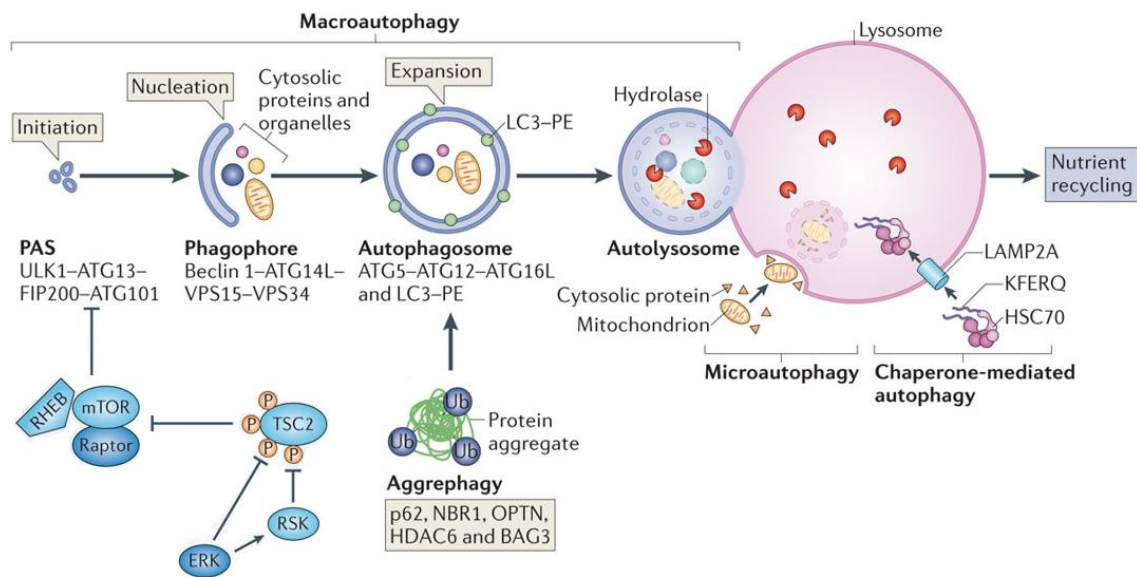
Figure 5 - Macroautophagy schematic pathway, displaying the major proteins involved in this mechanism as well as the regulation of autophagy by the Erk and mTOR pathways. Adapted from Fruman and Rommel (2014), and Kaur and Debnath (2015)

Beclin-1, a key protein in the activation of the nucleation step in autophagy and in the fusion of autophagosomes to lysosomes (Itakura et al. 2008; Sun et al. 2008; Matsunaga et al. 2009; Zhong et al. 2009), has recently been found to interact with ataxin-3 through its

polyQ domain (Ashkenaz et al. 2017). This interaction appears to protect beclin-1 from proteasome-mediated degradation and the presence of several disease-associated proteins that contain polyQ expansion mutations disrupts it, which might explain the reduced levels of beclin-1 seen in MJD. Other important autophagy-related proteins which are known to be altered in MJD include p62, an autophagy substrate, and LC3B-II, a protein found on the luminal and cytosolic surfaces of mature autophagosomes, thus being a good indicator of the number of autophagosomes (Nascimento-Ferreira et al. 2011; Onofre et al. 2016).

Therapeutic strategies for neurodegenerative disorders based on autophagy activation have reported promising results. Overexpression of beclin-1 was enough to stimulate the autophagic flux and promote functional benefits in *in vitro* and *in vivo* MJD models (Nascimento-Ferreira et al. 2011). Another approach to enhance autophagy consists in modulating the activity of mammalian target of rapamycin (mTOR), an inhibitor of autophagy. For example, inhibitors of mTOR have been shown to reduce toxicity in fly and mouse models of HD by increasing autophagy and thus attenuating huntingtin accumulation (Ravikumar et al. 2004). Extracellular signal-regulated kinase (Erk) activation is known to indirectly modulate the activity of mTOR and thus inhibit autophagy by preventing ULK1 activation by phosphorylation (Kamada et al. 2000; Kanazawa et al. 2004; Hosokawa et al. 2009; Jung et al. 2009). This modulation occurs through Erk-derived inhibition of TSC1, part of the TSC complex, which acts as a GAP for Rheb GTPase, an activator of mTOR (Inoki et al. 2002; Ma et al. 2005).

1.1.2.6 Current therapeutic strategies

Although no definitive treatment exists, some of the symptoms can be suppressed or reduced. Dopamine replacement therapy using levodopa or a dopamine agonist has been successful in cases displaying parkinsonian features, with dystonia and bradykinesia also being reduced through this approach (Tuite et al. 1995; Paulson et al. 2007). Sleep disturbances can be reduced with clonazepam or modafinil, while dysarthria and dysphagia can be ameliorated by the clinician as he can give advice to the patient on what and how he should eat to avoid complications. Double vision derived from impaired ocular motility can be helped with a prism, while occupational and physical therapy is essential to reduce gait symptoms as worsening of them can lead to use of walkers or wheelchairs (Paulson et al. 2007).

Medical counseling should be given not only to patients but also to possible candidates, as people that have a family history with MJD. Presymptomatic testing already exists and psychological and ethical guidance is essential to help in the decision-making

process that involves treatment and parenthood issues (Sequeiros et al. 1998; Drüsedau et al. 2004; Bettencourt and Lima 2011; Schuler-Faccini et al. 2014).

Recently, the number of clinical trials testing the potential therapeutic effect of certain compounds in MJD patients has been increasing, but results are still lacking for most (ClinicalTrials.gov identifiers NCT01811706, NCT00992771, NCT02147886 and NCT01104649). Still, trials using valproic acid (no ClinicalTrials.gov identifier found) and lithium carbonate (identifier NCT01096082) have shown very promising results (Saute et al. 2014; Lei et al. 2016) and further tests with these compounds are expected to occur. However, no therapy that could delay or stop disease progression has been developed so far.

1.2 Multipotent mesenchymal stromal cells

Mesenchymal stromal cells (MSCs) are multipotent progenitor cells capable of giving rise to tissues from the mesenchymal lineage. Their role was initially thought to be mainly due to the replacement of old and damaged cells (Wakitani et al. 1995; Pittenger et al. 1999) but, more recently, they are thought to mainly induce their effects by influencing neighbor cells by secreting bioactive factors or by inducing their secretion in host cells, thus being able to modulate the immune system and to promote tissue repair (Gao et al. 2001; Tremain et al. 2001). These characteristics led to their wide use in therapeutic approaches targeting several diseases with promising results (Woodbury et al. 2000; Chen et al. 2006).

1.2.1 History and nomenclature

MSCs' existence had been theorized since the nineteenth century but they were only identified in 1970, when plastic-adherent cells isolated from the bone marrow of guinea pigs were found to be able to form colonies of cells with fibroblastic morphology (Friedenstein et al. 1970). In the 1980s and 1990s, MSCs continued to be described and found to be a very heterogeneous population of cells with multipotent properties (Owen et al. 1988; Caplan et al. 1991), with quick expansion capacity (Moscoso et al. 2005) turning them very appealing for research, especially for therapeutic use.

Though MSCs are most commonly known as mesenchymal stem cells (MSCs), term coined in 1991 by Caplan (Caplan et al. 1991), and previously by many other different terminologies such as mesenchymal progenitor cells, colony-forming fibroblastic cells, stromal fibroblasts, marrow stromal stem cells and marrow stromal cells, in 2005, in order to standardize the terminology used throughout the scientific community, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) clarified

the nomenclature of these fibroblast-like, plastic-adherent cells, as multipotent mesenchymal stromal cells, as they do not always meet the stem cell criteria. Still, the acronym MSC can be used if the scientifically correct designation is mentioned (Horwitz et al. 2005).

1.2.2 Characterization

MSCs' populations are highly heterogeneous, not only in terms of differentiation capacity but also in terms of expression of surface markers they can express as there is no specific marker to distinguish purified populations of MSCs from other cells. Therefore, isolation protocols are based on the exclusion of possible contaminants, such as cells from the immune system and the hematopoietic stem cell (HSC) lineage, through negative selection of antigens specifically expressed by these, and also by positive selection using markers which MSCs appear to usually express (Deans et al. 2000; Bianco et al. 2001; Baddoo et al. 2003; Baksh et al. 2004; Peister et al. 2004).

Moreover, MSCs isolation and expansion methods widely differed between investigators, with comparison of results being difficult. In order to surpass these problems, minimal criteria to define MSCs were ultimately defined, with the Mesenchymal and Tissue Stem Cell Committee of the ISCT proposing three: plastic-adherence in standard culture conditions, surface expression of CD105, CD73 and CD90 (95%≥) and absence of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR (2%≤) markers, and finally, the capacity to differentiate into osteoblasts, adipocytes and chondroblasts *in vitro* (Dominici et al. 2006). These criteria were only appliable to human MSCs as those from other animals might display a different surface antigen expression, as seen in murines, where even different strains from the same species can show some differences (mostly in terms of CD106, CD34 and Sca-1) (Phinney et al. 1999; Sun et al. 2003; Tropel et al. 2004; Peister et al. 2004; Kolf et al. 2007).

1.2.3 MSCs' mechanism of action

1.2.3.1 Differentiation potential

MSCs' differentiation potential was initially thought to include only some of the most common lineages derived from the mesoderm such as bone, cartilage and adipose tissue. A hierarchical model of differentiation has been proposed for MSCs, in which several steps with small transcriptional changes occur, inducing the fate of a given cell into a more distinct pathway at each point. This could explain the already mentioned heterogeneity found within MSC populations, in which not all the cells are able to form osteoblasts, chondroblasts and

adipocytes (Karystinou et al. 2009). Differences in these levels also occur depending on the local and donor from which they are isolated (De Bari et al. 2008).

Later reports also indicated that MSCs are able to form tissues as diverse as muscle, hematopoietic-supporting stroma or even neurons (Wakitani et al. 1995; Pittenger et al. 1999; Woodbury et al. 2000; Reyes et al. 2001; Lu et al. 2004; Bertani et al. 2005). However, regarding neuronal fate, there is still some debate as they might not be able to become completely functional neurons because they might not be able to generate action potentials due to absence of certain ionic channels, even if they express typical neural markers, such as β-tubulin III, glial fibrillary acidic protein (GFAP), microtubule-associated protein (MAP) 2 and nestin (Alessandri et al. 2004; Tondreau et al. 2004). Thus, in addition to classical immunocytochemical and molecular characterization, electrophysiological testing has been tried to be performed (Wislet-Gendebien et al. 2005; Mareschi et al. 2006), but some technical limitations have challenged a definitive confirmation that MSCs can differentiate into mature neurons and establish neuronal circuits with neighboring cells (Liu et al. 2013). If this would be possible, it would allow MSCs to replace damaged neurons and attenuate synaptic dysfunctions that might originate from neurodegeneration.

1.2.3.2 Cell fusion

MSCs have also shown to be able to fuse with neuronal cells, adopting their phenotype. This mechanism might also be responsible for some of their neuroprotective effects, as in some cases MSCs could fuse with the host neuronal cells, as was observed with the Purkinje cells of host animals injected with MSCs (Chen et al. 2011, Kemp et al. 2011). While in these reports the authors referred to have some doubts whether fusion was indeed occurring since the Purkinje cells could be simply expressing the MSCs markers due to transfer of molecules into Purkinje cells, it was recently unequivocally shown in a SCA1 mice model that fusion in fact occurs. Interestingly, no fusion events were observed in wild-type or non-symptomatic Tg animals transplanted with MSCs (Huda et al. 2016). Still, as it occurs very rarely, this process does not appear to account significantly for MSCs' overall effect (Terada et al. 2002, Huda et al. 2016).

1.2.3.3 Paracrine activity

MSCs can also exert their protective effects through paracrine signaling mediated by the secretion of substances such as cytokines and neurotrophic factors that can act at several different cellular mechanisms in injured neuronal tissues (Tremain et al. 2001; Caplan et al. 2006).

1.2.3.3.1 Immunomodulation

Low immunogenicity is one of the key aspects that make MSCs ideal candidates for allogenic transplantations and treatment of autoimmune diseases, such as multiple sclerosis or Crohn's disease (Bartholomew et al. 2002; Sun et al. 2003; Djouad et al. 2009). Moreover, they express major histocompatibility complex class I vestigially (MHC-I), existing a high probability that T cell activation by MHC-I does not occur (Haynesworth et al. 1992; Le Blanc et al. 2003; Klyushnenkova et al. 2005). They do not express MHC class II (MHC-II) or costimulatory molecules such as CD40, CD80 or CD86. However, by a non-MHC-dependent way, they can modulate T cell activity and proliferation of these immune cells by a cell-cell interaction pathway (Krampera et al. 2003) or through the release of soluble factors (Le Blanc et al. 2003). An anti-inflammatory T helper 2 cell response can thus be promoted, through increased secretion of IL-4, while pro-inflammatory responses, mediated by T helper 1 cells, are inhibited by a decreased secretion of interferon-γ (IFN-γ) (Djouad et al. 2007). T cell activation can also be suppressed by the Notch ligand Jagged-1, which is known to be expressed by human MSCs (Liotta et al. 2008), or by the production of indoleamine 2,3-dioxygenase (IDO) or nitric oxide (NO) as they appear to suppress Stat5 phosphorylation and thus T cell proliferation (Munn et al. 1999; Mellor et al. 1999; Sato et al. 2007). Finally, the expression of the programmed death-1 (PD-1) receptor and its ligands PD-L1 and PD-L2 can also mediate this immunosuppression ability of MSCs by modulating the expression of several cytokine receptors (such as IL-12R) and other molecules also involved in cytokine signaling, such as the signal transducer and activator of transcription 5A (STAT5a) and 5B (Stat5b) (Augello et al. 2005).

Monocyte maturation into dendritic cells (DCs) is crucial in the transition from an innate response to an adaptive response by the presentation of antigens to B cells, with type-1 dendritic cells (DC1) being involved in T cell stimulation and type-2 dendritic cells (DC2) appearing to mainly fight infections (Djouad et al. 2007). MSCs can inhibit this pathway (Aggarwal et al. 2005; Jiang et al. 2005) alongside with decreased DC functionality with a consequent decreased release of tumor necrosis factor alpha (TNF-α) by DC1 and increased release of IL-10 secretion by DC2 (Djouad et al. 2007). Macrophages' toll-like receptor 2 (TLR2)/NF-κB signaling is also decreased by MSCs through secretion of tumor necrosis factor-α stimulated gene/protein 6 (TSG-6) (Choi et al. 2011). Natural killer (NK) cell proliferation can also be impaired (Poggi et al. 2005; Sotiropoulou et al. 2006), together with their cytotoxic activity, as they are less activated due to a downregulation of their surface receptors NKp30 and NKG2D (Spaggiari et al. 2006).

The possibility that an immune response is elicited by MSCs in certain conditions (Griffin et al. 2013) cannot be discarded and is indeed a hot topic now, though more evidences are clearly needed. Some authors showed that cultured MSCs pre-conditioned

with cytokines IFN-α and IFN-γ acquire a pro-inflammatory phenotype as these cytokines activate TLR3 and TLR4 and both are expressed by MSCs (Romieu-Mourez et al. 2009). More research done in *in vivo* settings is then definitely needed as MSCs can face an environment containing these molecules, and some studies in fact reported worsening of some immunological conditions when using MSCs (Chen et al. 2010; Grigoriadis et al. 2011). Nonetheless, most of the studies performed so far point for an immunomodulation capacity that counters pathological conditions.

1.2.3.3.2 Supportive role through secretion of neurotrophic factors

MSCs are known to secrete neurotrophic factors such as brain-derived neurotrophic factor (BDNF) (Auffray et al. 1996; Labouyrie et al. 1999; Chen et al. 2002), insulin-like growth factor 1 (IGF-1) (Sadat et al. 2007), nerve growth factor (NGF) (Chen et al. 2002; Crigler et al. 2006), glial cell line-derived neurotrophic factor (GDNF) (Kurozumi et al. 2005), vascular endothelial growth factor (VEGF) (Sadat et al, 2007; Tomar et al. 2010), platelet-derived growth factor (PDGF) (Suga et al. 2009) and hepatocyte growth factor (HGF) (Chen et al. 2002). These factors are then thought to interact with other cells directly from the interstitial space or by entering the cells through gap junctions or tunneling nanotubules, with the later also allowing passage of bigger cellular structures such as polyribosomes and mitochondria (Gerdes et al. 2008; Sanchez et al. 2017).
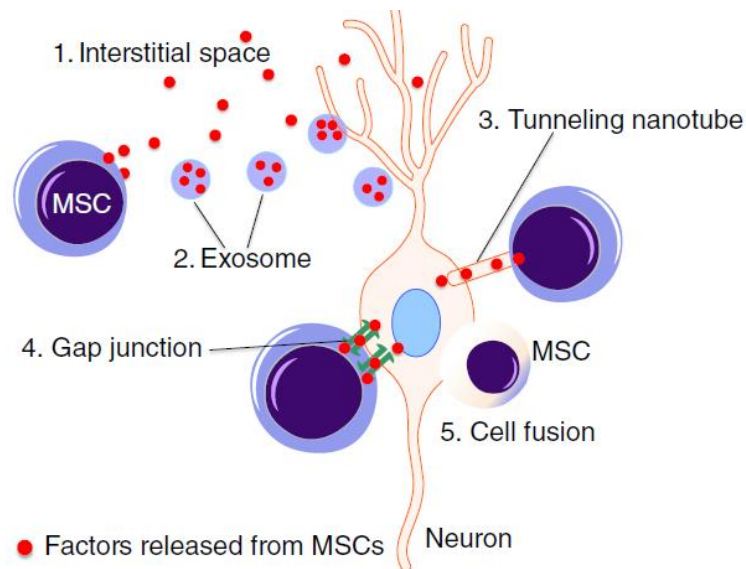

Figure 6 - Possible pathways for transfer of factors from MSCs to neurons. Adapted from Nakamura et al. (2004)

Intracellular changes induced by the mentioned factors and others derive from activation of diverse cellular pathways, such as the Erk/mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt pathways, which regulate features as diverse as protein synthesis, differentiation, cell division or even cell death. Intercommunication between different cellular pathways is common, thus becoming a hard task to know exactly which one is mediating an observed effect (Raman et al. 2007; Hoeffer et al. 2010; Jung et al. 2010; Aoki et al. 2011; Laplante et al. 2012).

Certain cellular pathways, such as the Akt pathway, have been reported to be differentially activated or to regulate proteins essential to the pathogenesis of certain neurodegenerative disorders, including HD and SCA1 (Humbert et al. 2002; Chen et al. 2003). Thus, modulating the activity of cellular pathways by using some of the previously mentioned factors could be a potential therapeutic approach. Still, delivery of certain factors to the brain in substantial quantities is challenging due to the existence of the blood-brain barrier and thus the use of MSCs as delivery vehicles is a promising approach to produce the desired effects in each specific case. Conditions such as hypoxia, inflammation or even the presence of the content from apoptotic cells are known to influence this process (Chen et al. 2002; Rosova et al. 2008; Giunti et al. 2012). Other approaches involve the engineering of the overproduction by MSCs of some of these factors or the use of MSCs' conditioned medium, with some studies already reporting benefits (Kurozumi et al. 2005; Timmers et al. 2008; Wilkins et al. 2009; Bai et al. 2012; Suto et al. 2016).

1.2.3.3.3 Extracellular vesicles in the modulation of MSCs' action

MSCs can also mediate beneficial effects through the release of micro-vesicles, such as exosomes, which can contain some of the previously mentioned factors but also other molecules such as RNAs (Théry et al. 2009; Lai et al. 2010). MSCs-derived exosomes can then act on other cells, even at distant locations, by releasing their contents on the interstitial space or by entering the cells and thus inducing neuroprotective effects (Simons et al. 2009).

Recent studies have focused on mapping MSCs' exosome-content to unravel how they might affect other cells and if they are influenced by the surrounding environment. Notably, a recent proteomic study identified several proteins specially enriched in the extracellular vesicles derived from MSCs, in comparison with their cellular content. Functional pathway characterization of the identified proteins showed they are known to be able to modulate functions such as inflammation, angiogenesis, blood coagulation, extracellular matrix remodeling and apoptosis (Eirin et al. 2016). Pre-clinical studies only using these micro-vesicles on the treatment of certain pathologies have reported benefits

similar to those observed when using MSCs themselves (Lai et al. 2010; Kordelas et al. 2014).

1.2.4 Therapeutic use of MSCs

Interest in cell-based therapy using MSCs surged due to their characteristics: they are easily obtained from adult tissues, their *ex vivo* expansion is relatively quick and cryopreservation is possible as it doesn't affect their differentiation and expansion potential (Baddoo et al. 2003; Moscoso et al. 2005). Their capacity to differentiate into a wide range of tissues led to the initial therapeutic trials with MSCs being focused on their cell-replacement ability to treat disorders involving bone defects, such as osteogenesis imperfecta (Bruder et al. 1998; Horwitz et al. 1999; Le Blanc et al. 2005), or ischemic injury to the heart (and later, to the brain) (Amado et al. 2005; Bang et al. 2005). But further studies soon after described that their mechanism of action can also include immunosuppression and release of protective factors, with their engraftment and differentiation potential apparently occurring only in a minor fraction of the used cells, leading to the possibility that their major effects derive from their paracrine signaling (Gao et al. 2001; Caplan et al. 2006; Liu et al. 2009; Wang et al. 2012).

1.2.4.1 MSCs in neurological disorders

The possibility of using MSCs as therapeutic agents in the treatment of neuronal disorders was first proven to be possible in 1998 as it was shown that after being injected in rats they have the capacity to engraft in the brain, with no signs of inflammatory or rejection reactions (Azizi et al. 1998). These findings, together with some reports of their differentiation into neural-like cells after transplantation (Kopen et al. 1999; Woodbury et al. 2000), led to the first pre-clinical tests in animal models of brain ischemia (Zhao et al. 2002; Kurozumi et al. 2005).

Intravenous or direct injection into the affected brain area showed significant improvements in motor coordination functions, with the effects probably coming from factors secreted by the MSCs and not through their differentiation as they were only able to persist for a few weeks but with the benefits persisting for at least 5 months after MSC delivery (Chen et al. 2003; Li et al. 2005). Further studies also using intravenous (Bang et al. 2005) or intraventricular (Zhang et al. 2008, Chen et al. 2012) injections also produced functional recovery, and rehabilitation programs seemed to improve this even more if coupled with this treatment. This combination therapy has also led to benefits in several trials with acute or chronic spinal cord injury patients (Yoon et al. 2007; Deda et al. 2008).

In terms of neurodegenerative disorders, MSCs have also been used as a therapeutic strategy, with amyotrophic lateral sclerosis (ALS) probably being the disease in which more studies have been done. Systemic delivery of MSCs in animal models of ALS has showed both neuropathological and motor benefits similar to more invasive approaches (Zhao et al. 2007; Morita et al. 2008; Uccelli et al. 2012), and with the main mechanism responsible for the cells' effect involving the action of some of its secreted factors, such as GDNF (Pastor et al. 2011; Marconi et al. 2013) and VEGF (Boido et al. 2014), but also their capacity to migrate towards injured motor neurons, with local inflammation generally being reduced (Boucherie et al. 2009; Kim et al. 2010; Uccelli et al. 2012; Kwon et al. 2014). Several clinical trials using MSCs in ALS patients have been performed and the published results indicate that while this approach appears to be safe and to delay disease progression (Mazzini et al. 2006; Mazzini et al. 2010; Karussis et al. 2010), repeated administration of MSCs might be needed in order to achieve a sustained effect (Staff et al. 2016; Syková et al. 2016).

Furthermore, promising results were also obtained in pre-clinical studies using MSCs in other neurodegenerative disorders, such as Parkinson's (PD), Alzheimer's (AD) and HD (Lu et al. 2005; Weiss et al. 2006; Sadan et al. 2008; Lee et al. 2009; Lee et al. 2010; Lin et al. 2011). In terms of clinical trials, there are only a few results published in which MSCs were used in Parkinson's patients, with no acute side effects derived from the therapy being reported (Venkataramana et al. 2010, Venkataramana et al. 2012). Similarly, the main mechanism of action of MSCs in these disorders is also thought to derive mainly from their paracrine activity (Park et al. 2008; Lin et al. 2011; Garcia et al. 2014; Kim et al. 2015; Song et al. 2015; Oh et al. 2016; Pollock et al. 2016).
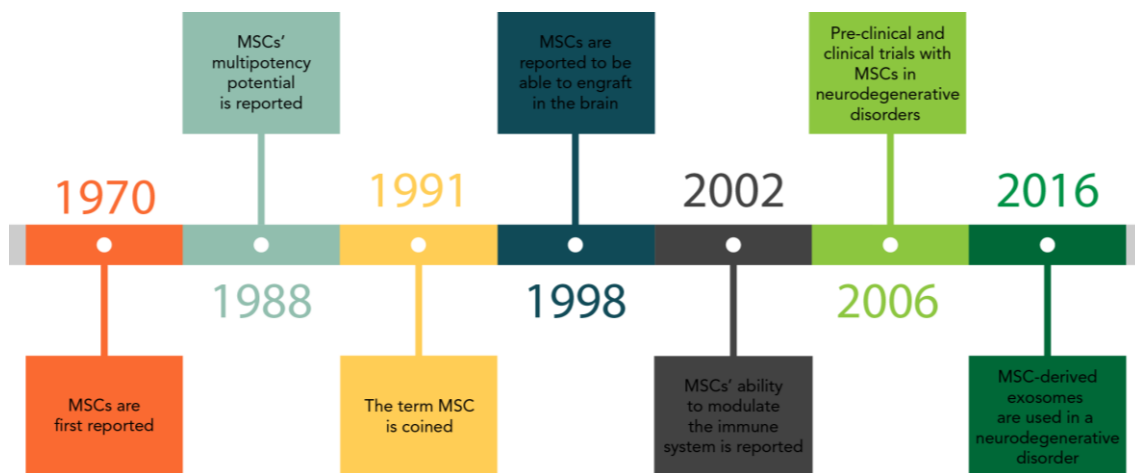

Figure 7 - Timeline of MSCs' history and use in neurodegenerative disorders.

Targeting of malignant brain tumors, such as gliomas, has also been hypothesized, with pre-clinical studies involving delivery of MSCs overexpressing IFN-β through the internal carotid artery leading to a decreased rate of tumor growth and with increased survival (Nakamizo et al. 2005). However, no clinical trials involving this strategy have been done.

1.2.4.2 MSCs in SCAs

Even though the first therapeutic approaches performed in SCAs were based on the use of RNAs to block the mutated form of the affected protein (Xia et al. 2004), the wide expansion of the research on MSCs soon led to their use in pre-clinical trials in order to test their neuroprotective effects, together with a few clinical trials and with future trials already scheduled to occur.

One of the first SCA animal models in which MSCs were used was a pharmacologically induced ataxic mouse. The authors injected cytosine β-D-arabinofuranoside intraperitoneally for three consecutive days, and intravenous injection of MSCs diminished the cerebellar atrophy, number of apoptotic cells and motor deficits of the animals, 8 weeks after transplantation (Zhang et al. 2011). Soon after, as Tg SCA animal models were developed, they quickly started to be more used, as they better mimic the pathological process observed in humans than the pharmacologically induced. Using *Lurcher* mutant mice, MSCs have been found to improve motor function if injected in the cerebellum (Jones et al. 2010). Motor deficits were also attenuated in a Tg mouse model of SCA2 after multiple intravenous injections of bone-marrow derived MSCs, but no benefits were observed when performing multiple intracranial injections. Both strategies could reduce the loss of Purkinje cells but more outcomes were reported with the intravenous delivery, which also lead to better engraftment of the cells in the brain (Chang et al. 2011). In SCA1 knockin mice, spinal motor neuron loss was attenuated with a single intrathecal injection of bone marrow-derived MSCs; moreover, peripheral neuronal pathology could also benefit from such a strategy (Mieda et al. 2015).

Intrathecal injection of MSCs in SCA1-Tg mice at 5 weeks of age (before symptoms occur) led to morphological improvements, since treated animals had a thicker cerebellar molecular layer and a single-layer of Purkinje cells in comparison with the control animals, which showed a very thin molecular layer and a multi-layered disorganization of Purkinje cells. In this study, MSCs were shown to migrate towards the cerebellum. Motor function was also improved from 11 to 20 weeks of age as tested using the rotarod test, with the treated animals showing results similar to wild-type mice in the rotarod test (Matsuura et al. 2014).

In MJD, both human umbilical cord-derived and mouse bone marrow-derived mesenchymal stem cells have been shown to induce neuropathological and motor benefits

in mouse models of the disease (Li et al. 2017; Miranda et al., *submitted*). Still, accordingly to what has been shown for other SCAs and in clinical studies, the effects only appeared to be sustained when applying multiple dosages of MSCs, as a single intracranial injection of MSCs only induced transient benefits (Miranda et al., *submitted*).

Despite the few number of pre-clinical studies, clinical trials have been testing the safety/efficacy of using MSCs to treat SCAs, and some reports already came out. In one study, intrathecal injection of MSCs in 14 SCA patients showed that it can indeed be a strategy to delay the progression of the neuronal deficits derived from the pathology since patients showed motor improvements 1 month after treatment. Still, benefits appear to be transient as 6 of the 14 of the patients showed disease progression occurring after around 4 months of stabilization (Dongmei et al. 2011). Another trial used 16 SCA1, SCA2 or SCA3 patients and MSCs were delivered both intravenously and intrathecally, and treated patients showed improvements in both quality of life and movement, while no serious side effects were observed for at least the first 6 months after the treatment. However, MSCs' effect in these trials also seemed to be transient, as 12 months after treatment there were no significant improvements observed in 3 of the 10 patients which had shown benefits in the first 6 months after treatment (Jin et al. 2013). Other recent clinical trials have reported similar findings regarding the safety (Tsai et al. 2017) and transitory action (Miao et al. 2015) of MSCs in MJD. In the meanwhile, more clinical trials have been completed but with no results reported (identifiers NCT01958177, NCT01649687 and NCT01489267), while others are currently recruiting (identifier NCT02540655, phase 2 clinical trials). Future clinical studies should focus not only on the feasibility of repeated injections for sustained benefits but also for the appropriate timing, dosage and best method of injection to be used, while also performing longer follow-ups of all patients to evaluate treatment efficacy and safety, and with placebo-controls being used.

Finally, cell-free approaches (conditioned medium or exosomes) might also be considered, as these might induce similar benefits as when using MSCs, as seen in a mouse model of SCA1 (Suto et al. 2016). In this approach, some of the complications derived from a cell-based strategy could thus be avoided but further studies should be done in order to identify the molecular effectors of the reported benefits.

Objectives

The main objective of the present study was to evaluate the effect of MSCs in the modulation of autophagy in *in vitro* and *in vivo* models of MJD and to unravel the putative therapeutic benefits resulting from autophagy modulation in this disease.

In order to accomplish that, through *in vitro* co-culture studies, we investigated the autophagic effects of mouse bone marrow-derived MSCs in a cellular MJD model, consisting in Neuro2-a cells expressing the mutant form of ataxin-3, by evaluating the levels of several proteins directly involved in the autophagic pathway and known to be abnormally expressed in MJD. Moreover, we evaluated the levels of proteins known to regulate the activity of the autophagic pathway and of soluble mutant ataxin-3.

Additionally, we also investigated the effects of repeated systemic injections of bone marrow-derived MSCs in transgenic MJD mice (Torashima et al. 2008), which express the mutant form of ataxin-3 in the cerebellum, in terms of autophagy, ataxin-3 levels and its ability to induce sustained motor functional benefits in this very severe model of MJD.

2 – Materials and methods

2.1 Cell cultures

2.1.1 Mesenchymal stromal cells

Mesenchymal stromal cells were previously isolated from the bone marrow of 6 to 8 weeks-old wild type C57BL/6 mice of both genders, sorted with CD45 antibody (e-Biosciences) for positive cells exclusion, and were expanded in DMEM/F-12 (Gibco) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), 20 ng/ml epidermal growth factor (EGF, PeproTech), 20 ng/ml fibroblast growth factor (FGF, PeproTech) and 2% B-27 (Gibco), at 37°C in 5% CO_2/air atmosphere and frozen. For the co-culture experiments, MSC were thawed and cultured in DMEM/F-12 (Gibco) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco), and incubated at 37°C in 5% CO_2/air atmosphere. All the experiments performed in this study used MSCs between passage 11 and 15.

2.1.2 Neuro-2a cells

Mouse neuroblastoma cell line (Neuro-2a cells, N2a) was obtained from the American Type Culture Collection cell biology bank (CCL-131) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco) (complete medium) at 37°C in 5% CO_2/air atmosphere.

Infection was performed using lentiviral particles expressing human full-length wild-type ataxin-3 with 27 glutamines or mutant ataxin-3 with 72 glutamines (Alves et al. 2008), which were produced in human embryonic kidney (HEK) 293T cells using a four-plasmid system (de Almeida et al. 2002).

2.2 Multipotency assay

Mouse Mesenchymal Stem Cell Functional Identification Kit (R&D Systems, catalog #SC010) was used for multipotency assessment. For adipogenesis and osteogenesis, cells were first seeded until 100% and 70% confluency, respectively, and media was then replaced by its respective differentiation medium. For chondrogenesis, a cell pellet of 250.000 cells was cultured in chondrogenic differentiation medium. For every condition, medium was changed every 3 days and cells were kept in culture for 21 days. Adipocytes and osteocytes were then fixed with paraformaldehyde 4% for subsequent immunocytochemistry staining. Chondrogenic pellets were fixed with zinc formalin solution overnight, embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek) and cryosectioned. A sodium citrate solution (10mM,

pH 6) was used for antigen retrieval, followed by immunohistochemical staining. Immunohistochemistry staining procedures were done according to the manufacturer's protocol. Primary antibodies used included goat anti-mouse fatty acid binding protein (FABP-4) for adipocytes, goat anti-mouse osteopontin for osteocytes and sheep anti-mouse collagen-II for chondrocytes, with all being included in the kit used. Secondary antibodies used included 488-conjugated donkey anti-goat (Invitrogen, cat. # A-11055) and 568-conjugated donkey anti-sheep (Abcam, cat. #ab175712). Cells were mounted on slides and visualized with a fluorescent microscope (Axio Imager Z2, with ApoTome2 and Stereo-Investigator, Zeiss).

2.3 Co-culture experiment design

Co-culture experiments of N2a cells expressing WT (N2A-WT) or mutant form (N2a-Mut) of ataxin-3 with MSCs were performed using cell culture inserts with pore size of 1.0 µm (Millipore, Ref. MCRP12H48). N2a cells were first seeded to the bottom chambers of 12-well plates (Corning® CellBIND®, Ref. CLS3336 SIGMA) at 75.000 cells/well. 24 hours later, MSCs were seeded on the top chambers at 40.000 cells/insert. The co-cultures lasted for 48h and the medium was changed after 24h.

2.4 Metabolic activity assay

Metabolic activity was assessed through the Alamar Blue assay. After the 48h co-culture protocol, transwell inserts containing MSCs were removed and the N2a cells were incubated with Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) medium containing 10% (v/v) Alamar Blue dye. Cells were incubated for 40 minutes at 37°C and the absorbance of the medium was measured at 570nm and 600nm. Metabolic activity was calculated as a percentage of the N2a WT-ATXN3 cells such as: metabolic activity (% of N2a WT-ATXN3 cells) = [(A570-A600) of cells*100 / (A570-A600) of N2a WT-ATXN3 cells)]. A blank control consisting of wells with only DMEM/F-12 medium containing 10% (v/v) Alamar Blue dye was run and the values obtained were subtracted from those obtained in the various groups.

2.5 Protein extraction and Western Blot

For N2A cell lysate collection, cells were washed with ice-cold PBS 1x twice and RIPA buffer (50 mM Tris HCl, pH 8, 150 mM NaCl, 1% NP-40 (nonyl phenoxypolyethoxylethanol), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate)

supplemented with protease inhibitors (Roche Diagnostics GmbH), 1 mM PMSF (phenylmethylsulphonyl fluoride, Sigma-Aldrich), 1mM sodium orthovanadate (Sigma-Aldrich), 5mM sodium fluoride (Sigma-Aldrich) and 10 µg/mL DTT (dithiothreitol, Sigma-Aldrich) was added. Cells were scratched mechanically, collected in eppendorfs, centrifuged at 5.000 g for 30 minutes at 4°C and the supernatant was stored at -20°C until use. Cerebellar tissue from Tg MJD mice was lysed in identical RIPA buffer, followed by sonication of two series of 4 s pulses. Tissue lysates were stored at -20°C until use.

Protein quantification was performed using the Bradford protein assay reagent (Bio-Rad). Forty µg (for *in vitro* studies) or fifty µg of protein (for *in vivo* studies) were separated on SDS-PAGE gels (10 or 15% running, respectively, and 4% stacking) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Immobilon®-P Millipore). Membranes were blocked by incubation in a 5% non-fat milk powder in 0.1% Tween 20 in Tris buffered saline (TBS-T) solution, and incubated overnight at 4°C with primary antibodies against mouse monoclonal anti-ataxin-3 antibody (1H9; 1:3000; #5360 Merck Millipore), mouse monoclonal anti-beclin-1 antibody (1:1000, BD Biosciences), rabbit monoclonal anti-p62 antibody (1:1000; #5117 Cell Signaling Technology), rabbit monoclonal anti-LC3B antibody (1:1000; #2775 Cell Signaling Technology), mouse monoclonal anti-ERK antibody (C-9, 1:1000; sc-514302 Santa Cruz Biotechnology), mouse monoclonal anti-p-ERK (E-4, 1:1000; sc-7383 Santa Cruz Biotechnology), rabbit monoclonal anti-mTOR (1:1000; #2972 Cell Signaling Technology), rabbit monoclonal anti-p-mTOR (Ser2448, 1:1000; #2971 Cell Signaling Technology), rabbit polyclonal anti-HA (1:1000, Abcam), mouse monoclonal anti-β-tubulin antibody (1:10000; T7816 Sigma-Aldrich), and mouse monoclonal anti-β-actin antibody (AC-74; 1:5000; A5316 Sigma-Aldrich). Protein bands were visualized after incubation with the correspondent alkaline phosphatase-linked anti-mouse or anti-rabbit secondary antibody (1:10000, Thermo Scientific Pierce) and enhanced chemifluorescence substrate (ECF) (GE Healthcare) through chemifluorescence imaging (VersaDoc Imaging System Model 3000, Bio-Rad).

Semi-quantitative analysis was done based on the optical density of the scanned membranes (Fiji, ImageJ; version 1.51; Schindelin et al. 2012; Schneider et al. 2012). Normalization of the specific optical density was performed using the amount of β-actin or β-tubulin loaded into each individual lane of each gel. Furthermore, phosphorylated proteins were normalized with the total amounts of the corresponding proteins.

2.6 *In vivo* experiments

2.6.1 Transgenic mouse model

The transgenic (Tg) MJD mouse model used consisted in animals with a C57BL/6 background expressing the N-terminal truncated human ataxin-3 with 69 glutamine repeats and with an N-terminal hemagglutinin (HA) epitope in the cerebellar Purkinje cells, driven by a L7 promoter (Oue et al. 2009; Torashima et al. 2008). Animals were maintained in the animal house facility of the Center for Neuroscience and Cell Biology (CNC) of the University of Coimbra through backcrossing of heterozygous males with C57BL/6 females (Nascimento-Ferreira et al. 2013), with genotype being confirmed by PCR. Animals were housed in temperature-controlled rooms and maintained on a 12h light/dark cycle with food and water available *ad libitum*. The animals were sacrificed by decapitation under anesthesia [mixture of ketamine (100 mg/kg, Clorketam 1000, Vétaquinol) and xylazine (10 mg/kg, Rompun, Bayer)], one week after the final behavioral tests have been performed. All animal experiments were in accordance with the European Community Council Directive (86/609/EEC) for the care and use of laboratory animals, and were approved by the Responsible Organization for the Animals Welfare (ORBEA_66_2015/22062015) of the Faculty of Medicine and CNC of the University of Coimbra.

In the present study two groups of animals were used: MSC-treated mice (MJD+MSC, systemic transplantation of MSC through the tail vein, n=9 females) and non-treated mice (the control group) (NT-MJD, saline injection with Hank's Balanced Salt Solution (HBSS) as control, n=9 females).

2.6.2 Intravenous injections of MSC

Mice were transplanted 1, 3, 5 and 9 weeks after the start of the experiment (week 0). The therapy began at 4.5-6 weeks of age and the number of cells injected at each time-point was $4.5-8 \times 10^7$/kg. Controls received an injection of a saline solution (Hank's Balanced Salt Solution).

2.6.3 Behavioral assessment

Motor tests were performed after acclimatization at 0, 2, 4 and 10 weeks after the start of the experiment (week 0). Animals were trained for each test, before the experiments started.

2.6.3.1 Rotarod

To assess for motor coordination and balance, a rotarod apparatus Letica Scientific Instruments, model LE 8200 (Panlab, Barcelona, Spain), was used. An acceleration speed from 4 to 40rpm in 5 minutes was used, and the latency to fall (amount of time the animal could stand in the rotated wheel) was recorded. Each animal performed 4 trials for each time point, with a minimum rest period of 15 minutes between each trial, for three consecutive days. The results show the average of the results of the first two days.

2.6.3.2 Beam walking test

Motor coordination and balance of the animals were also evaluated by assessing their ability to cross a narrow beam to reach an enclosed escape platform. The beam used was square, with 1m long and 9mm wide. The beam was placed horizontally and the time the animal took to cross it towards the platform was recorded. Each animal was subjected to 2 trials for each time point, with a minimum rest period of 15 minutes between each trial.

2.6.3.3 Footprint

The footprint test was performed to analyze gait. The animals' hind- and fore-feet were coated with blue and red nontoxic paints respectively, and they were allowed to walk along a fresh sheet of green paper 100 cm long and 10 cm wide runaway in an apparatus with 15 cm high walls. For each animal, a new fresh sheet of green paper was used.

Overlap footprint was measured to evaluate uniformity of step alternation. The distance between the centers of the hind footprint and the preceding front footprint was measured. Hind base width was measured as the distance between the left and right hind footprints. A sequence of 5 consecutive steps was used in order to perform 5 measurements for each animal. Footprints at the beginning and end of the run were excluded of the analysis as the animals were initiating or finishing their movement.

2.7 Statistical analysis

Statistical computations were performed using GraphPad Prism, version 5.0 (GraphPad Software, La Jolla, USA). Statistical significance was determined by unpaired Student's t-test and one-way or repeated measures ANOVA, followed by the adequate post-hoc test, for multiple comparisons. Correlations were assessed by Spearman's correlation coefficient. P-values were considered as statistically significant when <0.05; very significant when $p<0.01$ and extremely significant when $p<0.001$.

3 – Results

3.1 MSCs enhance autophagy of both cells and neuronal tissues expressing mutant ataxin-3

To minimize differences between MSCs' populations isolated and expanded through distinct protocols and to allow for comparison of results obtained from different laboratories, the Mesenchymal and Tissue Stem Cell Committee of the ISCT has proposed three minimal criteria to define a MSCs population: plastic-adherence in standard culture condition, selective surface expression of certain markers and the ability to differentiate into osteocytes, adipocytes and chondrocytes *in vitro* (Dominici et al. 2006).

The MSC population used in this study had previously been isolated from the bone marrow of 6- to 8-weeks old wild type mice of both genders with C57BL/6 background, according to the standard protocol of plastic adherence plus elimination of CD45 positive cells by sorting, before ex-vivo expansion. As expected, in culture, our population of MSCs grows in a monolayer and displays the typical fibroblast-like morphology. Their phenotype was also characterized through flow cytometry, demonstrating the lack of hematopoietic contaminants, such as CD11b and CD45, while being positive for typical mouse mesenchymal markers, such as CD73, CD105, Sca-1, CD106 and CD29 (Miranda et al., submitted). Moreover, after *in vitro* induction to differentiate into each of the three main mesodermal lineages (adipocytes, osteocytes and chondrocytes), they were positive for specific markers of each of these lineages (**Supplemental Fig. 1**). Thus, the previously used protocol can isolate a MSC population that displays all the key criteria that define a pure population of mouse MSCs.

To evaluate if MSCs could enhance autophagy in an already established *in vitro* model of MJD, we co-cultured N2a cells expressing the WT (N2a-WT) or mutant (N2a-Mut) form of ataxin-3 with MSCs, as described in **Fig. 7-A**. Since the transwell inserts used possess micropores that only allow passage of small molecules between both the upper and lower compartments, this system evaluates the paracrine effects of MSCs. N2a-Mut cells were already described to display severe impairments in terms of autophagy (Nascimento-Ferreira et al. 2011). Beclin-1 is a protein with a key role in an initial step of autophagy and its levels are decreased in N2a-Mut cells when comparing with N2a-WT cells (N2a-WT: 1 *versus* N2a-Mut: 0.693 ± 0.046, p=0.006), but the N2a-Mut+MSC group had significantly higher levels of beclin-1 than N2a-Mut (N2a-Mut+MSC: 0.91 ± 0.056 *versus* N2a-Mut: 0.693 ± 0.046, p=0.036) and similar levels to the N2a-WT (N2a-Mut+MSC: 0.91 ± 0.056 *versus* N2a-WT: 1, p=0.052) and N2a-WT+MSC (N2a-Mut+MSC: 0.91 ± 0.056 *versus* N2a-WT+MSC: 0.973 ± 0.052, p=0.752) groups (**Fig. 7-B**).
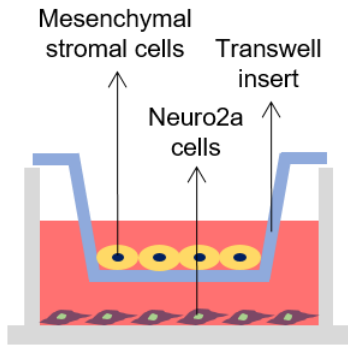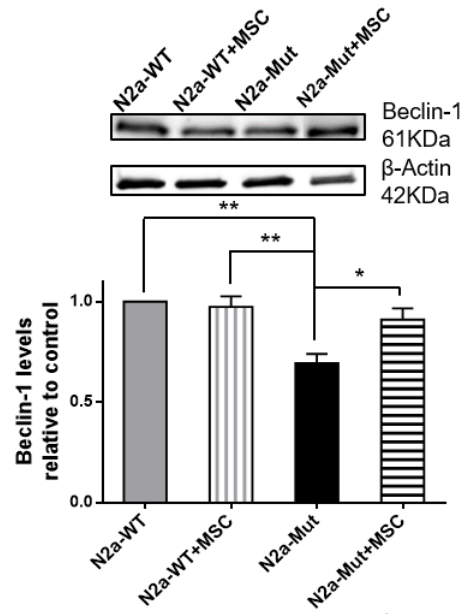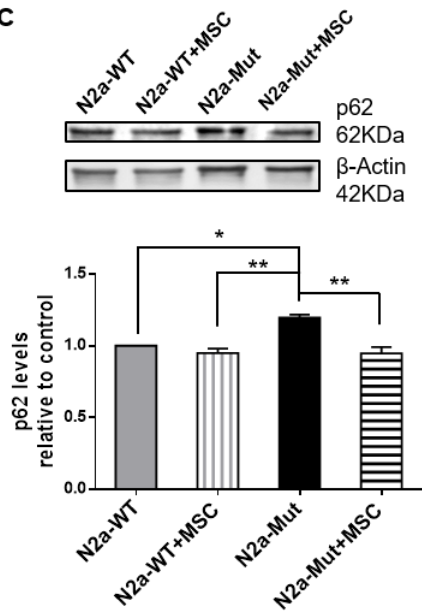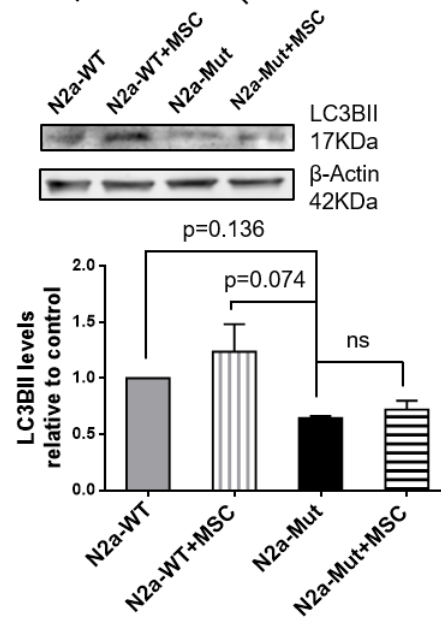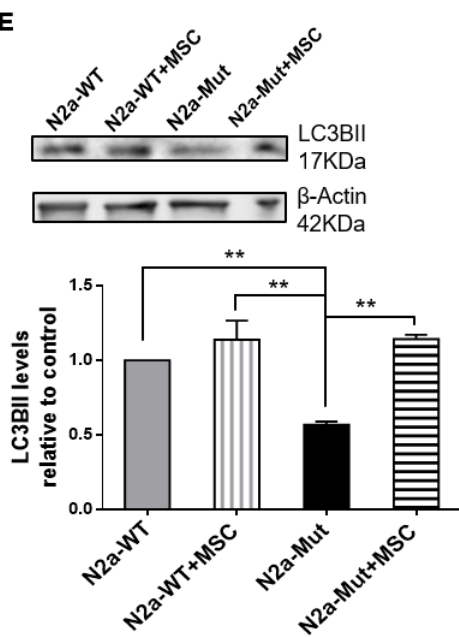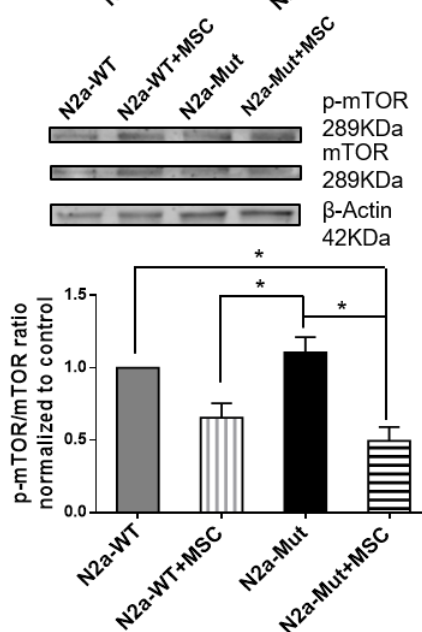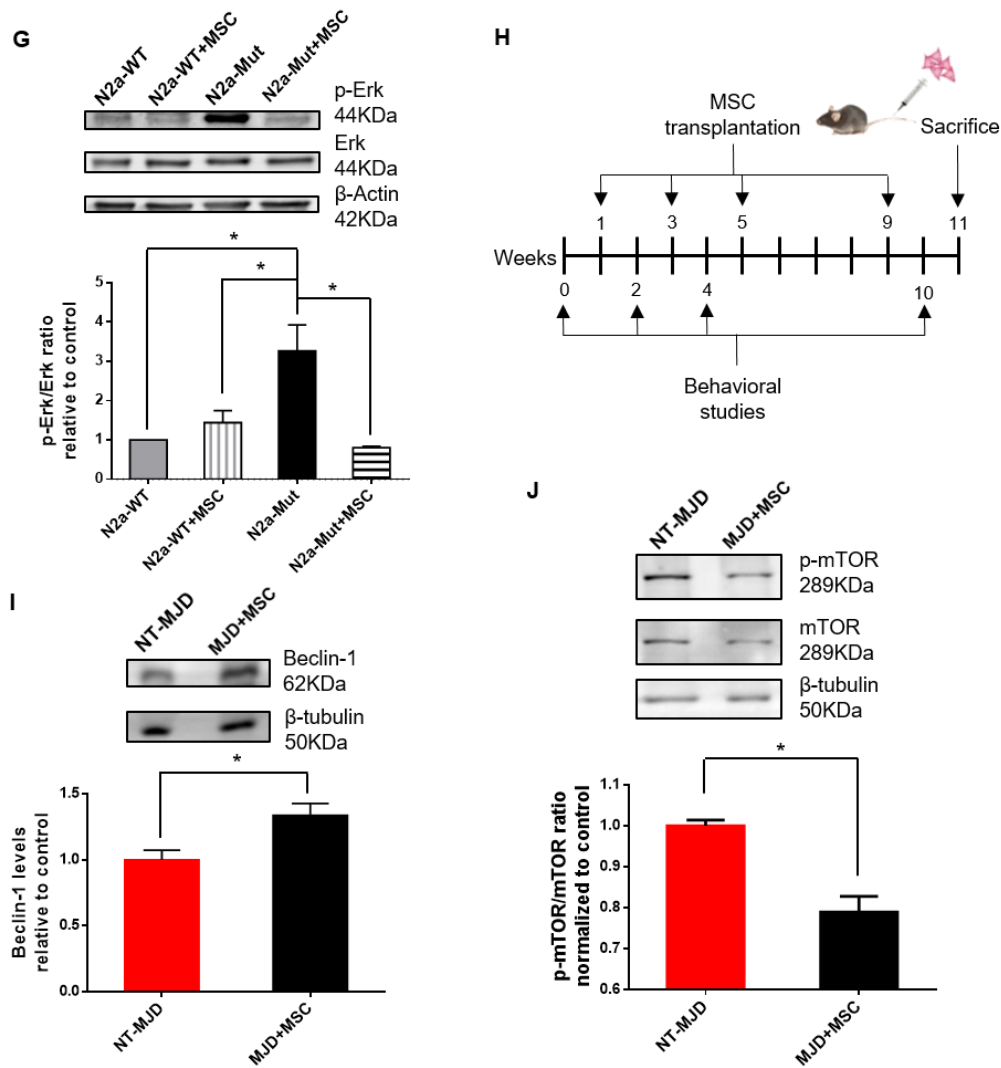
Figure 7 – MSCs increase the levels of autophagy-related proteins through modulation of mTOR and Erk in MJD models

A) Schematic view of transwell system used for co-cultures of N2a cells with MSCs. Levels of autophagic markers beclin-1 (**B**) p62 (**C**) and LC3BII at a basal state (**D**) or after chloroquine treatment (100µM for 3h) (**E**) are shown in N2a cells lentiviral-infected with ataxin-3 with 27 polyglutamines (N2a-WT) or 72 polyglutamines (N2a-Mut), grown for 48h either in the absence or in the presence of MSCs (N2a-WT+MSC and N2a-Mut+MSC groups, respectively) (n=3). **F)** Levels of mTOR activation (calculated by the ratio between p-mTOR and mTOR levels) in N2a-WT, N2a-WT+MSC, N2a-Mut and N2a-Mut+MSC (n=3). **G)** Activation of the Erk pathway (calculated by the ratio between p-Erk and Erk levels) in N2a-WT, N2a-WT+MSC, N2a-Mut and N2a-Mut+MSC (n=3). **H)** Schematic representation of the study design: transgenic MJD mice were divided in two groups, animals receiving four MSC transplants (MJD+MSC) or saline control solution (NT-MJD) through the tail vein. Levels of beclin-1 (**I**) or mTOR activation (**J**) in the cerebellum of MJD+MSC mice (n=3) in comparison with NT-MJD animals (n=4). Data is represented as mean±SEM. *One-way ANOVA, followed with Bonferroni's post-hoc test (***B-G****) and unpaired Student's t-test (****I-J****). ***p <0.001, **p <0.01 and *p <0.05.*

p62 is an autophagy substrate and thus, if the autophagic flux is impaired, it is expected to accumulate inside the cells. Indeed, N2a-Mut cells had significantly higher levels of p62 in comparison with all the other groups (N2a-Mut: 1.197±0.02186 *versus* N2a-WT: 1, p=0.0056; N2a-Mut: 1.197±0.022 *versus* N2a-WT+MSC: 0.095±0.031, p=0.001; N2a-Mut: 1.197±0.022 *versus* N2a-Mut+MSC: 0.947±0.043, p=0.001) (**Fig. 7-C**). N2a-Mut+MSC cells had similar p62 levels in comparison with the N2a-WT (N2a-Mut+MSC: 0.947±0.043 *versus* N2a-WT: 1, p=0.5793) and N2a-WT+MSC (N2a-Mut+MSC: 0.947±0.043 *versus* N2a-WT+MSC: 0.095±0.031, p=0.999) (**Fig. 7-C**) .

During a basal autophagic flux state, LC3B-II levels, a good indicator of autophagosome formation, are statistically similar between N2a-WT and N2a-Mut cells (N2a-WT: 1 *versus* N2a-Mut: 0.647±0.018, p=0.541) (**Fig. 7-D**). Still, in the presence of chloroquine (100 µM for 3h), an autophagy inhibitor which prevents autophagosome-lysosome fusion and subsequent proteolysis due to increase of lysosomal pH, LC3B-II levels increase due to autophagosome accumulation. In N2a-Mut cells, chloroquine incubation leads to lower levels of LC3B-II than in N2a-WT cells (N2a-WT: 1 *versus* N2a-Mut: 0.57±0.021, p=0.008) (**Fig. 7-E**). When co-cultured with MSC, after chloroquine treatment, N2a-Mut cells display significantly higher levels of LC3B-II than N2a-Mut cells (N2a-Mut: 0.57±0.021 *versus* N2a-Mut+MSC: 1.143±0.029, p=0.001), , reaching levels similar to N2a-WT (N2a-WT: 1 *versus* N2a-Mut+MSC: 1.143±0.029, p=0.464) or N2a-WT+MSC cells (N2a-WT+MSC: 1.14±0.127 *versus* N2a-Mut+MSC: 1.143±0.029, p>0.9999) (**Fig. 7-E**). Altogether, these results demonstrate that MSCs are able to enhance autophagy of N2a-Mut cells through a paracrine effect.

As autophagy is known to be inhibited by mTOR activity, we analyzed mTOR activation in this *in vitro* paradigm, through the ratio of the levels of its activated form and its total levels (p-mTOR/mTOR) in N2a cells. mTOR activation was significantly reduced in N2a-Mut+MSC cells when compared with the N2a-Mut group (N2a-Mut: 1.106+-0.107 *versus* N2a-Mut+MSC: 0.496+-0.095, p=0.015), although no differences were observed between the N2a-WT and N2a-Mut groups (N2a-WT: 1 versus N2a-Mut: 1.106+-0.107, p>0.9999) (**Fig. 7-F**).

Furthermore, we observed that the Erk cellular pathway, known to enhance the activity of mTOR, was significantly activated in N2a-Mut cells in comparison with N2a-WT cells (N2a-WT: 1 *versus* N2a-Mut: 3.268±0.658, p=0.013), whereas N2a-Mut+MSC cells had significantly lower levels of Erk activation than N2a-Mut cells (N2a-Mut: 3.268±0.658 *versus* N2a-Mut+MSC: 0.802±0.035, p=0.008), reaching similar levels of the N2a-WT (N2a-WT: 1 *versus* N2a-Mut+MSC: 0.802±0.035, p>0.9999) and the N2a-WT+MSC (N2a-WT+MSC: 1.441±0.307 *versus* N2a-Mut+MSC: 0.802±0.035, p>0.999) groups (**Fig. 7-G**). These

observations suggest that MSCs might enhance the activity of the autophagic pathway by decreasing Erk-derived mTOR activity.

To evaluate whether MSCs could drive similar effects *in vivo*, repeated intravenous injections of bone marrow-derived MSCs were performed post-symptomatically in a Tg MJD mouse model. The treatment consisted of 4 injections of $4.5\text{-}8\text{x}10^7$/kg MSCs through the tail vein (**Fig. 7-H**), starting at 4.5-6 weeks of age when the animals already display severe ataxia. Evaluation of the levels of beclin-1 in the cerebellum of both non-treated (NT-MJD) and treated (MJD-MSC) MJD animals revealed that beclin-1 was also significantly increased in MJD-MSC mice (NT-MJD: 1 ± 0.073 *versus* MJD+MSC 1.339 ± 0.088, p=0.031) (**Fig. 7-I**), corroborating the *in vitro* data. Moreover, mTOR activation was significantly decreased in the cerebellum of MJD+MSC animals when compared with NT-MJD mice (NT-MJD: 1 ± 0.014 *versus* MJD+MSC: 0.789 ± 0.039, p=0.007) (**Fig. 7-F**), suggesting that the observed increase in beclin-1 levels might be mediated by a reduction in mTOR activation. Taken together, these data show that MSCs are inducing autophagy and reducing mTOR activation, both in *in vitro* and in *in vivo* models of MJD.

3.2 MSCs reduce the levels of mutant ataxin-3 in MJD

In order to investigate if the observed MSC-induced increase in autophagy could promote a decrease in the levels of mutant ataxin-3, we performed Western blot analysis. I*n vitro*, after the co-culture protocol previously described, no statistically significant changes were observed in the levels of soluble mutant ataxin-3 between the N2a-Mut and N2a-Mut+MSC groups (**Fig. 8-A**; N2a-Mut: 1 *versus* N2a-Mut+MSC: 0.72 ± 0.161, p=0.157), even though a small tendency for its levels to decrease was observed in the N2a-Mut+MSC group. This suggests that even though an increase in the autophagic flux of N2a-Mut cells is induced by MSCs, the protocol performed might not be long enough to promote significant changes in terms of the levels of mutant ataxin-3. In the future, the levels of the aggregated form of the protein will also be determined.

Nonetheless, analysis of the levels of both soluble and aggregated forms of mutant ataxin-3 in the cerebellum of MJD+MSC mice showed a significant decrease (NT-MJD: 1 ± 0.111 *versus* MJD+MSC: 0.761 ± 0.035, p=0.039; **Fig. 8-B**), and a strong tendency to decrease, respectively (NT-MJD: 1 ± 0.172 *versus* MJD+MSC: 0.679 ± 0.118, p=0.098; **Fig. 8-C**), when compared with NT-MJD animals. Altogether, these observations suggest that MSCs can reduce the levels of mutant ataxin-3 in MJD through the activation of the autophagic pathway.
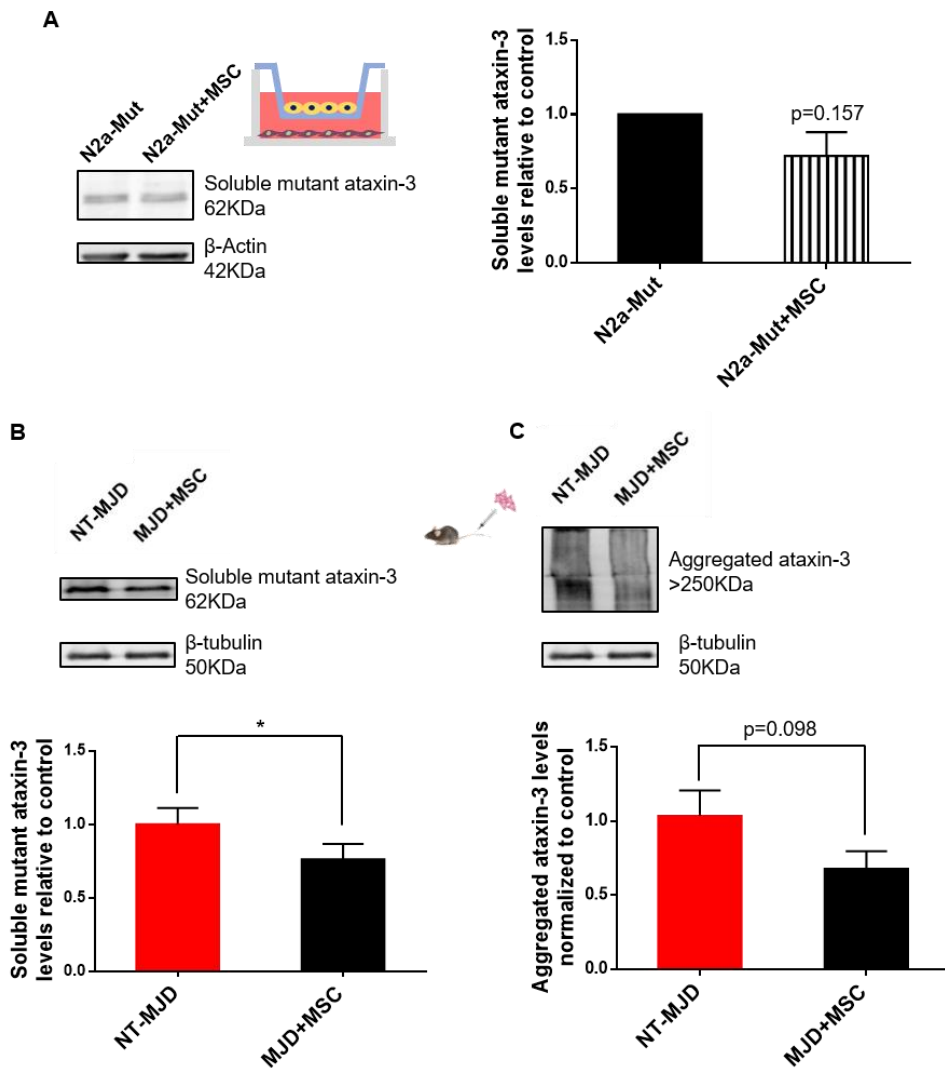
Figure 8 - Treatment with MSCs reduces the levels of ataxin-3 in MJD models

A) Levels of soluble mutant ataxin-3 in N2a-WT, N2a-WT+MSC, N2a-Mut and N2a-Mut+MSC after growing in a co-culture system for 48h (n=3). The levels of both soluble (**B**) and aggregated forms of ataxin-3 (**C**) were evaluated in the cerebellum of NT-MJD (n=7) and MJD+MSC mice (n=9*)*. Data is represented as mean±SEM.*Unpaired Student's t-test. ***p <0.001, **p <0.01 and *p <0.05.*

3.3 MSCs promote functional benefits in *in vitro* and *in vivo* models of MJD

To evaluate the metabolic activity of N2a cells, we performed the Alamar Blue assay after the co-culture protocol previously detailed. In comparison with N2a-WT cells, N2a-Mut cells had decreased metabolic activity (N2a-WT: 100 *versus* N2a-Mut: 64.48±5.023, p=0.003), while N2a-Mut+MSC cells showed higher metabolic activity levels than N2a-Mut cells (N2a-Mut+MSC: 85.63±618 *versus* N2a-Mut: 64.48±5.023, p=0.044), similar to the levels of the N2a-WT group (N2a-WT: 100 *versus* N2a-Mut+MSC: 85.63±6.178, p=0.192) (**Fig. 8-A**).
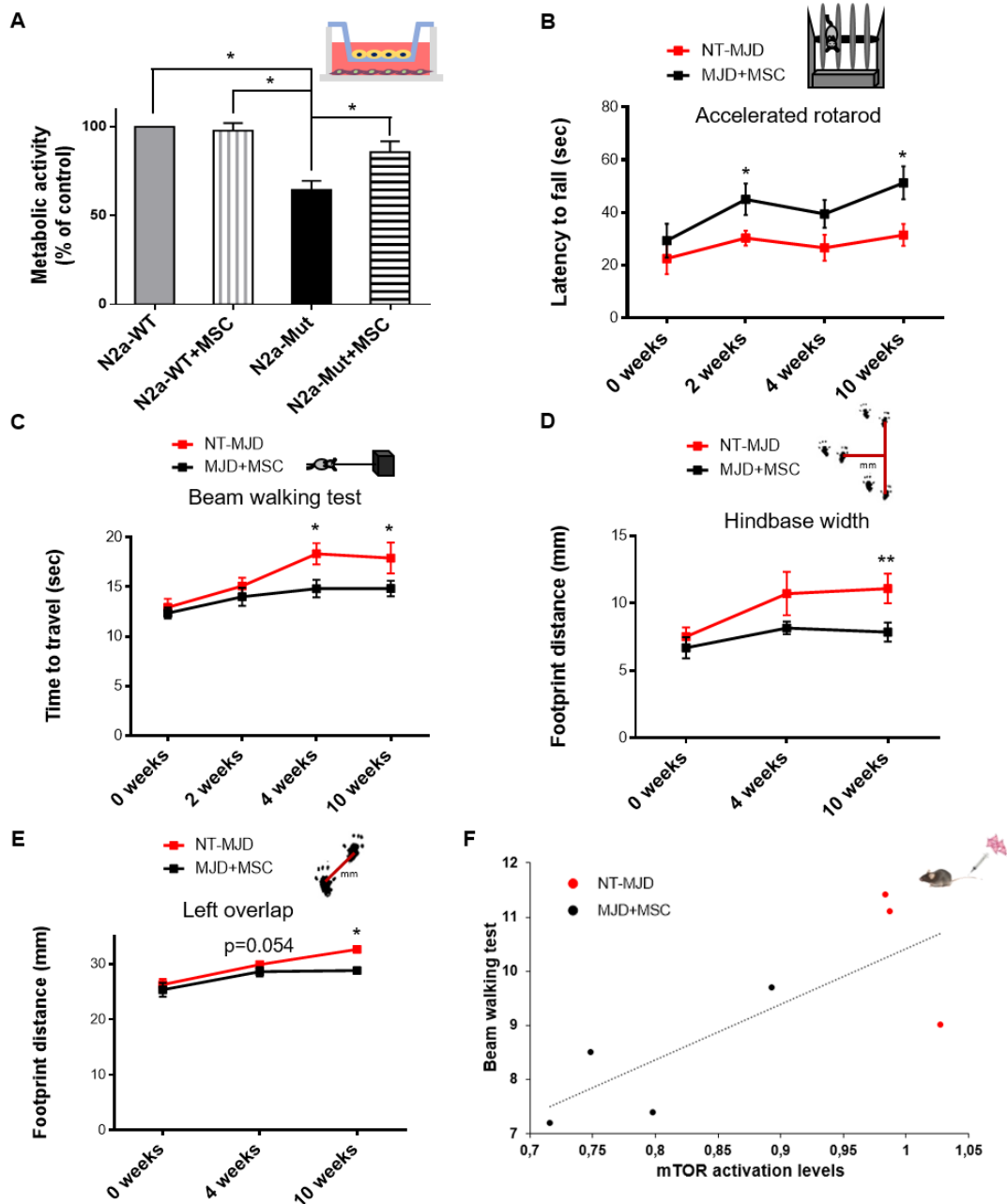
Figure 9 – MSCs-derived autophagy enhancement induces functional benefits in MJD models

A) Alamar Blue measurements in N2a-WT, N2a-WT+MSC, N2a-Mut and N2a-Mut+MSC cells grown in co-culture system for 48h (n=3). **B-E)** Motor performance assessment of NT-MJD and MJD+MSC transgenic MJD mice. Coordination and balance were evaluated through the accelerated rotarod (NT-MJD n=9; MJD+MSC n=9) **(B)** and beam walking (NT-MJD n=7; MJD+MSC n=9) **(C)** tests. Quantitative analysis of footprint patterns (NT-MJD n=7; MJD+MSC n=9); hindbase width **(D)** and left overlap **(E)** are represented. **(F)** Correlation between cerebellar mTOR activation levels and beam walking test performance of NT-MJD (n=3) and MJD+MSC (n=4) transgenic MJD mice. Data is represented as mean±SEM. **A)** *One-way ANOVA, followed with Bonferroni's post-hoc test.* **B-E)** *Repeated measures ANOVA* **F)** *Spearman correlation test ***p <0.001, **p <0.01 and *p <0.05.*

No changes were observed between N2a-WT cells co-cultured with MSCs (N2a-WT+MSC) nor (N2a-WT: 100 *versus* N2a-WT+MSC: 97.73±4.355, p=0.984) (**Fig. 9-A**).

To evaluate if the autophagy induction in mice treated with repeated intravenous injections of bone marrow-derived MSCs could drive sustained motor benefits in Tg MJD mice, we performed behavioral assessment through the rotarod, beam walking and footprint tests, at different time-points (**Fig. 7-H**). The rotarod test was used to assess both motor coordination and balance. One week after the first injection (week 2 of experimental assay), MJD+MSC mice (n=9 females) performed significantly better than NT-MJD (n=9 females) animals (week 2, MJD+MSC: 44.978±5.973 sec *versus* NT-MJD: 30.289±2.836 sec, p=0.041; **Fig. 9-B**). At week 4 (1 week after the second injection of MSCs), no significant differences between the two groups of animals occurred, though a tendency towards a better performance of the MJD+MSC animals was observed (week 4; MJD+MSC: 39.456±5.306 sec *versus* NT-MJD: 26.6±4.950 sec, p=0.0955; **Fig. 9-B**). At the last time-point in which motor performance was evaluated (week 10, 1 week after the last treatment), the treated group had higher latency to fall off the rotarod apparatus than the non-treated animals (week 10; MJD+MSC: 51.256±6.238 sec *versus* NT-MJD: 31.456±4.146 sec, p=0.018; **Fig. 9-B**).

The beam walking test was also used to evaluate motor coordination and balance. At week 2 (1 week after the first treatment), no significant differences were observed between treated (MJD+MSC, n=9 females) and non-treated (NT-MJD, n=7 females) animals (week 1; MJD+MSC: 14±1.023 sec *versus* NT-MJD: 15.1±0.831 sec, p=0.41202; **Fig. 9-C**). However, in both the following time points, MJD+MSC animals walked through the beam in significantly less time than the NT-MJD mice (week 4; MJD+MSC: 14.833±0.990 sec *versus* NT-MJD: 18.343±1.070 sec, p=0.0108; week 10; MJD+MSC: 14.844±0.883 sec *versus* NT-MJD: 17.914±1.560 sec, p=0.0247999; **Fig. 9-C**).

Gait analysis was done through the footprint test. Significant better performances of treated animals (MJD+MSC, n=9 females) in comparison with non-treated MJD mice (NT-MJD, n=7 females) were registered at week 10 (1 week after the last treatment) regarding hindbase width (week 10; MJD+MSC: 28.8±0.970 mm *versus* NT-MJD: 32.629±0.666 mm, p=0.004), and overlap between the front and hind footprint (week 10; MJD+MSC: 7.844±0.531 mm *versus* NT-MJD: 11.071±1.608 mm, p=0.016) (**Fig. 9.D-E**). Finally, a positive correlation between the cerebellar levels of mTOR activation and the time needed to perform the beam walking test of treated (MJD+MSC, n=4 females) and non-treated (NT-MJD, n=3 females) was observed (R=0.78, p=0.04) (**Fig. 9.F**).

In conclusion, the results obtained suggest that MSCs can induce functional benefits in MJD through activation of the autophagic pathway.

4 – Discussion

In the present study, we demonstrated that bone marrow-derived MSCs can enhance autophagy and modulate mTOR activation, both in *in vitro* and *in vivo* models of MJD. Furthermore, we showed that the observed increase in autophagy appears to mediate functional benefits through reduction of ataxin-3 levels.

Even though MSCs have already been tested in clinical trials, only a few pre-clinical studies have been done in SCAs (Jones et al. 2010; Chang et al. 2011; Matsuura et al. 2014). Pre-clinical trials are essential for any therapeutic strategy, not only to test the efficacy and safety of a certain approach, but also to optimize several of its parameters, such as the choice of the best delivery route, dosage and regularity of treatment. Moreover, the importance of knowing how MSCs are driving benefits is marked as novel therapeutic targets can be identified and more practical and/or better therapies could be designed, such as the use of cell-free approaches (i.e. MSCs' conditioned medium or MSCs'–derived products). Knowledge on the mechanism of action of MSCs in MJD is still very limited and pre-clinical trials are fundamental to unravel it. When first used as a therapeutic approach, MSCs were thought to mainly induce benefits by homing towards injured host cells and replacing them after differentiating (Bruder et al. 1998; Horwitz et al. 1999), but most recent findings appear to indicate that not only MSCs do not differentiate significantly but they sometimes are not found at the local of action, even when benefits are reported (Chen et al. 2003; Li et al. 2005). Thus, nowadays the idea that MSCs act mainly through the release of soluble factors now prevails, with several reports indicating an increment in certain neurotrophic factors, such as BDNF or VEGF after treatment with MSCs (Caplan et al. 2006; Sadan et al. 2008; Wilkins et al. 2009). Here, through *in vitro* co-cultures experiments without physical contact between cells, we have demonstrated that MSCs are exerting a paracrine effect in MJD which culminates in the promotion of the autophagic flux, impaired in MJD (Nascimento-Ferreira et al., 2011). In the future, we will investigate which are the factors responsible for MSCs' autophagy induction capacity as these may be used as a clinical strategy. Some authors proposed that reduced levels of mutant ataxin-3 might derive from an increment in Hsp70, mediated by IGF-1, and inhibition of the apoptotic pathway in MJD mice through the PI3K/Akt pathway (Li et al. 2017). Though we did not investigate the levels of apoptotic markers nor the Akt pathway in the present study, in the future we will address this pathway.

Protein clearance mechanisms as autophagy are especially relevant in neurodegenerative disorders in which mutant proteins aberrantly start aggregating and form insoluble inclusions, becoming crucial for the cells to be able to remove them, thus reducing the toxicity driven from this dysfunction (Walsh et al., 2002; Takahashi et al., 2008). In MJD, the accumulation over time of such mutant proteins that are prone to aggregate can be explained by severe impairments in the autophagic pathway (Nascimento-Ferreira et al., 2011). Indeed, restoring the autophagic flux has been shown to be a promising therapeutic approach in MJD (Nascimento-Ferreira et al. 2011; Nascimento-Ferreira et al. 2013).

Furthermore, MSCs have been shown to enhance autophagy in AD models (Shin et al. 2014). We observed that MSCs could restore the levels of several proteins involved in distinct steps of autophagy to normal levels *in vitro* and the levels of the autophagic protein beclin-1, known to be reduced in MJD (Nascimento-Ferreira et al., 2011) in the cerebellum of treated MJD mice.

Unexpectedly, we observed that N2a-Mut cells had significantly higher activation of the Erk pathway in comparison with N2a-WT cells, and co-cultures with MSCs reverted this increment. While the Erk pathway is mostly looked as an inducer of proliferation and survival effects (Raman et al. 2007; Aoki et al. 2011), it can also lead to activation of apoptotic pathways (Bhat et al. 1999; Pan et al. 2012). Accordingly, even though its activation is beneficial in some neurodegenerative disorders (Maher et al. 2010), its amplified activation appears to be a key component in the neuropathological process of other diseases (Zhu et al. 2001; Kirouac et al. 2017). This differential activity of the Erk pathway is still not completely characterized but it seems that its transient activation leads to neuroprotection, while an aberrant chronic activation induces prejudicial effects (D'Amato et al. 2003; Almeida et al. 2005; Cagnol et al. 2009; Submaraniam et al. 2010). Further experiments should evaluate the levels of Erk activation in the cerebellum of both WT and Tg MJD mice in order to verify if chronic Erk activation is occurring in the latter, as our observations in an *in vitro* model of MJD suggest.

Interestingly, Erk activation can reduce autophagy activation by indirectly activating mTOR. We observed that mTOR activation was significantly decreased in MSCs-treated N2a-Mut cells, in comparison with N2a-Mut cells, and in the cerebellum of MSCs-treated mice, when compared with non-treated animals, suggesting that the observed increase in autophagy might be attributed, at least in part, to the decline of mTOR activation. It is also important to remark that mTOR inactivation, by use of rapamycin or other molecules, has been suggested as an interesting strategy for treatment of several disorders in which autophagy has been found to be disrupted, such as HD (Ravikumar et al. 2008). However, therapies for neurodegenerative disorders based on autophagy activation do not always report benefits (Duarte-Silva et al. 2016), suggesting that our knowledge of autophagy regulation is still limited, as it also encompasses several mTOR–independent pathways (Sarkar et al. 2005), and additional testing will be required to optimize a viable therapeutic strategy based on such approach. Nonetheless, our hypothesis is supported by other reports, such as the previous study from our group where it was demonstrated that the overexpression of beclin-1 was enough to reduce mutant-ataxin-3 and drive motor benefits in MJD models (Nascimento-Ferreira et al. 2011), and another study where the authors reported that MSCs were capable of enhancing autophagy and increasing β-amyloid clearance in models of AD (Shin et al. 2014).Further experiments should be done to confirm

the importance of the modulation of Erk and mTOR in the effects driven by MSCs in MJD, while also analyzing additional pathways also involved in autophagy modulation, such as Akt.

As a proof of benefits driven by autophagy induction, the levels of soluble mutant ataxin-3 levels were decreased in the cerebellum of treated MJD mice, while aggregated ataxin-3 also showed a tendency to be decreased. It is however important to note that, despite some controversy, the soluble form of mutant ataxin-3 appears to be more toxic than its aggregated form (Simões et al. 2012; Simões et al. 2014). In N2a-Mut cells, although MSCs did not significantly decrease the levels of soluble mutant ataxin-3, it is possible that a longer co-culture period with MSCs is needed to achieve a stronger effect. Altogether, these results suggest that MSCs' might be inducing a decrease in the levels of ataxin-3 by restoring the autophagic flux. Moreover, we also observed a positive correlation between the time taken to perform the beam walking test and cerebellar mTOR activation in Tg MJD animals, which supports the idea that the observed functional benefits are, at least in part, derived from reduced mTOR activity and subsequent increase in the autophagic flux, thus reducing mutant ataxin-3 toxicity and subsequent cerebellar neurodegeneration.

In summary, our findings demonstrate that MSCs can promote functional benefits through autophagy enhancement in models of MJD, thus showing this cellular therapy can be a viable therapeutic approach for this fatal disorder, when delivered as a repeated treatment. We hope that this study helps not only in the development of a therapy for MJD patients, but also for other neurodegenerative disorders, since the mechanism of action of MSCs described here might be similar throughout distinct pathologies.

5 – Conclusion and future perspectives

In this study we showed that MSCs are able to induce neuroprotective effects in MJD models by promoting an increase in autophagy, mediated by Erk and mTOR modulation. Additionally, we demonstrate that repeated systemic treatment with bone marrow-derived MSCs is able to sustainably alleviate motor impairments in Tg MJD mice, in accordance with previous results from our group which showed that a single treatment with MSCs only promoted transient motor benefits. This experimental assay thus shows that a continued therapy with MSCs is a viable therapeutic approach for MJD, with further experiments being needed to identify which factors are responsible and additional cellular mechanisms which might be involved. We intend to characterize changes in terms of activation of receptor tyrosine kinases (RTKs) in N2a-Mut cells co-cultured with MSCs in our established co-culture design. Moreover, the identification of soluble molecules, such as neurotrophic factors, in increased concentrations in the conditioned medium in the presence of MSCs is also planned. All these assays will allow us to further unravel the mechanism of action of MSCs, with the possibility that a novel therapeutic target for MJD might be found.

In the future we also intend to test the capacity of MSCs in enhancing autophagy in fibroblasts derived from MJD patients, another MJD model developed in our group, which display severe autophagy impairments (Onofre et al. 2016), thus allowing us to infer about the potential of this treatment in a model that more closely resembles MJD patients. Furthermore, it would be interesting to test if exosomes released by MSCs are able to induce similar benefits to those observed when administering MSCs, as a cell-free therapy could be preferred. In conclusion, our study supports the idea that an MSC-mediated increase in autophagy is a promising strategy for the treatment of MJD.

6 – Supplemental figures
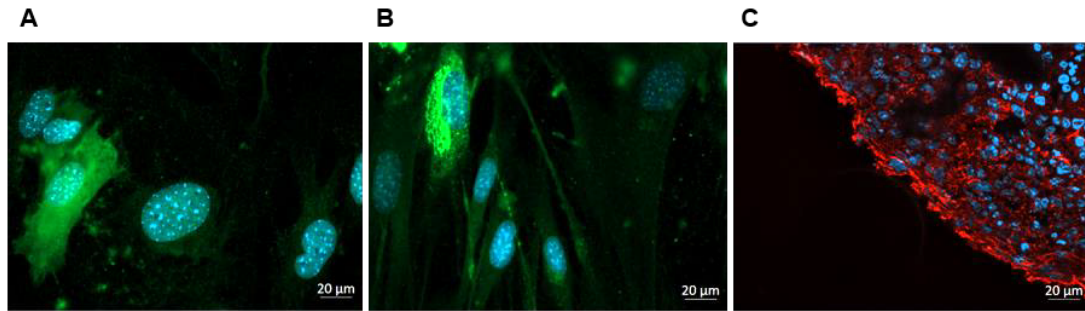
Supplemental figure 1 – MSCs in vitro differentiation into the three main mesodermal lineages

Fluorescence photomicrographs showing MSCs expression of specific markers for adipocytes (fatty acid-binding protein 4 (FABP4), in green) (**A**), osteocytes (osteopontin, in green) (**B**) and chondrocytes (collagen II, in red) (**C**) respectively, after 21 days in contact with the appropriate induction media. DAPI is shown in blue.

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