Filipa Alexandra Ferreira de Brito

Pharmacological modulation of mutant ataxin-3 translation and it potential therapeutic effect in Machado Joseph disease

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À minha mãe

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A todos os meus mais sinceros agradecimentos.

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ABBREVIATIONS

a.u. - arbitrary units

A1 - Adenosine receptors 1

A3 - Adenosine receptors 3

ADA - enzyme adenosine deaminase

AONS - Antisense oligonucleotides

AR - Androgen receptor

Atx3 - Ataxin-3

Atx3 agg - Aggregates of ataxin-3

Atx3 endo - Mice endogenous ataxin-3

Atx3 mut - Human mutant form of ataxin-3

Atx3-27Q - Human wild-type ataxin-3

Atx3Q72 - Human full-length mutant ataxin 3 with 72 glutamines

BA - Behavioural assessment

bp - based-pairs

BSA - Bovine serum albumin

CAG - Cytosine-Adenine-Guanine

CHOP - C/EBP homologous protein

CHX - Ciclohexamide

CM - Cordyceps militaris (Fr.) L.

CNC Centre for Neuroscience and Cell Biology

CNS - Central nervous system

COX-2 - Cytochrome c oxidase subunit II

CS - Cordyceps sinensis (Berk.) Sacc

CT - Control

DMEM - Dulbecco's modified Eagle's medium supplemented

DMJ - Doença de Machado-Joseph

DMSO - Dimethyl sulfoxide

DR3 - Death Receptor-3

DRPLA - Dentatorubral-pallidoluysian atrophy

DUB - Deubiquitinating enzyme

ECF - Enhanced Chemifluorescent substrate

ER - Endoplasmic reticulum

ER stress - Endoplasmatic Reticulum stress

ERAD - Endoplasmic reticulum-associated degradation

FBS - Fetal bovine serum

GFAP - Glial fibrillary acidic protein

HA - Hemagglutinin

HD - Huntington's disease

HDAC - histone deacetylase

HDAC3 - Histone deacetylase 3

HDAC6 - histone deacetylase 6

HEK - Human embryonic kidney cell line 293T

i.p. - Intraperitoneal injection

IBA-1 - Ionized calcium-binding adapter molecule 1

IL - Interleukin

JD - Josephin domain

LV - Lentiviral

MAP2 - Microtubule-associated protein 2

MDA - Melandialdehyde oxidation

MJD - Machado- Joseph disease

MMP - Mitochondrial membrane potential

MTD - Maximally tolerated dose

MTOC - Microtubule-organizing center

mTOR - Mammalian target of rapamycin

MW - Molecular weight

NES - Nuclear export signal

NeuN - Neuronal nuclei

NF-κB - Nuclear factor-κB

NIIs - Intranuclear neuronal inclusions

NIIs - Neuronal intranuclear inclusions

NIs - Neuronal inclusions

NLS - nuclear localization signal

NT - Non-treated

ON - Overnight

PBS - Phosphate-buffered saline

PCAF - p300/CBP-associated factor

PFA - Paraphormaldehyde

PK - multiple dose pharmacokinetics

PNA - Peptide nucleic acid

polyQ - polyglutamine track

PVDF - Polyvinylidene difluoride

RD - Recommended dose

RNAi - interference RNA

ROS - Reactive oxygen species

RT - Room temperature

SBMA - Spinal bulbar muscular atrophy

SCA3 - Spinocerebellar ataxia type 3

SCAs - Spinocerebellar ataxias

SEM - Standard error of the mean

shRNA- Short hairpin RNA

SNP - Small nucleotide polymorphism

SOD - Superoxidade dismutase

ssRNA - Stranded silencing RNA

TAF4 - TATA box binding protein (TBP)-associated factor 4

TBS-T - Tris buffered saline

TBT - TATA box binding protein

TCM - Traditional Chinese Medicine

Ub - Ubiquitin

UIM - Ubiquitin interacting motifs

UPP - Ubiquitin-proteosome pathway

UPS - Ubiquitin-proteasome system

VCP/p27 - Valosin-containing protein/p27

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ABSTRACT

Machado-Joseph disease (MJD) or spinocerebellar ataxia type 3 (SCA3) is described as the most common ataxia worldwide. It is associated with the expansion of a (CAG)n tract in the coding region of the causative gene MJD1. This abnormal over-repetition is translated into an expanded polyglutamine tract within ataxin-3 protein, resulting in severe clinical features leading to neurodegeneration and premature death. Despite important progresses, the mechanisms accounting for neuronal degeneration are still largely unknown and there is no available treatment. One of these mechanisms, the translation dysfunction may be important in MJD, as well as in other spinocerebellar ataxias, originating a cascade of events leading to neurodegeneration and cell death. The aim of this project is to evaluate the pharmacological inhibition of translation as a therapeutic approach for MJD. For a pharmacological attempt of overcomes this dysfunction we tested an adenosine analogue, which is already described as a translation inhibitor, cordycepin. Preliminary *in vitro* and *in vivo* results performed in the lab, point to a beneficial effect of the drug in MJD-associated abnormalities and together with the literature form a great indicator of the potential of this drug. Cordycepin was tested *in vitro* as well as in two *in vivo* models, in a lentiviral (based in the local expression of Atx3-72Q) and in a transgenic (cerebellar expression of Atx3-69Q) mouse model. The *in vitro* experiments allowed us to find an accurate concentration and treatment time, as well as to detect the beneficial effect of the drug in reducing the levels of mutant protein. With the lentiviral model, the neuropathological features were evaluated and it was detected a redunction in the number of inclusions accompanied by a decrease in neuronal loss. The transgenic model allowed us to detect a rescue of the motor deficits phenotype upon 6 week treatment with cordycepin.

Altogether these results suggest that cordycepin has the ability to ameliorate some characteristic features of MJD, partially by the inhibition of translation. Taking this into account and the fact that a pharmacological method facilitates the application of the approach to MJD clinics in a very short time frame, cordycepin could play a promissory role in the treatment of MJD or even be extended to other disorders, as it is generally accepted that spinocerebellar ataxias share common pathogenesis.

Key words: Machado Joseph disease (MJD)/Spinocerebellar ataxia type 3 (SCA3), Cordycepin/3'deoxyadenosine, translation inhibition

RESUMO

A doença de Machado-Joseph (DMJ) ou ataxia espinocerebelosa do tipo 3 é descrita como a mais comum forma de ataxia. Está associada com a expansão de uma cadeia de poliglutaminas na região codificante do gene causador da doença, MJD1. O anormal número de repetições é traduzido numa proteína ataxina-3 com uma cadeia de poliglutaminas expandida, o que resulta em características clínicas severas que levam à neurodegenescência e à morte prematura do doente. Apesar de todos os esforços e progressos ainda há muito por descobrir e os mecanismos responsáveis pela degeneração neuronal estão ainda por desvendar não existindo até ao momento tratamento disponível para esta doença. Um dos mecanismos envolvidos na doença pode ser uma disfunção da tradução, podendo ser importante quer na DMJ quer em outras ataxias espinhocerebelosas, ao originar uma cascata de eventos que leva à neurodegenerescência e morte celular. O objetivo deste projeto é avaliar a inibição farmacológica da tradução, como uma potencial abordagem terapêutica na DMJ. Para tentar ultrapassar esta disfunção farmacologicamente, foi testado um análogo da adenosina, que está descrito como sendo um inibidor da tradução, a cordicepina. Resultados preliminares tanto *in vitro* como *in vivo* efetuados no nosso laboratório, demonstram um efeito benéfico da cordicepina em alterações associadas à doença. Estes dados juntamente estudos publicados são grandes indicadores do potencial deste fármaco. Foram feitas experiências *in vitro* e em dois modelos *in vivo*, um lentiviral, baseado na expressão local de Atx3- 72Q e outro transgénico, com uma expressão cerebelar da forma truncada de Atx3-69Q. As experiências *in vitro* permitiram-nos determinar a concentração e tempo de tratamento ótimos, bem como detetar a redução nos níveis de proteína mutante. Foi ainda possível detetar uma melhoria nas características neuropatológicas, como a diminuição do número de agregados e diminuição da perda neuronal usando o modelo lentiviral. Já com o modelo transgénico foi possível verificar uma recuperação no fenótipo motor após 6 semanas de tratamento com cordicepina. Estes dados sugerem que a cordicepina tem a capacidade de melhorar algumas das características desta da DMJ, e que em parte essa melhoria se deve à inibição da tradução. Além disso uma abordagem farmacológica permite uma mais rápida aplicação deste tipo de tratamentos para a clínica. Deste modo a cordicepina pode ter um papel promissor no tratamento da doença de Machado-Joseph e a sua aplicação pode ainda ser estendida a outras doenças, visto ser aceite que as ataxias espinocerebelosas partilham mecanismos comuns de patogenicidade.

Palavras-chave: Doença de Machado-Joseph, Ataxia espinocerebelosa do tipo 3, Cordicepina/ 3' desoxiadenosina, inibição da tradução.

CHAPTER 1 - INTRODUCTION

1.1 Triplet repeat diseases

Triplet repeat disorders also known as trinucleotide repeat expansion disorders, are a set of genetic neurodegenerative diseases that share a mutual pathogenic mechanism, the unstable expansion of trinucleotide repeats (Koshy and Zoghbi, 1997; Gatchel and Zoghbi, 2005). Normally, these repetitions display a stable transmission inheritance pattern with low levels of length variation. On the contrary, under disease condition, where a mutation exceeds a certain threshold of triplet repetitions it becomes unstable and the transmission occurs in a non-Mendelian pattern increasing the severity of the disease, and decreasing the age of onset of the following generations Depending on the affected gene and the type of trinucleotide extension the features are different for each disease (Paulson and Fischbeck, 1996; Koshy and Zoghbi, 1997). They are classified in two different categories, regarding the location of the repetitions: whereas it appears in a non-coding or coding region of the gene. Mutations that occur in a non-coding region (introns, 3'UTR or 5'UTR) are involved in untranslated sequences, possibly by an alteration in the gene expression. Characteristically they present a high level of repetitions than those that occur in the coding regions (Paulson and Fischbeck, 1996; Tsuji, 1997; Cummings and Zoghbi, 2000; Gatchel and Zoghbi, 2005).One type of triplet diseases is characterized by the abnormal expansion within of the coding region of a Cytosine-Adeninde-Guanine (CAG) tract, leading to an abnormally long polyglutamine track (poly Q). This class of disorders is designated as polyglutamine diseases (La Spada et al., 1994; Paulson and Fischbeck, 1996; Koshy and Zoghbi, 1997; Tsuji, 1997; Cummings and Zoghbi, 2000; Ross and Poirier, 2004; Gatchel and Zoghbi, 2005; Riley and Orr, 2006).

1.1.1 Polyglutamine diseases

The polyglutamine diseases (polyQ) are a group of nine inherited diseases, including Huntington's disease (HD), dentatorubral-pallidoluysian atrophy (DRPLA), spinal bulbar muscular atrophy (SBMA) and the spinocerebellar ataxias (SCAs) type 1, 2, 3, 6, 7 and 17 (see Table 1) (Zoghbi and Orr, 2000; Havel et al., 2009). They have result from the expansion of an unstable trinucleotide repetition of CAGs, which translates into a long polyQ tract, conferring a toxic function to the related protein (Cummings and Zoghbi, 2000). All these disorders, with exception of SBMA that is linked to chromosome X, are autosomal dominant, being the HD and SCA3 the most prevalent worldwide (Fan et al., 2014). There is a strong correlation between the expanded repeat size and the age at onset of the disease, as well as the clinical presentation of the symptoms (Maciel et al., 1995; Zoghbi and Orr, 2000). Although these polyglutamine proteins have different functions and are located in different subcellular regions, these

disorders share common pathological features: progressive neuronal cell loss, decline in physical and psychological functions and the accumulation of polyQ proteins with formation of inclusions (Zoghbi and Orr, 2000; Gatchel and Zoghbi, 2005). Moreover, it is hypothesized that they share a common mechanism of pathogenesis based in the "toxic gain of function" of the poly tract itself (Nagai et al., 2007). This inclusions occur mainly in a nuclear form and this nuclear accumulation of polyQ proteins could lead to gene transcriptional deregulation, proteotoxic stress, which could result in disruptions of the quality control systems and mitochondrial dysfunction, contributing decisively to the neuropathology (Ross and Poirier, 2004; Gatchel and Zoghbi, 2005; Havel et al., 2009). These inclusions are usually ubiquitinated and considered a common pathological hallmark in brains of patients and animal models of this disorders (Paulson et al., 1997a; Skinner et al., 1997).

Table 1 - Molecular characteristics of Polyglutamine neurodegenerative diseases

Disease	Gene and locus	Protein	Number of CAG repetitions		Localization of inclusions	Affected brain regions
			Normal	Pathogenic		
Dentarubral-pallidoluysian atrophy (DRPLA)	DRPLA 12q	Atrophin 1	6-35	49-88	Nuclear	Cerebellum, central cortex, basal ganglia, Luys body
Huntington's disease (HD)	HD 4q16.3	Huntingtin	6-35	36-121	Nuclear and cytoplasmatic	Striatum and cerebral cortex
Spinal and bulbar muscular atrophy (SMBA)	AR Xq11-12	Androgen receptor (AR)	9-36	38-62	Nuclear and cytoplasmatic	Anterior horn and bulbar neurons, dorsal root ganglia
Spinocerebellar ataxia type 1 (SCA1)	SCA1 6p22-23	Ataxin 1	6-44	39-82	Nuclear	Cerebellar Purkinje cells, dentate nucleus, brainstem
Spinocerebellar ataxia type 2 (SCA2)	SCA2 12q23-24	Ataxin 2	15-31	36-63	Nuclear and cytoplasmatic	Cerebellar Purkinje cells, brainstem, frontotemporal lobes
Spinocerebellar ataxia type 3 (SCA3)/ Machado-Joseph disease (MJD)	SCA3/ MJD1 14q24.3-31	Ataxin 3	10-51	55-84	Nuclear	Cerebellar dentate neurons, basal ganglia, brain stem, spinal cord
Spinocerebellar ataxia type 6 (SCA6)	CACNA1A 19p13	α1A Ca+2 channel	4-18	21-33	Cytoplasmatic	Cerebellar Purkinje cells, dentate nucleus, inferior olive
Spinocerebellar ataxia type 7 (SCA7)	SCA7 3p12-p21.1	Ataxin 7	4-35	37-306	Nuclear	Cerebellum, brain stem, macula, visual cortex
Spinocerebellar ataxia type 17 (SCA17)	TNP 6q27	TATA box binding protein (TBP)	25-42	47-63	Nuclear	Cerebellar Purkinje cells, inferior olive

Adapted from (Zoghbi and Orr, 2000)

1.2 Spinocerebelar ataxias

Spinocerebellar ataxias (SCAs) are a group of autosomal-dominant hereditary neurodegenerative progressive disorders characterized by the slowly degeneration of the cerebellum, often escorted by degenerative changes in the brainstem and other parts of the central nervous system (Taroni and DiDonato, 2004; Paulson, 2007). More than 30 types of spinocerebellar ataxias are currently described (Di Gregorio et al., 2014; Obayashi et al., 2014). They are numbered in the order of discovery of the defective gene, and it is possible that the number of known SCAs continues to grow regarding its minor prevalence, and the fact that are a lack of genetic diagnosis in most countries. SCAs are phenotypically and genetically very different, nevertheless some common features could be found as gait ataxia and incoordination of eye movements, speech, and hand movements (Zoghbi, 2000). The pathogenic mechanism that cause neurodegeneration are still poorly understood, and can be triggered by different causes such as a toxic gain of function in polyglutamine-expanded genes, an RNA defect in polyglutamine and/or noncoding repeat expansions, and/or a likely loss of function (La Spada and Ranum, 2010). Several molecular mechanisms could help to explain SCAs neuropathological features, like for example transcriptional regulation, protein clearance mechanism deregulation or alterations in calcium homeostasis. (Di Gregorio et al., 2014). The disease onset is usually between the 30-50 years of age, progressively evolving to a fatal state, however some childhood onset is described. Typically of trinucleotide repetitions disorders, like SCAs, most cases demonstrate an increase of severity of symptoms over the time (Schöls et al., 2000; Zoghbi, 2000; Taroni and DiDonato, 2004; Dueñas et al., 2006; Carlson et al., 2009).

1.3 Spinocerebellar ataxia type 3/ Machado-Joseph Disease

Spinocerebellar ataxia type 3 (SCA3) is also known as Machado-Joseph disease (MJD) (MJD [OMIM 109150]), a polyglutamine neurodegenerative disorder whose CAGn trinucleotide repeat expansion is localized in the MJD1/ATXN3 gene, encoding for an extended tract of glutamine repeats in the ataxin-3 protein. It was firstly described in Azorean Portuguese descents (from the islands São Miguel (Machado family) and Flores (Joseph family)) and named after the first patients described with it (Nakano et al., 1972; Rosenberg et al., 1976). Currently has an worldwide distribution comprising many ethnic backgrounds (Fig.1) (Gaspar et al., 2001; Subramony et al., 2002). Because of its variable phenotypic expression was initially identified as different pathologies: Machado disease (Nakano et al., 1972), Joseph disease (Rosenberg et al., 1978), Nigro-spino-dentatal degeneration with nuclear ophthalmoplegia (Woods and Schaumburg, 1972). Later it become accepted as a single genetic disease with a large

range of phenotypic variability (Coutinho and Andrade, 1978). Upon the identification of the causative gene MJD1 (Kawaguchi et al., 1994) the diagnosis became more precise, and indicated Spinocerebellar ataxia type 3 and Machado-Joseph disease as the same pathology (Cancel et al., 1995; Stevanin et al., 1995; Twist et al., 1995). Nowadays, MJD is considered the most frequent form among the autosomal dominantly inherited cerebellar ataxias in Europe, Japan and United States (Riess et al., 2008). Nevertheless, it is not homogenously distributed, for example, in Portugal, although relatively rare in the mainland (1:100 000), achieves the highest global incidence in Azorean Islands, particularly in Flores Island (1:239) (Bettencourt and Lima, 2011).
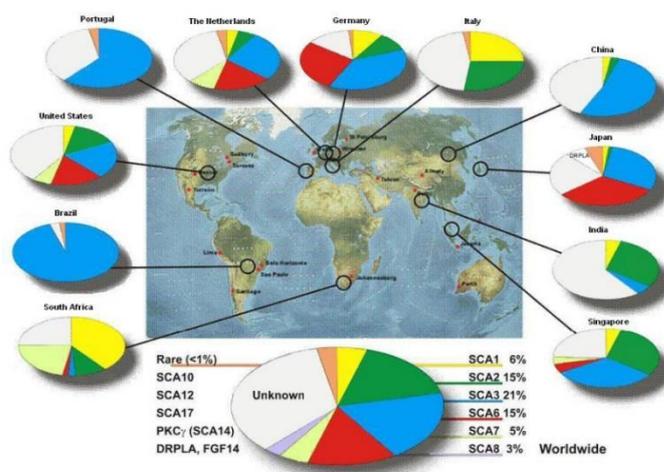
Figure 1 - Worldwide distribution of Spinocerebellar ataxias subtypes (SCAs). Adapted from (Bird, 2015).

1.3.1 Clinical features

Machado-Joseph disease is a multisystem neurodegenerative disorder characterized by an extensive variety of clinical manifestations. Common features such as ophtalmoplegia, dystonia, dysphagia, facial and lingual fasciculation-like movements, as well as progressive cerebellar ataxia that leads to a dysfunction motor coordination affecting gaze, speech, gait, and balance are considered a clinical hallmark of the disease (Lima and Coutinho, 1980; Taroni and DiDonato, 2004; Paulson, 2007, 2012; D'Abreu et al., 2010). Various systems such as the cerebellar, pyramidal, extrapyramidal, motor neuron and oculomotor systems are also affected causing brisk deep tendon reflexes, Babinski sign and spasticity, peripheral neuropathy with amyotrophy, oculomotor abnormalities with nystagmus and eyelid retraction (Rosenberg, 1992; Sequeiros and Coutinho, 1993; Sudarsky and Coutinho, 1995). In addition, beyond the typical signs, some clinical data shows that a non-motor involvement can also affect MJD patients, including sleep disorders, olfactory dysfunction and peripheral neuropathy, weight loss and affective, psychiatric and cognitive disturbances. Nevertheless, these cognitive deficiencies are mild and unlikely to develop

relevant dementia. (Rüb et al., 2008; Paulson, 2012; Pedroso et al., 2013). The age of onset of the disease is high variable, although symptoms onset usually occurs around 40 years. The reported homozygous cases are a big contribution for this wide range of onset that vary from 4 to 70 years old (Carvalho et al., 2008; Lysenko et al., 2010). Based in the diverse phenotypic variability, the Machado-Joseph disease is classified into five sub phenotypes, with the possibility of one type evolving to another during the course of the disease. These disease phenotypic sub-types illustrate the extreme clinical heterogeneity of the disease, and take mainly into account the symptomatology and the age of onset (Fowler, 1984; Riess et al., 2008; Paulson, 2012). Regarding MJD treatment, as the effective causative approaches are still missing, there are only symptomatic strategies available, like levodopa treatment for patients that display parkinsonian features, antispasmodic drugs to help reduce spasticity, and drugs that help with other symptoms such as sleep disturbances and cramps, urinary dysfunctions (Schöls et al., 1998; Buhmann et al., 2003; Freeman and Wszolek, 2005; Cecchin et al., 2007). This together with the non-pharmacological approaches, like wheelchairs for the gait problems, use of prism glasses for blurry or double vision, speech therapy to treat dysarthria and dysphagia, physiotherapy for retard the muscle atrophy, psychiatric concealing to assist the mental state of the patient, support their everyday activities and are the great allies to ameliorate the life conditions of the patients (D'Abreu et al., 2010).

1.3.2 Neuropathological features

The majority of the brains from MJD patients in a advanced disease stage present low weight when compared with brains from individuals without any neurological or psychiatric disease (Iwabuchi et al., 1999). MJD neuropathological alterations involves involves neuronal loss in selective brain regions, including the cerebellum (spinocerebellar pathways and dentate nucleus) (Fig.2A), brainstem (pons and medulla oblongata) (Fig.2A), basal ganglia (globus pallidus, caudate and putamen, substantia nigra) and spinal cord (Sudarsky and Coutinho, 1995; Dürr et al., 1996; Klockgether et al., 1998; Wüllner et al., 2005; Alves et al., 2008b; Rüb et al., 2008). Neuronal dysfunction and degeneration was described in the cerebellar hemispheres and vermis (spinocerebellar pathways and dentate nucleus), whole brainstem (including midbrain, pons and medulla), basal ganglia (globus pallidus, caudate and putamen), thalamus, substantia nigra, striatum, pontine nucleus, spinal cord (including anterior horn cells and Clarke's column) and cranial nerves (III-XII), as well as the visual, auditory, vestibular, somatosensory and urination-related systems, with relative preservation of the cerebellar cortical neurons and minimal loss of Purkinje cells (Sudarsky and Coutinho, 1995; Dürr et al., 1996; Paulson et al., 1997b; Muñoz et al., 2002; Alves et al., 2008b; Rüb et al., 2008) (Fig.2B). Furthermore, progressive hypometabolism activity such as axonal dysfunction, glucose

uptakes deficits in cerebellum, brainstem and cerebral cortex and the involvement of dopaminergic and cholinergic systems have been also reported (Coutinho and Andrade, 1978; Sudarsky and Coutinho, 1995; Dürr et al., 1996; Goti et al., 2004; Wüllner et al., 2005; Bichelmeier et al., 2007; Alves et al., 2008b; Rüb et al., 2008; Riess et al., 2008; Bettencourt and Lima, 2011; Matos et al., 2011; Costa and Paulson, 2012; Nóbrega, C. and de Almeida, 2012). A common feature of the polyglutamine diseases is the accumulation of insoluble intracellular inclusions containing the misfolded disease protein (Fig.2C) (Paulson, 1999). In MJD, the mutant ataxin-3 protein, with its polyQ extended tract undergoes conformational alterations and aggregate in ubiquitinated intranuclear neuronal inclusions (NIIs). These inclusions act as a neuropathologic hallmark of MJD in the brain, appearing in both affected and unaffected areas within more than one inclusion per neuron, being it cytoxicity still controversial (Paulson et al., 1997a; Chai et al., 1999b; Schöls et al., 2004). NIIs are spherical and eosinophilic structures with a size varying from 0.7 to 3.7μm; non-membrane bound elements, heterogeneous in their composition containing a mixture of granular and filamentous structures. Various proteins are found within the inclusions, both normal and pathogenic ataxin-3, as well as ubiquitin, molecular chaperones, proteasomal components, transcription factors and other polyQ proteins like Ataxin-2 and TBP (TATA box binding protein) (Paulson et al., 1997a; Perez et al., 1998; McCampbell et al., 2000; Uchihara et al., 2001; Yamada et al., 2001; Schmidt and Lindenberg, 2002; Hayashi et al., 2003). It was also described this type of aggregates in the cytoplasm of neurons of several non-affected areas however they display a ubiquitin-negative profile (Yamada et al., 2002, 2004, 2008; Hayashi et al., 2003).
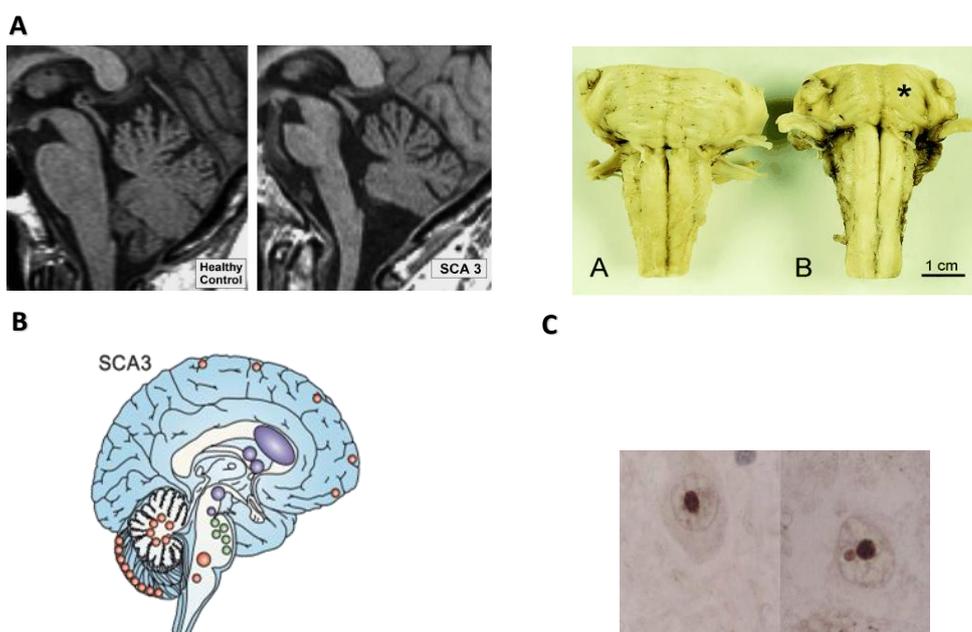
Figure 2 - Neuropathological features of Machado-Joseph disease (MJD). (A) On the left image T1-weighted images of the brain stem in patients with SCA3 and in a healthy control subject showing visible brain stem atrophy in Spinocerebellar ataxia type 3 (SCA3)/MJD. Adapted from (Eichler et al., 2011). On the right image, anterior aspect of the pons and medulla oblongata of a subject without neurological (A.A) or psychiatric historical comparison to a patient with SCA3/MJD (A.B), the patients have the same age. Note the remarkable atrophy of the pons (asterisk). Adapted from Rüb et al., 2002.(B) Schematic representation indicating the principal sites of neuronal loss and organ dysfunction in SCA3/MJD. Large dots indicate severe neuronal loss. Blue dots indicate involvement of extrapyramidal nuclei. Green dots indicate cranial nerve involvement. Adapted from (Taroni and DiDonato, 2004). (C) Neuronal intranuclear inclusions, revealed with ubiquitin immunohistochemistry assay, in two MJD patients. Adapted from Muñoz et al., 2002.

1.3.3 Genetic/molecular features

1.3.3.1 MJD1/ ATX3 gene

The MJD1/ ATX3 gene was firstly mapped in 1993 by Takiyama. The locus responsible for Machado-Joseph disease is localized in the long arm of chromosome 14 (14q32.1) (Fig. 5) containing 11 exons and span 48,240 based-pairs (bp). The (CAG)n tract is located in exon 10 in the 5' region (Takiyama et al., 1993; Ichikawa et al., 2001). ATX3 gene encodes the ataxin-3 protein whose function has been reported as linked to ubiquitin-mediated proteolysis (Burnett et al., 2003; Donaldson et al., 2003; Doss-Pepe et al., 2003; Chai et al., 2004; Durcan et al., 2011). Homozygous cases are rare, but when described patients show a much more severe phenotype (Lang et al., 1994; Carvalho et al., 2008; Lysenko et al., 2010).

Figure 3 - Schematic representation of the localization of MJD1/ ATX3 gene in chromosome 14 (14q32.1). Adapted from Twist et al., 1995.

1.3.3.2 ATX3 protein

Ataxin-3 is an intracellular deubiquitinating (DUB) enzyme belonging to the group of cysteine proteases, with an overall molecular weight of 42 kDa, containing 339 amino acids, and a variable number of glutamines (Kawaguchi et al., 1994; Nicastro et al., 2005). It is present, in mice and humans, in the different body tissues and

cell types, with a wide distribution and a variable expression levels in the brain. This discrepancy between widespread expression of ataxin-3 and selective neuronal degeneration is common to many neurodegenerative diseases in which selective tissue vulnerability cannot be explained by a restricted disease protein expression. Concerning the subcellular localization, ataxin-3 has been reported both in the cytoplasm (predominantly) and in the nucleus, and even in mitochondria (Paulson et al., 1997b; Schmidt et al., 1998; Trottier et al., 1998; Ichikawa et al., 2001; Costa et al., 2004; Pozzi et al., 2008; Paulson, 2012). Ataxin-3 biological function is not fully understood yet, however there are described different characteristics and properties, that allow to speculate about it cellular role. The evidences support its participation in several pathways, whose deregulation can compromise cell functioning and survival, like activities related to protein homeostasis maintenance (such as clearance of misfolded and damage proteins via UPP, ERAD and aggresome formation) (Burnett et al., 2003; Doss-Pepe et al., 2003; Albrecht et al., 2004; Chai et al., 2004; Berke et al., 2005), transcriptional regulation (Evert et al., 2003; Rodrigues et al., 2007a), cytoskeleton regulation (Rodrigues et al., 2010) and myogenesis (do Carmo Costa et al., 2010).

1.3.3.2.1 Structure

Ataxin-3 is composed by a conserved and structured globular N-terminal, designated Josephin domain (JD), responsible for the catalytic site with deubiquitinating activity, whereas the C-terminal is a flexible tail containing two Ubiquitin-interacting motifs (UIMs), followed by the polyQ sequence of variable length (Fig. 6) (Chow et al., 2004). In healthy individuals the trinucleotide repeat range from 10 to 51 CAG, while MJD patients exhibit repetitions between 55 to 87 CAG (Maciel et al., 2001). Nevertheless, atx3 can display different isoforms due to alternative splicing over 56 human alternative splicing variants are described, with 26 forms found exclusively in MJD patients (Bettencourt et al., 2010). So, depending on the protein isoform, the flexible tail can contain an extra UIM after the polyQ region (Goto et al., 1997; Ichikawa et al., 2001), which is the most common isoform found in human brain (Harris et al., 2010). It is hypothesized that alternative splicing of ataxin-3 may alter the properties of the encoded protein and thereby contribute to selective neurotoxicity (Harris et al., 2010). The JD is an important functional region, which presents two binding surfaces for Ub. It has the ability to cleave isopeptide bonds between Ub monomers. It belongs to the papain-like cysteine protease family, comprising ubiquitin protease activity, with the structurally conserved C14, H119, N134 catalytic triad forming the cleavage pocket (Burnett et al., 2003; Chow et al., 2004; Mao et al., 2005; Nicastro et al., 2005) establishing atx3 as deubiquitinating enzyme (DUB) The two conserved UIMs that are located in the N-terminal of the polyQ region are two α-helical structure separated by a short flexible linker region and act cooperatively when binding Ub, with greater affinity when the

two UIM are combined (Song et al., 2010). A highly conserved, putative nuclear localization signal (NLS) was found upstream of polyQ stretch (Albrecht et al., 2004, Tait et al., 1998). This signal may determine the rate of transportation of ataxin-3 into the nucleus, but display a weak nuclear import activity (Antony et al., 2009). It was also found 2 nuclear export signals (NES), NES 77 and NES 141, which localizes in the Josephin domain. There is the hypothesis that another NES (NES 174), localized following the Josephin domain, can also influence the nuclear export activity (Albrecht et al., 2004; Antony et al., 2009).
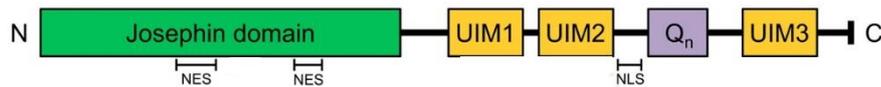
Figure 4 - Schematic diagram of Ataxin-3 protein. Atx3 is mainly composed of a globular N-terminal catalytic domain, the JD, with DUB activity, followed by a flexible C-terminal tail containing 2 or 3 UIMS and a polyQ (Qn) sequence of variable length. Additionally, ataxin-3 was described to enclose one functional nuclear localization signal (NLS) in the region linking the second UIM to the polyQ tract and two nuclear export signal (NES) within the JD. Adapted from Matos et al., 2011.

1.3.3.2.2 Function

Although the biological function of ataxin-3 has not yet been completely understood, it seems to participate in many cellular pathways. Some of the functions and biological roles of ataxin-3 seems to be linked to:

DUB activity – As previously referred atx3 behaves as a deubiquitinating enzyme (DUBs) and as an ubiquitin-binding protein, showing preference for chains of no less than four ubiquitin monomers, correlating with the type of chains necessary for the targeting of proteins for proteosomal degradation (Schmidt and Lindenberg, 2002; Burnett et al., 2003; Mao et al., 2005; Nicastro et al., 2005; Schmitt et al., 2007; Winborn et al., 2008). This class of enzymes is known for their ability to remove mono- or polyubiquitinated chains from target proteins. In this way ataxin-3 can edit the ubiquitin chain production by ubiquitin ligases and remodel or disassemble heavily ubiquitinated species (Reyes-Turcu and Wilkinson, 2009), thus regulating the ubiquitination status of proteins before their presentation to the proteasome. Upon inhibition of the catalytic activity of atx3 there is an increase of polyubiquitinated proteins (mainly localized in the nucleus) similarly to what happen when the proteasome is inhibited (Paulson, 2012).

Involvement in UPP pathway – There is evidences that atx3 is likely operating through the ubiquitin-proteosome pathway (UPP), one of the mechanism of turnover of short-duration or damaged proteins, once it binds to polyUb

chains through the UIMs (Burnett et al., 2003; Chai et al., 2004; Miller et al., 2004), and its engagement with polyUb proteins targeted for proteasomal degradation. Atx3 interacts with ubiquitined proteins, and many atx3 interactors have been suggested, participating in substrate delivery to the proteasome (Doss-Pepe et al., 2003; Zhong and Pittman, 2006). In other hand, atx3 can bind to proteasome itself and to proteins connected with the shuttling of polyUb substrates for further degradation – for example, valosin-containing protein/p27 (VCP/p27) and Rad23 – particularly involved in endoplasmic reticulum-associated degradation (ERAD) (Hirabayashi et al., 2001; Doss-Pepe et al., 2003).

Alterations in ERAD mechanism- Endoplasmic reticulum-associated degradation system (ERAD) is responsible for the ubiquitination of misfolded proteins and unassembled complexes present in the secretory route, and for their exportation into the cytosol from the endoplasmic reticulum (ER), to be degraded by the ubiquitin-proteosome system (UPS) (Burnett et al., 2003; Albrecht et al., 2004; Zhong and Pittman, 2006). Noteworthy that the complex VCP/atx3 interacts with components of ER membrane, in order to control both exportation and degradation of misfolded proteins from ER (Wang et al., 2006; Zhong and Pittman, 2006). Moreover, this complex directly transports polyUb substrates to the proteasome or to other transporting proteins, upon editing by atx3 (Wang et al., 2000; Doss-Pepe et al., 2003).

Aggresome formation - Atx3 is also implicated in regulating aggresome formation, relying on its DUB activity (Burnett and Pittman, 2005; Heir et al., 2006). In fact, aggresomes are misfolded proteins transported to perinuclear localization that form aggregates near the microtubule-organizing center (MTOC). Their physiological importance stands out when proteasome is overwhelmed or compromised, since those defective proteins are then degraded by lysosomes, leading to the maintenance of cellular homeostasis. The interaction of atx3 with other components involved in the aggresome formation, such as dynein and histone deacetylase 6 (HDAC6), part of the complex responsible for the delivery of misfolded proteins to MTOC (Burnett and Pittman, 2005; Heir et al., 2006; Rodrigues et al., 2010), as well as the interaction with tubulin and microtubule-associated protein 2 (MAP2) (Mazzucchelli et al., 2009), other elements of the cytoskeleton, supports the atx3 role in this cellular process. Supposedly atx3 is recruited to bind and trim ubiquitin chains on misfolded ubiquitinated proteins, protecting them before they reach the MTOC for aggresome formation or to stabilize proteins involved in the trafficking of misfolded proteins by its DUB activity (Burnett and Pittman, 2005).

Involvement in transcriptional regulation Another biological characteristic of atx3 concerns its capability to regulate transcription, as it can bind DNA and interact with transcriptional factors (activators and repressors) (Li et al., 2002; Evert et al., 2006). Several mechanisms may be involved, influencing the regulation of the expression of many genes. Interestingly, as many others DUBs, atx3 may also mediate the turnover of transcription regulators, which could then influence the repressor complex formation and subsequent activity (Evert et al., 2006; Rodrigues et al., 2007a)

Cytoskeleton and myogenisis impairment - Besides its contribution to cellular homeostasis and transcription regulation, atx3 is also a player in other cellular mechanisms, including myogenesis and the cytoskeleton organization. Strikingly, its absence leads to morphologic alterations, which are accompanied by the disorganization of several cytoskeleton constituents (microtubules, microfilaments and intermediate filaments) and components (tubulin, MAP2 and dynein) (Mazzucchelli et al., 2009) and also, loss of cell adhesions and increased cell death (Rodrigues et al., 2010). Considering myogenesis, ataxin-3 appears to be critical for the regulation of integrin subunit levels and once more for the organization of the cytoskeleton (do Carmo Costa et al., 2010). Still, its involvement may be conducted through an indirect way (Mazzucchelli et al., 2009; Rodrigues et al., 2010).

It was shown absence of atx3 leads to an enhanced stress response in *C. elegans,* this phenotype correlates with an alteration of the proteomic profile with several molecular chaperones abnormally up regulated during heat shock and recovery (Feder et al., 1992; Krebs and Feder, 1997). Long term deregulation of molecular chaperones (HSPs) can be detrimental for cell growth, division and viability and along with the proteotoxic stress may contribute to neuronal demise in the context of MJD (Rodrigues et al., 2011). Nevertheless, despite the implication of atx3 in many cellular pathways, knockout models of ataxin-3 orthologs in mouse and C. elegans indicate that could be an non-essential protein, as viability or fertility were not affected, and no obvious phenotype was displayed (Rodrigues et al., 2007b; Schmitt et al., 2007). Furthermore, silencing endogenous ataxin-3 in wild-type rat brain was not toxic and did not impair the function or integrity of striatal GABAergic neurons (Alves et al., 2010).
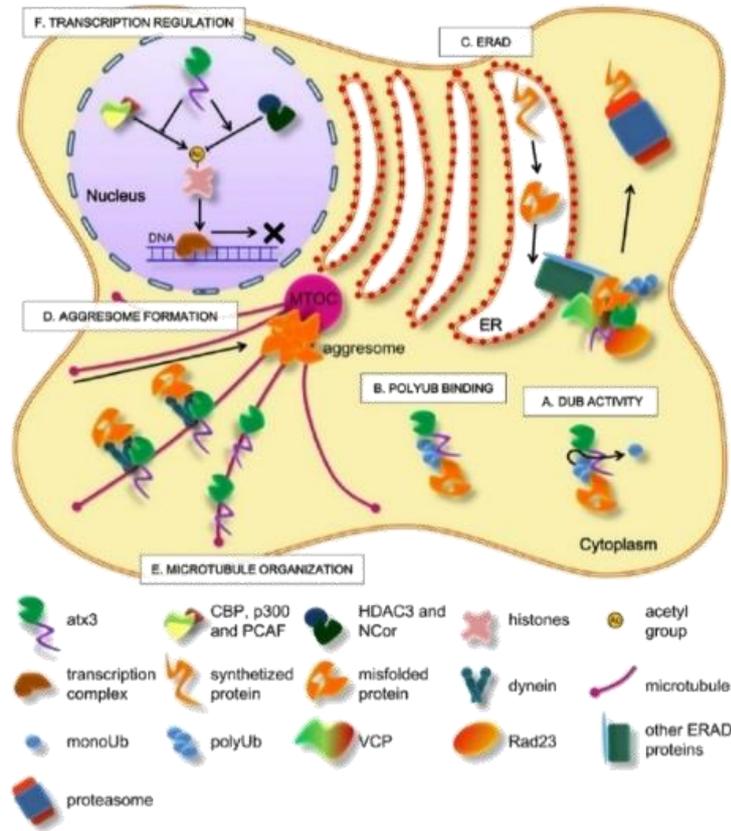
Figure 5 - Overview of the proposed biological function of ataxin-3 and its activity. (A) Atx3 displays DUB activity. In line with this, **(B)** it can interact with polyUb chains, which is a major feature suggesting axt3 involvement in protein homeostasis system through signal transduction mediated by Ub, such as UPP. **(C)** Atx3 was also proved to interact with Rad 23 and to VCP/p27 and participate in homeostasis processes in which these proteins are present, for instance, the ERAD. **(D)** Further establishment of atx3 as a promoter for aggresome formation was explained by **(E)** interactions with cytoskeletal partners (tubulin and dinein), otherwise impossible if the interplay between these proteins were not acquired. Moreover, the organization of the cytoskeleton itself is brought by atx3. **(F)** Some reports described atx3 as a player in transcription regulation owing to its interactions with regulators of histone de/acetylation. Adapted from Matos et al, 2011.

1.3.4 Pathogenesis mechanism

The genetic of MJD is well described, contrasting to the molecular basis, which is still poorly understood, and controversial. Nevertheless, like for other Polyglutamime disorders several molecular mechanisms seem to be implicated in MJD pathogenesis (Fig.4). It is known that the presence of mutant ataxin-3, with it polyglutamine expanded tract, triggers several events that lead to neurodegeneration in specific areas of the brain. It was shown that expansion of the polyQ tract in ataxin-3 affects its properties through a conformational change, acquiring toxic properties, which can result in altered molecular interactions (Paulson et al., 1997a). The presence of neuronal inclusions (NIs) is a common feature among polyQ diseases, containing the mutant protein, and thus suggesting that

misfolded proteins are pathological features for the disease. The abundance of these neuronal inclusions has been correlated with the CAG repeat size and disease severity, and it was proposed that they could also impair axonal transport and nuclear function (Paulson et al., 1997a; Seidel et al., 2010; Nóbrega and de Almeida, 2012).

Besides NIs, it has been shown that polyQ monomers of ataxin-3 acquire β-strand conformations and later assemble into oligomers. These β-rich ataxin-3 monomers and oligomers may be toxic for neurons (Bevivino and Loll, 2001; Nagai et al., 2007; Takahashi et al, 2008). The proteolytic cleavage of mutant protein may also play a role in MJD, producing smaller fragments that contain the expanded polyQ tract, which could easily entry the nucleus, and in this way undergo conformational changes that are important to aggregation (Wanker, 2000; Ross et al., 2003; Simões et al., 2012). The interaction between the mutant fragments and the full length protein lead to a misfolding event of the polyQ tract, which facilitates their incorporation into fibrillar aggregates (Ikeda et al., 1996; Haacke et al., 2006). The toxic fragments of atx3 were described *in vitro*, in transgenic models and also in MJD patients (Yamamoto et al., 2001; Goti et al., 2004), however failed to be found in other studies (Cemal et al., 2002; Berke et al., 2004; Chou et al., 2006). These findings together with the discovery of a fragment that, even with a absent polyQ tract, was able to induce MJD neurological phenotype in a mice model (Hübener et al., 2011) indicates that the toxicity is not exclusively due to the presence of the expanded polyQ, but also by post transcriptional modifications in the aminoacid residues (Fei et al., 2007; Tao et al., 2008; Mueller et al., 2009).

Also important for the MJD pathological mechanism could be the protein-protein interactions. Interestingly, normal atx3 is found in the inclusions of other diseases (SCA1, SCA2 or DRPLA) (Uchihara et al., 2001), and the activity of other proteins, like ataxin-2, in MJD neurodegeneration suggested that a normal polyglutamine protein can modulate the toxicity activity of another mutant form (Lessing and Bonini, 2008).

Moreover, NIs may compromise the recruitment of several key proteins such as transcription factors, proteasomal components and chaperones, thus affecting various cellular pathways (Paulson et al., 1997a; Chai et al., 1999a, 1999b; McCampbell et al., 2000; Schmidt and Lindenberg, 2002; Riley and Orr, 2006).

Mutant atx-3 could also contribute for an impairment in the ubiquitin-proteasome system (UPS), once the atx3 protein act as a polyubiquitin-binding protein by recruiting poly-ubiquitinated subtracts through their ubiquitin interactive motifs (UIM), and a loss of function could lead to an accumulation of misfolded proteins enhancing neuronal degeneration and cell death (Li and Chin, 2007). Impairment in autophagy was also reported (Nascimento-Ferreira et al., 2011) thus turning the cellular quality control system inefficient.

Mitochondrial dysfunction (damage of mitochondrial DNA through a decrease in the activity of antioxidant enzyme by mutant atx-3 (Yu et al., 2009)), as well as abnormal a Ca^{2+} signalling (mutant atx3 binds and activates

intracellular calcium channel (Bezprozvanny, 2009; Kasumu and Bezprozvanny, 2012)) could also be implicated in MJD and appear to play an important role in the pathogenesis mechanism. Recent data from our group also suggest that mutant atx3 drive an abnormal alteration in the levels of important translation components, leading thus to a deregulation of translation possibly increasing the mutant ataxin-3 levels and other proteins aggravating MJD pathology (Nóbrega et al., 2015 submitted).
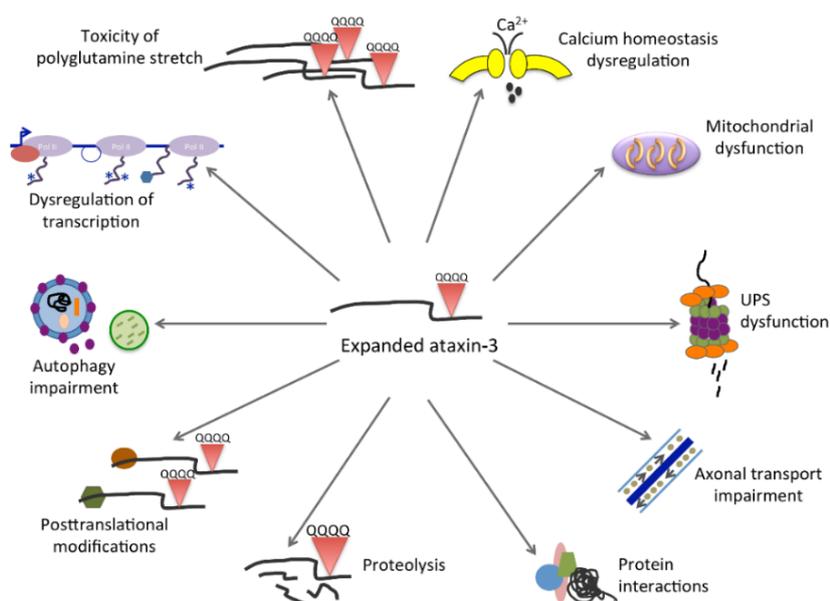
Figure 6 - Mechanisms of pathogenesis in MJD. Expanded polyglutamine proteins might mediate pathogenesis through a range of mechanisms. Adapted from (Nóbrega and de Almeida, 2012).

1.3.5 Therapeutic strategies

As mentioned before, to date, no effective treatment has been developed for MJD or other polyglutamine disorders, and only symptomatic treatment is available. For MJD, some pharmacological strategies have been performed to reduce symptoms like depression, Parkinsonism, restless legs syndrome and sleepiness (Takei et al., 2004; D'Abreu et al., 2010; Paulson, 2012). A few clinical trials have been carried out, however a well-designed clinical study showing the symptomatic effect for progressive ataxia is lacking (Correia et al.; Mello and Abbott, 1988; Azulay et al., 1994; Sakai et al., 1995; Schulte et al., 2001; Monte et al., 2003; Wilder-Smith et al., 2003; Ogawa, 2004; Bettencourt and Lima, 2011). There is a necessity for MJD therapies directed at preventing or slowing the progression of neurodegeneration. For that, further investigation of the molecular players of the cascade beyond neuronal dysfunction and cell death in MJD is vital for the development of a therapeutic approach. Currently, potential therapeutic positions pointing several known processes are addressed in order to try to find an ideal therapeutic strategy.

Activation of clearance mechanism: Up-regulation of autophagy may be a potential strategy to deal with the accumulation of the mutant proteins. Studies demonstrate that overexpression of beclin autophagic protein led to increase clearance of mutant ataxin-3 and prevented neurodegeneration (Nascimento-Ferreira et al., 2011, 2013). It was also shown a reduction of aggregates and soluble mutant ataxin-3 levels with a stability in the wild-type levels of the protein through upregulation of autophagy, with rapamycin or temsirolimus (Ravikumar et al., 2004; Menzies et al., 2010). Clearance through UPS seems to also be a remarkable target for disease therapeutic, once increasing the proteasome activity, leads to a amelioration of the neuronal death and of the neurological phenotype in MJD mouse models (Wang et al., 2013). Based on this, drugs that target these pathways are a promising therapeutic to treat the disease.

Altering toxic protein levels: The direct silencing of the causative gene appears to be a potential strategy for MJD (Boy et al., 2009). Use of interference RNA (RNAi) has been successfully reported for gene silencing in cases of genetic diseases (Bonini and La Spada, 2005). Targeting small nucleotide polymorphism (SNP) specific for a majority of MJD patients with RNAi was able to silence specifically mutant ataxin-3 and decrease the inclusion formation and neuronal dysfunction (Alves et al., 2008a, 2010; Nóbrega et al., 2013, 2014). This proves that direct silencing of atx3 could be an efficient approach to MJD treatment. However some issues as to be concerned regarding this type of technic. A safety delivering system, a specific target for the mutant protein and the evaluation on the long-term effect are questions to be addressed and answered before the application of this technic in a human context.

Modulation of Calcium homeostasis: It was suggested an involvement of calcium deregulation in MJD pathology. Deranged Ca^{2+} signalling may play an important role in MJD pathology and calcium signalling stabilizers such as dantrolene could be considered as potential therapeutic drugs for treatment of MJD patients, as it improved mice motor performance and prevented neuronal cell loss in pontine nuclei and substantia nigra regions (Chen et al., 2008b). Caffeine, operating through A2a receptors, by blocking them, demonstrated a decrease in neuropathology in MJD models (Gonçalves et al., 2013). The mechanism of actuation has not been elucidated yet, but it might function through normalized glutamatergic transmission (Popoli et al., 2007) and prevention of calcium-dependent proteolysis and aggregation of the mutant protein (Koch et al., 2011).

Prevention of transcriptional deregulation: Expanded ataxin-3 has been shown to repress transcription, as mRNAs related to signal transduction, calcium mobilisation and neuronal differentiation are found downregulated. This suggests that transcriptional deregulation might play a central role in neurodegeneration mechanism of polyQ

disorders (Chou et al., 2008). Use of histone deacetylase (HDAC) inhibitor (sodium butyrate) show reverse mutant atx3-induced histone hypoacelylation and transcriptional downregulation in the cerebellum, as well as improved ataxic symptoms of MJD mouse models. This suggests that transcriptional modulators could be an effective treatment to MJD.

Inhibition of proteolytic cleavage: The fragments that result from mutant ataxin-3 were proposed to contribute to neurotoxicity (Ikeda et al., 1996; Goti et al., 2004; Colomer Gould, 2005; Haacke et al., 2006). As caspases are involved in several brain functions, influencing apoptosis, synaptic plasticity, dendritic development and memory formations, it inhibition would not be applicable (Troy and Salvesen, 2002; Li and Sheng, 2012; Troy and Jean, 2015). Inhibition of calpains has demonstrate a reduction of mutant ataxin-3 toxicity in several in vitro and in vivo models (Haacke et al., 2007; Koch et al., 2011; Simões et al., 2012; Hübener et al., 2013), however a lack of specificity among calpain isoforms and other proteolytic enzymes exhibit the necessity of further investigations on this field.

Prevention of aggregation: Ataxin-3 aggregates are a hallmark of MJD, and tt is therefore likely to be involved in the pathogenic process. Actually, the use of molecular chaperones has demonstrated a reduction in aggregation and toxicity of expanded polyglutamine tracks in several mouse models, ameliorating their phenotype (Cummings et al., 2001; Adachi et al., 2003). Evidences demonstrate that some chaperones increase the solubility of expanded polyQ and alleviates toxicity (Chan et al., 2000; Adachi et al., 2003), probably resulting from proteasome degradation (Bailey et al., 2002; Verhoef et al., 2002). Different chemical compounds have been tested in polyQs disorders to prevent aggregation; however the needed concentrations for an efficient aggregates clearance are cytotoxic (Yoshida et al., 2002) and there is a lack of phenotypic improvement (Smith et al., 2003; Schilling et al., 2004; Wood et al., 2007). A useful tool to target specifically aggregation is focused in vector encoding to small antibody fragments, the intrabodies. It has been demonstrated for other polyQ, Huntington's disease, that they are capable of reduction aggregation and improve neurological symptoms. Discover an intrabody that could bind mutant ataxin-3 and consequently decrease aggregation could be a potential candidate for a strategy relying in toxicity inhibition. Although, despite the numerous approaches of preventing aggregation, no promise candidate for clinic evaluation has been identified yet.

Prevention of mutant ataxin-3 formation: Wild-type ataxin-3 functioning displays an indispensable role to cell maintenance (Rodrigues et al., 2007a; Schmitt et al., 2007; Alves et al., 2010). An allele-specific downregulation target to mutant ataxin-3 form is the most accurate and favourable strategy for a therapeutic application in MJD as well as for other monogenetic neurodegenerative disorders (Miller et al., 2003; Rodriguez-Lebron and Paulson, 2005). This specificity could be achieved through RNAi pathway using shRNAs directed through a single SNP unique to the mutant form, present in over 70% of MJD patients. With this strategy was possible to specifically silence mutant ataxin-3 and work as a neuroprotector in MJD rodent models. (Gaspar et al., 2001; Alves et al., 2008a; Nóbrega et al., 2013). However optimization of RNAi delivery therapeutic in the central nervous system need to be addressed in order to use it as a therapeutic strategy. Or by the targeting of the expanded polyglutamine tract directly. Antisense oligonucleotides (AONs), peptide nucleic acids (PNAs) as well as RNAi-based approaches with abasic substitutions and stranded silencing (ssRNAs), are able to specific silencing, in vitro, mutant ataxin-3, by binding to expanded CAG repetetions, resulting in a translational blockage of mutant ataxin-3 (Evers et al., 2011; Hu et al., 2011; Liu et al., 2013a, 2013b). Other strategy to specifically silence mutant ataxin-3 was remove the polyQ repetitions through exon skipping in this case the toxic polyQ expansion can be removed while the protein levels are not altered and but retained the main functional domains and ubiquitin binding capacity (Evers et al., 2013) All this oligonucleotide-based approaches together with knowledge from it use in other neurodegenerative disorders , regarding safety, potency and oligonucleotide distribution in the brain turn this approach as a potential therapeutic treatment for MJD.

1.4 Fungi in Traditional Chinese Medicine

For many centuries, fungi have been used as folk medicine in Oriental practice. However in the last decade the overwhelming interest of western research community in it potential pharmaceutical value has increased considerably, due to the enormous amount of benefits linked to this "biotherapeutics". These natural compound could act as anti-oxidant, anti-diabetic, anti-microbial, anti-tumor, anti-cancer, hypocholesterolemic, immunodulatory, anti-allergic, could also improve respiratory and renal functions and treat heart disease, including arrhythmias (Zhu et al., 1998; Patel and Goyal, 2012; Yang et al., 2012). Taken this into account, it has been carried a lot of investigations to find the main compounds responsible for each one of this benefits and to unveil their underlying mechanisms of biological action.

1.4.1 *Cordyceps spp.*

The genus Cordyceps is a entomopathogenic fungus, frequently used in Traditional Chinese Medicine (TCM) ((Ng and Wang, 2005; Tuli et al., 2013). Cordyceps has been used by over 300 years in eastern Asia for its tonic and medicinal proprieties and was introduced in Western society during the 17th century (Yue et al., 2013). The taxonomy of this genus is controversial, in some cases it is classified over the morphological aspect, in other for the host affiliation, and even classified using some phylogenetic studies with ribosomal. In Sung et al., 2007, was studied several genes and purposed a new classification dividing the genus Cordyceps in three major clades. It belongs to the phylum Ascomycota classified in the order hypocreales (Wang et al., 2008) containing over 400 species, including *Cordyceps sinensis* (Berk.) Sacc. (CS) and *Cordyceps militaris* (Fr.) L.. (CM) (Fig.8). Several studies using these two species have been conducted, showing in addition to its anti-tumoral and immunodulatory activity (Kim et al., 2008; Rao et al., 2010; Patel and Goyal, 2012; Yang et al., 2012), to ameliorate renal fibrosis (Zhang et al., 2012), reverse memory impairments (Lee et al., 2011) and induced apoptosis and autophagy in gliobastoma cells (Yang et al., 2012) among others beneficial effects. It is described the presence of many bioactive constituents in the fungi of this genus: polysaccharides, cordycepin and cordycepic acid, mannitol, aminophesnol and ergosterol. Since the encouraging researches regarding this group several studies concerning different compounds have been performed (Yue et al., 2013).

Figure 7 - Cordyceps militaris. ©Copyright Malcolm_Storey 2011-2112

1.4.2 Cordycepin

Cordycepin or 3'deoxyadenosine (figure 8) is a derivative of the nucleoside adenosine, differing from it by the lack of an oxygen in the 3' position of its ribose (Paterson, 2008). Cordycepin has received special attention duo to its broad-spectrum biological activity (Cunningham et al, 1950). Its involvement is described in various biochemical and molecular processes such as purine biosynthesis (Overgaard, 1964; Rottman and Guarino, 1964b), DNA/RNA synthesis (Holbein et al., 2009) or mTOR (mammalian target of rapamycin) transduction (Wong et al., 2010).
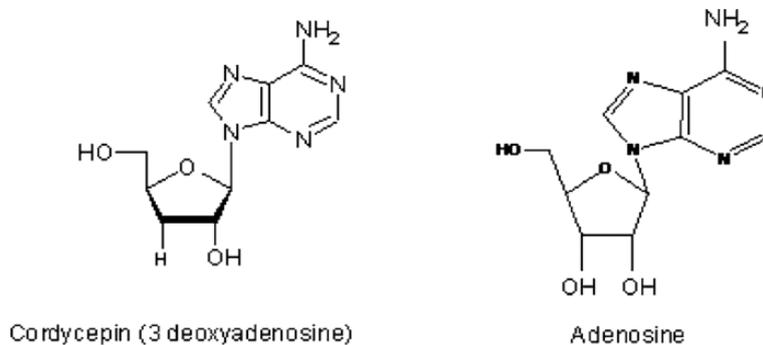
Figure 8 - Representation of chemical structures of the bioactive compounds, cordycepin and adenosine, existent in *Cordyceps militaris*, figure adapted from Tuli et al., 2013.

1.4.2.1 Mechanism of action

Purine biosynthesis inhibition: The structural similarity of cordycepin and adenosine or deoxyadenosine suggests the possibility that this compound might function as anti-metabolic and interfering with nucleic acid synthesis by this incorporation into RNA molecules causing its premature termination. It also, is converted into 5' mono, di- and tri-phosphates forms, which inhibit the activity of enzymes essential to de novo biosynthesis of purines, such as 5-phosphoribosyl-1-pyrophosphate amidotransferase and ribose-phosphate pyrophosphokinase (Klenow, 1963; Overgaard, 1964; Rottman and Guarino, 1964b).

RNA synthesis/ Polyadenylation inhibition: As adenosine is a nitrogenous base and act as a nuclear nucleoside, essential for various molecular processes in cells, such as DNA or RNA synthesis. As it only differ from cordycepin by the lack of an hydroxyl moiety in the 3' position, it is normal if some enzymes do not distinguish between both compounds incorporating cordycepin instead of normal nitrogenous bases. Some studies with mutant poly(A) yeast demonstrate that cordycepin also play an important role in the polyadenilation metabolism. It was observed that it

interferes at different levels with several mRNAS: the bigger the poly(A) tail more effective is its inhibition (Horowitz et al, 1976; Müller et al., 1977; Chen et al., 2008a; Holbein et al., 2009; Wong et al., 2010).

mTOR signal transduction interference/ translation inhibition: It is known that cordycepin shorten polyadenylation elongation of mRNAs affecting their stability (Holbein et al., 2009; Kitamura et al., 2011). At high doses it even inhibits the cell attachment and reduce the focal adhesion between cells (Holbein et al., 2009). Results in fibroblasts and HeLa cells demonstrate that cordycepin is a strong inhibitor of translation in mammalian cells. However, the direct translation inhibition fail to be proven indicating that the effect is indirect. One possible explanation for this is the shutdown of the mTOR signalling pathway, which plays an important role in the regulation of protein synthesis. Cordycepin activates AMPK pathway, which mediates mTOR signalling through the activity of Akt protein kinase. Conversely, in the presence of a AMPK inhibitors the translation inhibition cordycepin-mediated is blocked and AKT is dephosphorylated. This indicates that cordycepin act inhibiting the translation process mediated by the activation of AMPK, leading consequently to an inhibition of cell proliferation and cell growth (Wong et al., 2010; Ferrari et al., 2013; Tuli et al., 2013). Upon cordycepin treatment it is also described a phosphorylation of the eukaryotic translation initiation factor 2α (elf2α) (Kitamura et al., 2011; Kadomatsu et al., 2012) which could also explain a translational suppression.

1.4.2.2 Cordycepin as an anti-cancer agent

The anti-tumor effects of cordycepin have been observed in a variety of cancer cells, as revealed by several in vitro and in vivo studies. From the in vivo studies its was proven an increased in the survival time and the inhibition of the tumor growth (Jagger et al., 1961). Some of the effects of cordycepin included both the suppression of cell growth/tumor proliferation and the reduction of cell viability (He et al., 2010; Choi et al., 2011; Kadomatsu et al., 2012; Lee et al., 2012, 2013, 2014). However concerning the mechanism of actuation the results are not totally in concordance, meaning that under different stress conditions or even dependent of the type of cell type the drug could act through different mechanisms. Some claim that cordycepin apoptotic activity is due to a mitochondria-mediated pathway (Choi et al., 2011), or through TNF-α -induced pathway (Kadomatsu et al., 2012), through Death Receptor-3 (DR3) pathway (Kadomatsu et al., 2012; Lee et al., 2013) or even through enhanced expression of pro-apoptotic molecules and ROS-mediated activation of caspases (He et al., 2010; Lee et al., 2014). Others hypothesize an anti-apoptotic property dependent of Adenosine receptors 1 (A1) (Jin et al., 2014) or of Adenosine receptors 3 (A3) (Kitamura et al., 2011; Kadomatsu et al., 2012). A possibility of the autophagy

involvement it is not set aside, as a complement cell mechanism (Choi et al., 2011) or as a pro-survival compensatory mechanism (Lee et al., 2014).

1.4.2.3 Cordycepin and it immunodolatory effect

In the past few years, several reports have shown that cordycepin has a potential immunodolatory effect. Inflammatory cytokines mediate a well-defined inflammatory response in many cells types through transcription factors families, determining which gene respond to inflammatory stimuli. It is described the inhibition effect of cordycepin in some inflammatory genes via supressing the transcription factor NF-κ B activation (Kim et al., 2006; Shin et al., 2009; Jeong et al., 2010; Ren et al., 2012; Peng et al., 2015), an important transcription factor involved in maturation and survival of T-lymphocytes that induced expression of genes products mediating innate and adaptive immunity (Ahn et al., 2010). It influence in some interleukin, is also reported, decrease of IL-1β , a proinflammatory cytokine (Noh et al., 2009; Shin et al., 2009; Jeong et al., 2010; Zhang et al., 2014), up regulation of the anti-inflammatory IL-10 expression (Zhou et al., 2002, 2008) display an important evidence of cordycepine effect in the immune response. However for the regulation of the timing of the immune response, the stability of mRNAs produced, by that inflammatory genes is the main participant (Hao and Baltimore, 2009). And even in this field cordycepin display an important role once it is able to specifically inhibit the induction of inflammatory mRNAs by citokines without affecting the housekeeping mRNAs expression. Cordycepin can affect this mRNAs post-transcriptionally, when the mRNAs disappears during or immediately after the nucleus export or affect directly the mRNA production by inhibit the transcription elongation (Kondrashov et al., 2012b). Effects in microglia overactivation impairments were also detected demonstrating a part of cordycepin in neuroinflamation (Peng et al., 2015). Several studies regarding immunological related diseases shows a amelioration due to the immunodolatory effect of cordycepin, such as osteoporosis (Zhang et al., 2014) and asthma (Yang et al., 2015).
Demonstrating a potential biomedical application of cordycepin in inflammation associated disorders.

1.4.2.4 Cordycepin and it neuroprotective properties

About the neuroprotective action of cordycepin and its molecular mechanism there are a few data reported until now. Studies in ischemic/reperfusion cerebellar models demonstrated that in the presence of cordycepin neuronal degeneration and brain slice injury were prevented. Moreover it was reported less cell death, less neuronal loss, low glutamate and aspartate levels (that at higher doses lead to neurotoxicity and cell damage), decreased in

the Melandialdehyde oxidation (MDA) levels (marker of ER stress) and increase in the antioxidant activity of the Superoxidade dismutase (SOD). It is also believed that cordycepin exert a neuroprotective effect against oxidative toxicity induced by glutamate. This was demonstrated in HT22 cells by the decrease in the neuronal cell death caused by high glutamate levels. Cordycepin block cell death through the inhibition Reactive Oxigen Species (ROS) production and the influx of Ca2+; and thus recovering the mitochondrial membrane potential (MMP), demonstrating an involvement of the mitochondrial pathway in the glutamate-induced apoptosis. A descrease in the apoptotic behavioural of glutamate-injured cells, was detected by the reduction of Endoplasmatic Reticulum stress (ER stress) and of the activation of MAPK pathway. It appears that the Adenosine receptor A1 (mainly expressed in regions such as hippocampus and cerebellar cortex and which activation it is described to promote neuron recovery) plays a part in this neuroprotective effect of cordycepin. In a way that the inhibition of the A1 receptor reverse the cordycepin's recovery of toxicity glutamate-induced, whereas the blockage of the others adenosine receptors not altered the function and ameliorations of the compound (Jin et al., 2014).

It was shown a role in the inflammation route by preventing the induction of the expression of the Matrix metaloproteinase 1 and 3 (this last one described as involved in neuroinflammation) and by blocking p38 and JNK phosphorylation (Cheng et al., 2011). In adition, it was demonstrated a supportive evidence of the protective role of cordycepin against the impairments of brain caused by neuroinflammation. Microglial cells are normally activated in response to brain injury or immunological stimuli to protect the central nervous system (CNS) however when over-activated conversely amplifies the inflammatory effects and mediates cellular degeneration, leading to exacerbated neuroinflammation and neuronal death. Cordycepin acts through the inhibition of TNF-α , IL-1β , iNOS and COX-2. Cordycepin treatment was able to recover the neuronal death caused by microglial over-activation and successfully rescue the inflammation-induced impairments of neural growth and development in the hippocampal cultured neurons. These data suggest that cordycepin could penetrate the brain and act as an anti-inflammatory agent in treating brain injury thus demonstrating a neuroprotective role against neuroinflammation-induced impairments (Peng et al., 2015). Taking all this together we might it is easy to assume that cordycepin could act as a potential future therapeutic agent for neuronal disorders.

1.4.2.5 Cordycepin and others properties

Regarding all the properties and effects, cordycepin is related to many other different activities: anti-fungal (Sugar and McCaffrey, 1998), anti-bacterial (Rottman and Guarino, 1964a), anti-viral (Wu et al., 1972; White and Dawson, 1979; Xu et al., 2005), anti-diabetic (Yun at al, 2003; Shin et al., 2009) and anti-parasitic (Trigg et al.,

1971; Rottenberg et al., 2005; Vodnala et al., 2009; Dalla Rosa et al., 2013). Taking all this into account and the low toxicity associated with the use of cordycepin it is right to assume that this compound demonstrate a strong pharmacological and therapeutic potential to attenuate many dreadful diseases in future. Although further investigations need to be focused on the study of the specific mechanistic of this wonderful insight into the potential of this medicinal mushroom on human health for ethno-pharmacological use.

1.4.2.6 Clinical trials

There are currently two clinical trials finished or ongoing testing the use of Cordycepin in humans. The one that it is already completed were a two-part, open-label, Phase I/II study in subjects with relapsed or refractory TdT-positive leukemia for which no standard therapies were expected to result in durable remission. The objectives of this study were firstly to define the maximally tolerated dose (MTD) and recommended dose (RD) for administration of cordycepin. In the second phase, the objectives were to assess the safety, multiple dose pharmacokinetics (PK), and clinical outcomes of cordycepin in combination with pentostatin at the RD. Pentostatin is an anticancer chemotherapeutic drug, that is classified as a purine analog, a type of antimetabolite. It is proved that when co-administered tend to extend the half-life of cordycepin potentiating it effect, as cordycepin is converted by ADA to an inactive form (Adamson et al., 1977; North and Cohen, 1978). In OncoVista, Inc, Study of Cordycepin plus Pentostatin in Patients with Refractory TdT-Positive Leukemia, In: ClinicalTrials.gov [Internet], Bethesda (MD): National Library of Medicine (US). 2000- [Cited 2015.05.26]. Available from: https://clinicaltrials.gov/ct2/show/NCT00709215 NLM Identifier: NCT00709215.

The second trial, currently active, attempts to test drugs in chemotherapy treatment using different ways to stop tumor cells from dividing so they stop growing or die. In the Phase I trial they propose to study the effectiveness of chemotherapy consisting of cordycepin plus pentostatin in treating patients with refractory acute lymphocytic or chronic myelogenous leukemia. With the goals of evaluate the safety, maximum tolerated dose, adverse effects, and toxicities of cordycepin/pentostatin treatment. In Boston Medical Center, Chemotherapy With Cordycepin Plus Pentostatin in Treating Patients With Refractory Acute Lymphocytic or Chronic Myelogenous Leukemia, In: ClinicalTrials.gov [Internet], Bethesda (MD): National Library of Medicine (US). 2000- [Cited 2015.05.26]. Available from: https://clinicaltrials.gov/ct2/show/NCT00003005 NLM Identifier: NCT00003005.

OBJECTIVES

The general objective of this work was to evaluate the effect of treatment with Cordycepin in Machado Joseph disease models, concerning its different properties and potential neuroprotective effect, specifically it capability to inhibit/reduce translation.

The specified objectives are:

- To evaluate the optimal dose for cordycepin treatment in neuroblastoma cell line.

- To investigate the effect of cordycepin in the translational levels in a cellular model of MJD.

- To investigate the effect of cordycepin in the levels of mutant and endogenous Atx3 in a MJD cellular model.

- To evaluate the effect of treatment with cordycepin at a neuropathological level in a lentiviral MJD mouse model.

- To evaluate motor coordination and activity profile by motor behaviour test upon cordycepin treatment in a transgenic MJD mouse model.

CHAPTER II - MATERIALS AND METHODS

2.1 IN VITRO EXPERIMENTS

Lentiviral vectors

Viral vectors encoding for human full-length mutant ataxin 3 with 72 glutamines (Atx3Q72) and human wild-type ataxin-3 (Atx3-27Q) (Alves et al., 2008b), were produced in human embryonic kidney (HEK) 293T cells using a four-plasmid system described previously (de Almeida et al., 2001). The lentiviral particles were produced and resuspended in 0.1M phosphate-buffered saline (PBS) with 0.5% bovine serum albumin (BSA), and samples were matched for particle concentration by measuring HIV-1 p24 antigen content (RETROtek, Gentaur, Belgium) Viral stocks were stored at -80 C until use.

Neuroblastoma cell culture

Mouse neuroblastoma cell line (Neuro-2A cells) obtained from the American Type Culture Collection cell biology bank (CCL-131) were incubated in Dulbecco's modified Eagle's medium supplemented (DMEM) with 10% foetal bovine serum (FBS), 100 U/ml penicillin and 100mg/ml strepto- mycin (Gibco) (complete medium) at 37 C in 5% CO_2/ air atmosphere.

Neuroblastoma cell cultures infection

Cells were plated and infected with lentiviral particles expressing human full-length mutant ataxin 3 with (Atx3Q72) or with human wild-type ataxin-3 (Atx3-27Q) at the ratio of 10ng of p24 antigen/10^5 cells. The infection was performed with 400μl of new medium, hexadimethrin bromid (Sigma-Aldrich) (8μg/ml) and the corresponding lentivirus vectors. The multiwells were incubated at 37°C in 5% CO_2/air atmosphere. After 8h of infection 600μl of fresh medium was added to the cells. At least two weeks post-injection, cordycepin treatment was performed. Transduced cells were stored at -80°C in 10% Dimethyl sulfoxide (DMSO) 90%FBS for posterior use.

Cordycepin treatment

Neuro-2A cells were plated and 24h after were treated with growing concentrations of drug during different times of treatment. Cordycepin (100mg/ml, Sigma, USA) diluted in DMSO, 1μM, 10μM, 20μM, 50μM, 100μM during 12h, 24h, 48h, 72h, 96h. Upon choosing the ideal concentration and time point, the procedure was repeated only with the defined set.

Cell Viability/ Alamar Assay

Cell viability under the different experimental conditions was assessed by a modified Alamar blue assay. At several time-points after transfection or infection, the cells were incubated with DMEM containing 10% (v/v) Alamar blue dye. After a 1h incubation period at 37°C, the absorbance of the medium was measured at 570 nm and 600 nm. Cell viability was calculated as a percentage of the control cells (non-transfected/infected), according:

Cell viability (% of control) = [(A570 − A600) of treated cells x 100/(A570 − A600) of control cells].

Puromycin Translation Rate Assay

Neuro-2A cells were plated and 24h after were incubated with cordycepin at 20 µM, 100µM and 200µM for 1h of treatment. After 45min of the start of the experiment puromycin (10µg/ml) was added to all conditions. Ciclohexamide (CHX, 10µg) was used as a positive control and a well of cells non-treated as a negative control. Cells were collected and translation inhibition measured by Western Blot (Schmidt et al., 2009).

Protein extraction and western blotting

Cells were lysed in RIPA-buffer solution (50mM Tris HCl pH 8, 150nM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (Roche diagnostics) followed by sonication of 4s ultra-sound chase (1 pulse/s). Protein concentration was determined with the PierceTM BCA Protein Assay Kit (Thermo). Sixty micrograms of protein extract were resolved in SDS-polyacrylamide gels (10%). The proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (GE Healthcare) according to standard protocols. The membranes were blocked by incubation in 5% non-fat milk powder in 0.1% Tween 20 in Tris buffered saline (TBS-T) for 1 h at room temperature, and were then incubated overnight at 4 C with mouse monoclonal anti-ataxin 3 (Millipore, 1:5000), mouse anti-puromycin (Millipore, 1:20000), mouse anti-tubulin (Sigma; 1:5000) mouse anti-actin (Sigma; 1:5000) antibodies. Blots were washed three times in TBS-T, for 10 min each, and incubated with the secondary antibody goat anti-mouse (1:10 000; Vector Laboratories) for 2h at room temperature. Blots were washed three times in TBS-T, for 10min each and the bands were visualized with Enhanced Chemifluorescent substrate (ECF) (GE Healthcare) and chemifluorescence imaging (Versadoc Imaging System Model 3000, Bio-Rad). Semi-quantitative analysis was carried out based on the optical density of scanned membranes (Quantity One®1-D image analysis software version 4.4; Bio-Rad). The specific optical density was then normalized with respect to the amount of β-actin or α-tubulin loaded in the corresponding lane of the same gel.

2.2 IN VIVO EXPERIMENTS

2.2.1 Lentiviral mouse model

Animals

8-week-old C57/BL6 male mice (Charles River) were used in this experiment. The animals were housed in a temperature-controlled room maintained on a 12h light/ 12h dark cycle. Food and water were provided ad libitum. The experiments were carried out in accordance with the European Community directive (86/609/EEC) for the care and use of laboratory animals. The researchers received adequate training (FELASA-certified course).

Stereotaxic injection in the striatum

Concentrated viral stocks were thawed on ice and resuspended by repeated pipetting. Lentiviral vectors encoding for mutant ataxin-3 (ATX-3 72Q) were stereotaxically injected into the right hemisphere of the striatum in the following coordinates: antero-posterior: +0.6mm; lateral; -1.8mm; ventral: -3.3mm; mouth bar: 0. Animals were anesthetized by administration of a mixture of ketamine (100mg/kg, Clorketam 1000, Vétaquinol) with xylazine (10mg/kg, Rompun®, Bayer) by intraperitoneal injection (ip). Particle contents of the viral vectors were determined by p24 antigen ELISA (RETROtek, Gentaur, France) and a single 1 µl injection of 400,000ng were injected at a rate of 0.25 µl/min by means of an automatic injector (Stoelting Co., Wood Dale, IL, USA) into the mouse brain area through a 34-gauge blunt-tip needle linked to a Hamilton syringe (Hamilton, Reno, NV, USA). After injection, the syringe needle was left in place for an additional 5 min to allow the viral vector diffusion and to minimize backflow. Mice were kept in their home cages for 4 weeks of treatment before being sacrificed.

Treatment with Cordycepin

One week after lentiviral vectors injections, animals started the treatment with cordycepin (3'-deoxyadenosine) acquired from Sigma-Aldrich, (EUA). Otherwise indicated, all reagents were diluted in DMSO (100mg/mL) with NaCl 0,1% as vehicle. A daily intraperitoneal 40µl injection of 20mg/Kg of Cordycepin-NaCl 0,1% was administrated to the treated group (n=9), whereas a 40µl injection of the vehicle (NaCl 0,1%) was administrated to the control group (n=9) (five days a week). The concentrations were adjusted to the weight of each animal.

Immunohistochemical procedure

After an anaesthetic overdose the transcardial perfusion of the mice was performed with a phosphate solution followed by fixation with 4%paraphormaldehyde (PFA). The brains were removed and post-fixed in 4% PFA for 24h and cryoprotected by incubation in 25% sucrose/ phosphate buffer (PBS) for 48 h. The brains were frozen and store at -80ºC. Subsequently 20μm coronal sections were cut using a cryostat (LEICA CM3050 S) at -21°C. Slices throughout the entire striatum were collected in anatomical series and stored in 48-well trays as free-floating sections in PBS supplemented with 0.05 μM sodium azide. The trays were stored at 4°C until immunohistochemical processing.

The immunohistochemical procedure for light microscopy was initiated by incubating free-floating sections for 1 h at 37ºC in PBS containing 0.1% diphenylhydrazine, to inhibit endogenous peroxidases. The sections were incubated at RT for 1h in blocking solution (0.1% TritonX 100 containing 10% Normal Goat Serum (NGS, Gibco) in PBS), and then with the appropriate antibodies: anti-ubiquitin (1:500; Millipore, O/N 4ºC), NeuN (1:1000, Chemicon International. O/N 4ºC) diluted in the blocking solution. After O/N incubation three washings were performed and the sections were incubated with the corresponding biotinylated secondary antibody (1:200; Vector Laboratories Inc., CA, USA) diluted in the blocking solution for 2h at RT. After three washes, bound antibodies were visualized by the ABC amplification system (Vectastain ABC kit, Vector Laboratories, West Grove, USA) and 3,3′- diaminobenzidine tetrahydrochloride (peroxidase substrate kit, DAB, Vector Laboratories, CA, USA) as the substrate. The sections were mounted, hydrated (with H2O mQ) and then dehydrated by passing through an increased degree of ethanol solutions (ETOH 75%, 96% and 100%) and xylene solution, and coverslipped with Eukittw (O. Kindler GmbH & CO, Freiburg, Germany).

Fluorescence immunohistochemical procedure was also performed. Free-floating sections were incubated at RT for 1h in blocking solution (0.1% Triton X-100 containing 10% (NGS, Gibco) in PBS), and then in the blocking solution containing the appropriate e antibodies: anti-IBA-1 (1:1000, Wako, O/N 4ºC), anti -GFAP (1:1000, DAKO, O/N 4ºC),. After three washes, the sections were incubated with the corresponding secondary antibodies coupled to fluorophores (1:200; Molecular Probes, OR, USA or Vector Laboratories) diluted in blocking solution for 2h at RT. The sections were washed three times and then mounted in Mowiol Reagent in microscope slides. Staining was visualized using Zeiss Axioskop 2 plus, Zeiss Axiovert 200 and Zeiss LSM 510 Meta imaging microscopes (Carl Zeiss Microimaging, Germany), equipped with AxioCam HR color digital cameras (Carl Zeiss Microimaging) using 5x, 20x and 40x Plan-Neofluar objectives and the AxioVision 4.7 software package (Carl Zeiss Microimaging). Quantitative analysis of fluorescence was performed with a semiautomated image-analysis software package (Image J software, USA).

Evaluation of the volume of the NeuN depleted volume

The extent of ataxin-3 lesions in the striatum was analysed by photographing, with a x20 objective, eight NeuN stained sections per animal (20 µm thickness sections at 200 µm intervals), selected so as to obtain complete rostrocaudal sampling of the striatum, and by quantifying the area of the lesion with a semi-automated image analysis software package (Image J software, USA). The volume was then estimated with the following formula: volume=d(a1+a2+a3!), where d is the distance between serial sections (200 µm) and a1+a2+a3 are NeuN depleted areas for individual serial sections.

Quantification of ubiquitin inclusions

Coronal sections showing complete rostrocaudal sampling (1 of 8 sections) of the striatum were scanned with a x20 objective using a Zeiss Axiovert 200 M imaging microscope Zeiss (Zeiss, Germany) motorized for X, Y and Z displacements using the image acquisition and analysis system PALM Robot Software (version 4.0). The analysed areas of the striatum encompassed the entire region containing ATX-3 and ubiquitin inclusions, as revealed by staining with the anti-ataxin-3 and anti-ubiquitin antibodies. All inclusions were manually counted using a semi-automated image-analysis software package (Image J software, USA) Section lighting was similar for all acquisitions and was automatically corrected using blank images. Images were automatically segmented for the quantification of dark objects (aggregates/inclusions), using the same parameters defining the light intensity threshold. For all images, objects touching one of the X or Y borders of the fields of view were not counted. For each animal, the estimated total number of inclusions was estimated.

2.2.2 Transgenic mouse model

A Machado Joseph disease transgenic mouse model (C57BL/6 background) expressing the N-terminal-truncated human ataxin-3 with 69 glutamine repeats together with an N-terminal hemagglutinin (HA) epitope in cerebellar Purkinje cell, driven by the L7 promoter was used (Torashima et al., 2008), and maintained in the animal house facility of the Centre for Neuroscience and Cell Biology (CNC) of the University of Coimbra by backcrossing heterozygous males with C57BL/6 females (Nascimento-Ferreira et al., 2013). The animals were housed in a temperature-controlled room and maintained on a 12h light/dark cycle. Food and water were available *ad libitum*. The experiments were carried out in accordance with the European Community Council Directive (86/609/EEC) for the care and use of laboratory animals. Genotype was confirmed by PCR.

Treatment with cordycepin

In this study two groups of animals were used: the treated group received a daily intraperitoneal 40 µl injection of growing doses of Cordycepin-NaCl 0,1% (15 mg/Kg in the first week, 20mg/Kg in the second week and then 25mg/Kg), and the control group received a 40 µl injection of the vehicle (NaCl 0,1%). The concentrations were corrected according to the weight of each animal. The treatment was performed for 6 weeks and the 2 groups included animals of both genders in the same proportion.

Behaviour Assessment

Mice were subjected to behaviour tests every 2 weeks during the 6 weeks of treatment, one week before the beginning of the treatment behaviour tests were also executed to be used as time 0. All tests were performed after at least 1h acclimatization. For each time point, to evaluate statistical significance was used GraphPad software (La Jolla) and tests performed according to the most adequate ones for each type of assessment test, and Welch's correction was performed when variances were significantly different. Data are represented as mean ±SEM.

Rotarod

Motor coordination and balance were evaluated in a rotarod apparatus (Letica Scientific Instruments, Panlab, Barcelona, Spain). Mice were placed on the rotarod at a constant speed (5rpm), or at accelerated speed (from 4 to 40rpm in 5 minutes) for a maximum of 5 minutes, and the latency to fall (the amount of time they could stand in the rotated wheel) was recorded. Mice were subjected to 4 trials for each test at each time point, with a minimum of 15 minutes rest between trials. For statistical analysis, the mean latency to fall off the rotarod of 3 trials was used (the trial with the most distant value from the average was not considered).

Swimming

Coordination of limbs used during voluntary locomotion was assessed by the swimming test. Mice were placed in one extremity of a tank filled with water at 23°C and allowed swimming along a 60-cm-long, 14-cm-wide tank until they achieve the platform (located at the other extremity and with the water level at the platform level). The latency to navigate along the tank (time between they were dropped in the water and they reached the platform) was recorded. Mice were subjected to 3 trials for each test, with a minimum of 15 minutes rest between trials. For statistical analysis, the mean latency of 3 trials was used.

Footprint pattern analysis

Gait analysis was performed by the footprint test. Hind and front feet were coated with blue and red nontoxic paints respectively, and the animals were encouraged to walk along a 100-cm-long, 10-cm-wide runaway (with 15-cm high walls) over a fresh sheet of beige paper. The footprint patterns that were analysed were (1) Stride length the average distance of forward movement between each stride. (2) Hindbase width and (3) frontbase width were measured as the average distance between left and right hind or front footprints, respectively. These values were determined by measuring the perpendicular distance of a given step to a line connecting its opposite preceding and proceeding steps. (4) Distance from left or right front footprint/hind footprint overlap was used to measure uniformity of step alternation. When the center of the hind footprint fell on top of the center of the preceding front footprint, a value of 0 was recorded. When the footprints did not overlap, the distance between the center of the footprints was recorded. A sequence of seven consecutive steps (six measures) was chosen for evaluation, excluding the footprints at the beginning and end of the run where the animal was initiating and finishing movement, respectively. All measurements were performed by the same operator blindly. Mean values for each measure were calculated and statistical analysis was performed using the GraphPad software (La Jolla, USA). Data are presented as the mean ±SEM.

Western blot

Mice cerebella were removed after a sodium pentobarbital overdose and stored at -80°C. Tissue were lysed in RIPA-buffer solution (50mM Tris HCl pH 8, 150nM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (Roche diagnostics) followed by sonication of 4s ultra-sound chase (1 pulse/s). Total protein lysates in were stored at -80°C, and protein concentration was determined by Pierce™ BCA Protein Assay Kit (Thermo). Sixty micrograms of protein extract were resolved in SDS-polyacrylamide gels (10%). The proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (GE Healthcare) according to standard protocols. The membranes were blocked by incubation in 5% non-fat milk powder in 0.1% Tween 20 in Tris buffered saline (TBS-T) for 1h at room temperature. The immunobloting procedure was performed as described previously with the respective primary antibody (1C2, 1:1000, Millipore), followed by incubation with the corresponding alkaline phosphatase-linked secondary antibody. Bands were visualized with Enhanced Chemifluorescence substrate (ECF, GE Healthcare) and chemi-fluorescence imaging (VersaDoc Imaging System Model 3000, Bio-Rad). Membranes were washed with TBS-T for 20min and then reprobed with mouse monoclonal anti- β -actin antibody (1:5000, Sigma) Semi-quantitative analysis was carried out based on the optical density of scanned membranes (Quantity One®1-D

image analysis software version 4.4; Bio-Rad). The specific optical density was then normalized with respect to the amount of β-actin or α-tubulin loaded in the corresponding lane of the same gel.

2.3 Statistical analysis

Statistical analysis was performed using Student's t-test or ANOVA for multiple comparisons, using the GraphPad software (La Jolla, USA). Values of $p < 0.05$ were considered statistically significant.

CHAPTER III — RESULTS

3.1. Optimal concentration and time point of actuation by cordycepin

To define the ideal concentration and time of exposure to the cordycepin treatment we performed a dose-response curve, N2a cells were treated with a crescent concentration of cordycepin during different times of treatment (1 µM, 10 µM, 20 µM, 50 µM and 100 µM for 12h, 24h, 48h, 72h and 96h of treatment). Our main goal was to identify the optimal range that would promote a decrease of the mutant atx3 protein, without cell death. For that, cell viability was measured using a resazurin assay. Resazurin is a cell permeable redox indicator that can be used to monitor viable cell number. Resazurin can be dissolved in physiological buffers (resulting in a deep blue colored solution) and added directly to cells in culture in a homogeneous format. Viable cells with active metabolism can reduce resazurin into the resorufin product which is pink and fluorescent. The quantity of resorufin produced is proportional to the number of viable cells which can be quantified using a microplate fluorimeter equipped with a 560nm excitation / 590nm emission filter set.
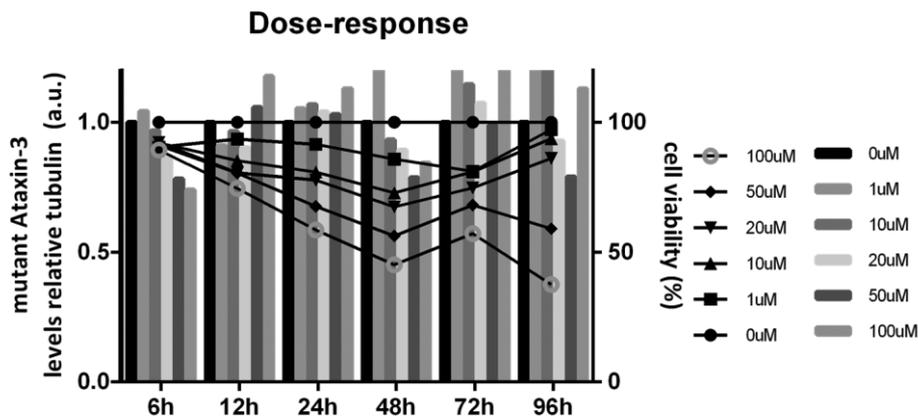
Figure 9 - Optimal concentration and time point for the treatment with cordycepin. Optical densitometry analysis for mutant ataxin 3 of N2a cell lysates. Each atx3 lane was normalized according to the tubulin loading control band. Results were expressed as ataxin-3/tubulin ratio (Left Y axis). Cell viability measured by a resazurin assay. (Right Y axis) Values were normalized for the untreated condition in each time point. Results from 4 different independent experiments.

Taking into account cell viability results, the decrease amount of mutant protein levels (Fig.9) and the published data (Wong et al., 2010; Jin et al., 2014), we choose the concentration of 20 µM of cordycepin during 48h of treatment as the optimal set for the evaluation of the drug potential.

3.2. Cordycepin decreases the levels of mutant ataxin-3 in N2a cells

Upon the identification of the optimal concentration and time of treatment, new experiments were performed using those conditions (20 μM during 48h) to a further investigation of cordycepin effect. We observed a decrease of ± around 30% in the levels of mutant ataxin-3 upon cordycepin treatment (Fig. 10 A-B). This result suggests that cordycepin mediates an important effect in the clearance of the mutant form of the protein. This was even more significant, as the atx3 endogenous levels were not altered (Fig.10 A-C)
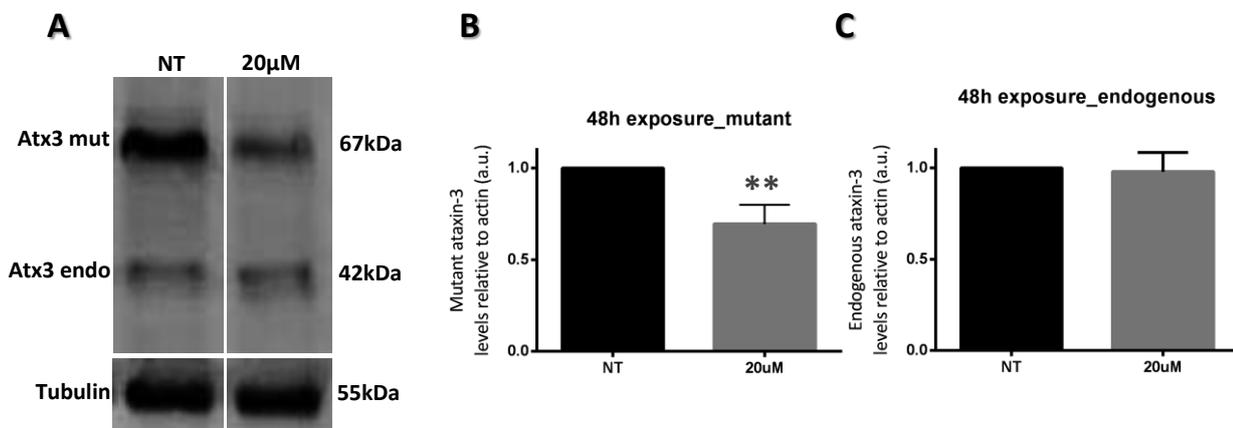
Figure 10 – Cordycepin decreases the levels of mutant ataxin-3 in N2a cells. (A-B) Western blotting analysis of lysates of N2a cells treated with [20 μM] of cordycepin for 48h and N2a cells non-treated (NT). (A) Representative Western Blot probed for ataxin-3 and tubulin. Note the presence of endogenous Atx3 (MW: 42kDa), mutant Atx3 (MW: 67kDa) and tubulin (MW: 55kDa). (B-C) Optical densitometry analyses for mutan and endogenoust ataxin-3. Significant decrease of mutant Atx3 levels and no difference in the levels of endogenous atx3 were observed. Each atx3 lane was normalized according to the tubulin loading control band. Results were expressed as ataxin-3/tubulin ratio, and normalized to non-treated values. Values are expressed as mean ± SEM of 4 independent experiments. *P<0,05; **P<0.01 (Unpaired Student's t-test).

3.3 Cordycepin decreases the translation levels in N2a cells

Recent studies point to an effect of cordycepin at translational level (Wong et al., 2010). Aiming to clarify by which mechanism cordycepin induces clearance the of mutant ataxin-3 protein we performed an assay to detect protein synthesis levels. This puromycin-based assay described by Schmidt et al., 2009, is a useful and simple method to measure translation levels. Puromycin is an aminonucleoside antibiotic produced by *Streptomyces alboniger*, and a structural analog of aminoacyl tRNAs, which is incorporated into the nascent polypeptide chain and prevents elongation (Nathans, 1964). When used in minimal amounts, puromycin incorporation in neosynthesized proteins reflects directly the rate of mRNA translation in vitro. Then we used a monoclonal antibody against puromycin to directly monitor translation levels. For that, N2a cells were treated with 20 or $200\,\mu M$ of cordycepin for 1h30min, and CHX was used as positive control.
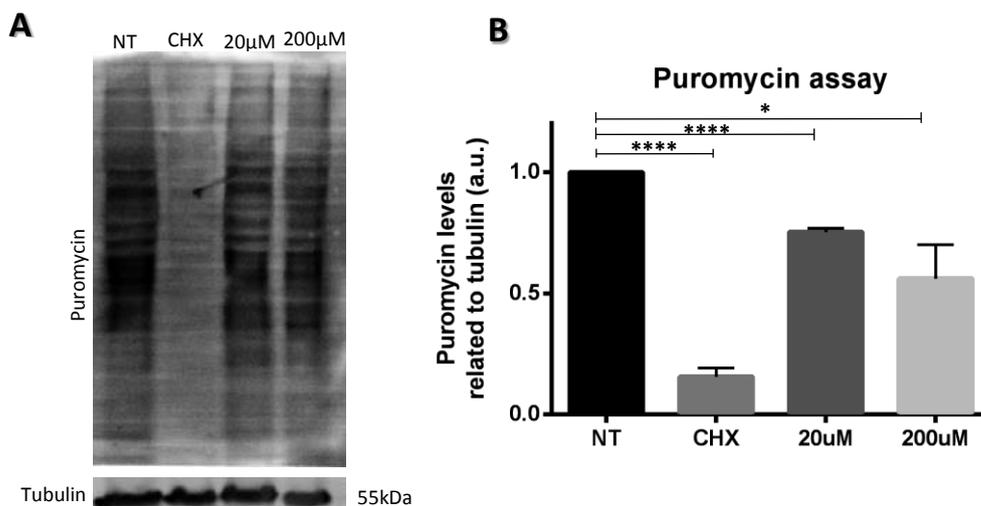
**Figure 11 – Cordycepin decreases the translation levels in N2a cells.** Western blotting analysis of lysates of N2a cells treated with 20 or $200\,\mu M$ of cordycepin for 1h30min, N2a cells non-treated (NT) or treated with cycloheximide (CHX). (A) Representative Western Blot probed for puromycin. (B) Optical densitometry analysis for puromycin levels. A significant decrease of puromycin incorporation in the cells treated with cordycepin is observed. Each puromycin line was normalized according to the tubulin loading control band. Results were expressed as puromycin/tubulin ratio. Values are expressed as mean ± SEM of 4 independent experiments. *$P<0,05$; ****$P<0.0001$ (Unpaired Student's t-test).

Upon cordycepin treatment for only 1h30min we could detect a significant decrease in the levels of translation (Fig. 11 A-B). These results taken together with the decrease of the mutant ataxin-3 levels, and the maintenance in the levels of the endogenous protein (among the preservation of other structural proteins, against the decrease of cytosolic ones (data not shown)) could mean that cordycepin is acting in the inhibition of the translation in a somehow specific manner.

3.4 Cordycepin reduce the levels of mutant ataxin-3 and the number of aggregates in a lentiviral model of MJD

To test the pharmacological effect of cordycepin *in vivo* we used a MJD lentiviral based model (Alves et al., 2008b). We studied the effect of the drug in the mutant ataxin-3 aggregates and soluble levels by western blot analysis of the striatal punches.
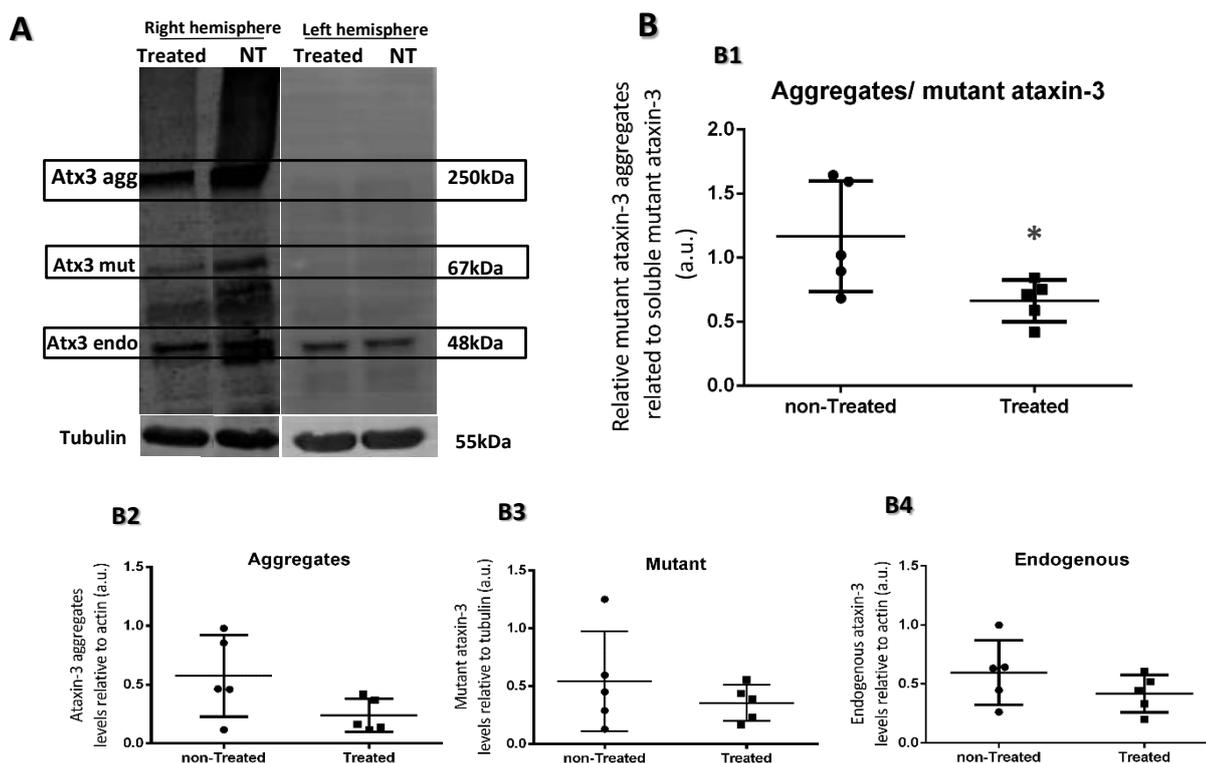
Figure 12 – Cordycepin decreases the levels of ataxin-3 aggregates and of mutant ataxin-3 in a lentiviral mouse model of Machado-Joseph disease. (A-B) Western blotting analysis of striatal lysates punches: the right striatal hemisphere was injected with LV encoding for mutant ataxin-3, whereas the left hemisphere was not injected acting as internal control. Animals were treated with 20 μM/Kg of cordycepin, injected daily for 4 weeks of treatment (Treated) and control animals of the same model were treated with a saline solution (NaCl 0,1%) (non-Treated (NT)). The presence of aggregates and mutant form of the disease was only detected in the LV injected side. (A) Representative Western Blot probed for ataxin-3. Note the presence of endogenous Atx3 (MW: 48kDa), mutant Atx3 (MW: 67kDa), ataxin-3 aggregates (MW: 250kDa) and tubulin (MW: 55kDa) on the right hemisphere, and endogenous Atx3 (MW: 48kDa) and tubulin (MW: 55kDa) on the left hemisphere. (B) Optical densitometry analysis for ataxin-3 levels. (B1) Optical densitometry analysis for mutant ataxin-3 aggregates/soluble levels ratio. (B2) Optical densitometry analysis for mutant ataxin-3 aggregates levels. (B2) Optical densitometry analysis for soluble mutant ataxin-3 levels. (B3) Optical densitometry analysis for endogenous ataxin-3 levels. A significant decrease of the mutant ataxin-3 aggregates/soluble ratio was detected upon cordycepin treatment. No significant differences were detected in the endogenous levels of ataxin-3. Each atx3 band was normalized according to the tubulin loading control band. Results were expressed as ataxin-3/tubulin ratio. Values are expressed as mean ± SEM. n = 5. *P<0,05 (Unpaired Student's t-test).

We observed a tendency to a reduction in the aggregates and mutant soluble forms of mutant ataxin-3, although the endogenous levels remain unaffected (Fig 12). The levels of mutant ataxin-3 aggregates/soluble ratio were significantly decreased upon cordycepin treatment (Fig. 12B), whereas only a significant decrease was observed when those levels were analysed alone (Fig. 12 B2 and B3). No difference were found between the two groups of animals concerning the ataxin-3 endogenous levels (Fig. 12 B3). These results corroborate the *in vitro* results indicating some specificity of cordycepin for mutant ataxin-3 form.

3.5 No changes were observed in the weight of the animals upon cordycepin treatment

The weight of the animals was evaluated every week, and no significant differences were observed between the two groups (Fig. 13). Moreover, no visible macrohistological differences were observed in the animals (non-treated vs treated) upon sacrifice. These data could indicate a good tolerance of the animals to the cordycepin treatment.
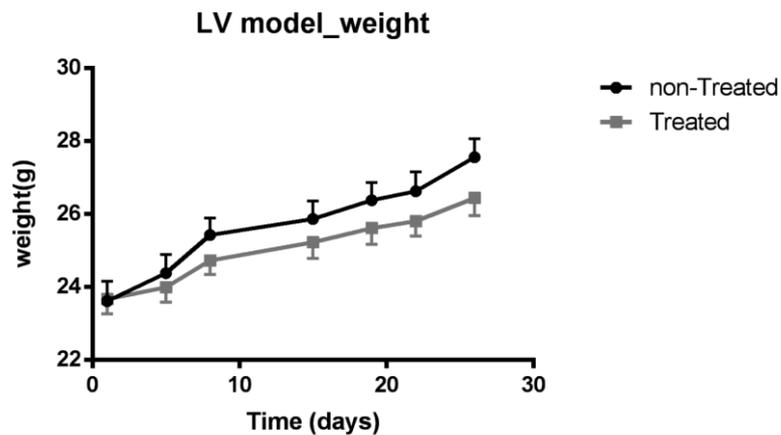
Figure 13 – Cordycepin has no impact in the lentiviral mouse model weight. Upon over 4 weeks of treatment no significant differences were observed between the animals treated daily i.p. with [20mg/Kg] of cordycepin and the animals treated with a saline solution (NaCl 0,1%). Animal's weight normalized for the initial weight. n =8 for each condition (Unpaired Student's t-test).

3.6 Cordycepin does not lead to astroglial or microgial activation

Inflammation plays a two faced role in neurodegeneration. Can act as a first league mechanism defence or as causative of cell death (Amor et al., 2010). In this study to further evaluate if cordycepin could affect inflammation we performed an immunohistochemical analysis for some markers: iba-1 for microglia and GFAP for astrocytes. No differences were identified; which could indicate that cordycepin does not act in neuroinflammation in this particular model (Fig. 14).
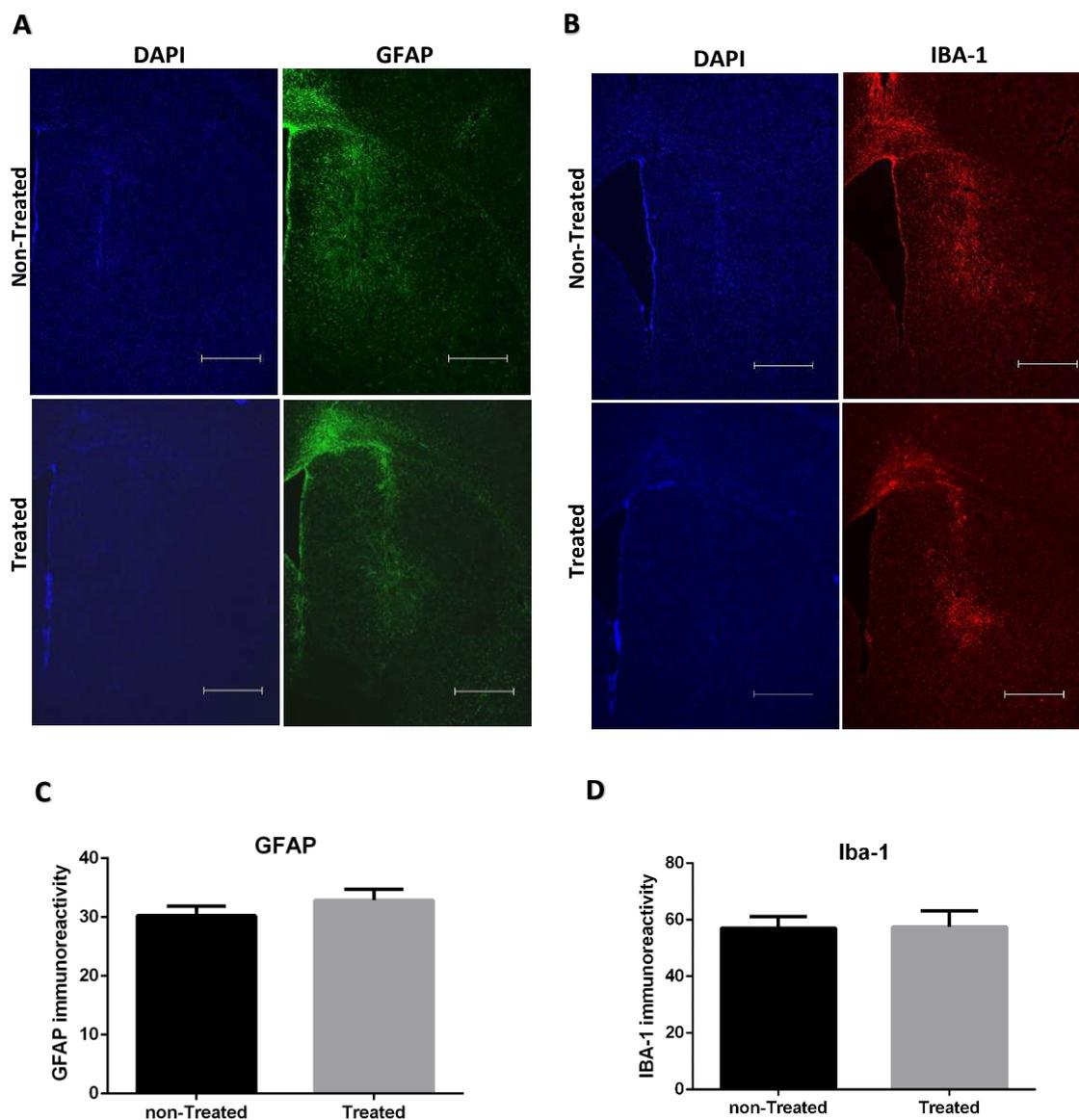
Figure 14 - Cordycepin has no effect in the astroglyosis or microgliosis (A-D) Immunohistochemical staining of mice striatal sections for microglia and astrocytes markers (A) Immunohistochemical staining for Glial fibrillary acidic protein (GFAP) a marker for glial activation. (B) Immunohistochemical staining for ionized calcium-binding adapter molecule 1 (IBA-1), a neuronal inflammation marker. (C) Quantification of immunoreactiviy GFAP upon 4 weeks treatment with cordycepin. Values are expressed as mean ±SEM. (D) Quantification IBA1 immunoreactivity upon 4 weeks of treatment of cordycepin. Values are expressed as mean ±SEM. Scale bar: 500 μm. No differences were detected between the two groups. The images were obtained with the same settings, and quantification performed semi-manually with ImageJ. n =5 (Unpaired Student's t-test).

3.7 Cordycepin decreases the neuronal loss and improves clearance of aggregates in the MJD-lentiviral mouse model

To further investigate the effect of cordycepin in the neuropathological features we performed immunohistochemistry staining the aggregates and neuronal markers. For the evaluation of inclusions we probed the histological sections for ubiquitin and detected a significant reduction in the number of ubiquitinated aggregates in the animals treated for 4 weeks with cordycepin when compared to the controls (Fig.15B). These results are in agreement with the reduction of aggregates that we already demonstrated in the western blot analysis of the striatum punches from the LV model animals treated with cordycepin. According to these results we next investigate if the clearance of inclusion result in a neuroprotective effect. The lentiviral expression of mutant ataxin-3 produces a depletion of the neuronal nuclei protein NeuN, which is quantifiable (Alves et al., 2008b). We performed an imunohistochesmitry assay for that marker (Fig.15C). Importantly, cordycepin led to a robust reduction of neuronal loss, thus revealing a neuroprotective effect.
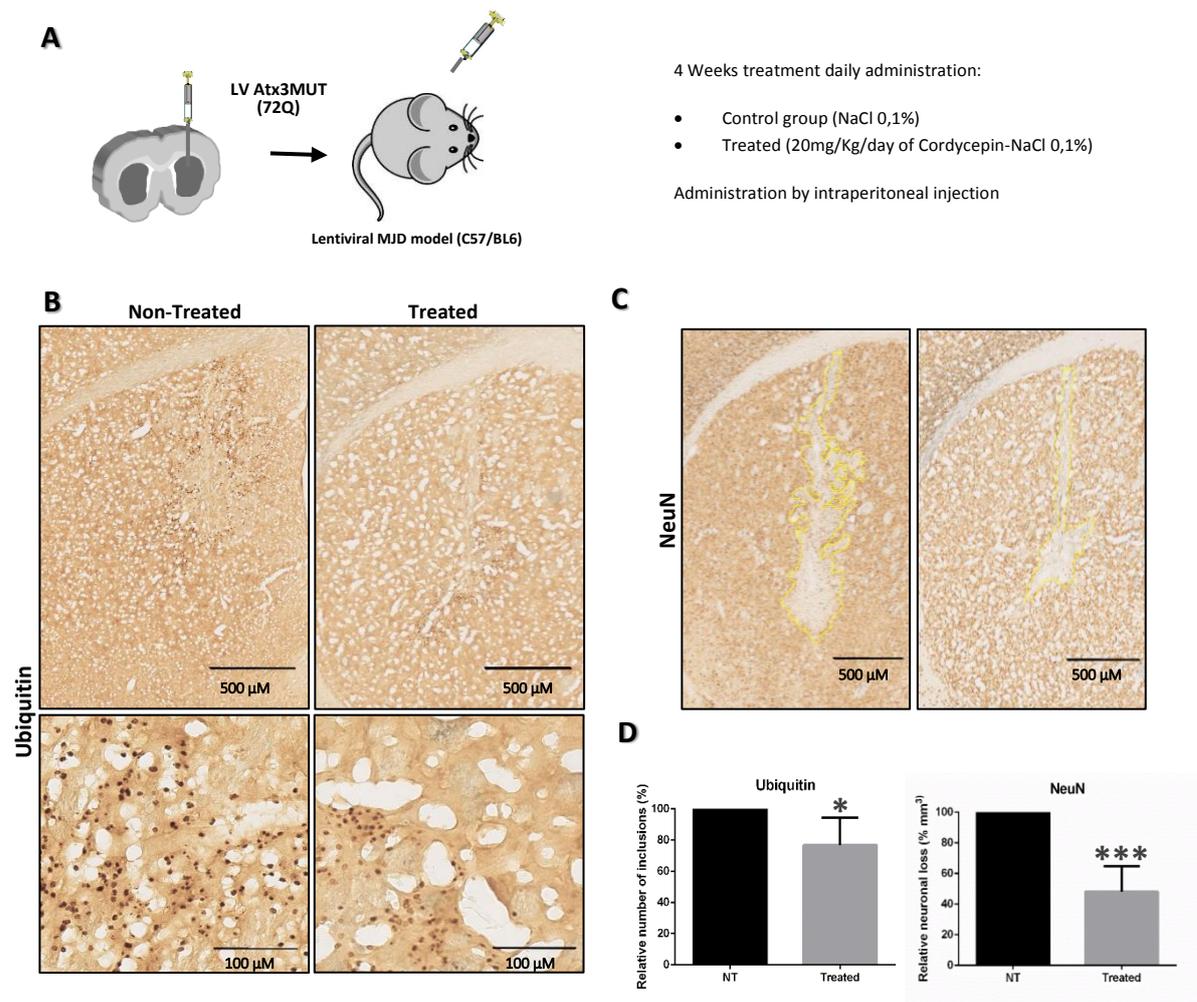
Figure 15 - Cordycepin decreases the neuronal loss and improves clearance of mutant ataxin-3 aggregates. (A-E) Schematic representation and analysis of lentiviral-based MJD mice model treated during 4 weeks with 20mg/Kg daily by ip injection (n=4) or with the saline solution (NaCl 0,1%) as controls (n=4). (A) Schematic representation of stereotaxic unilateral injection of lentiviral vectors in mice striatum. Lentiviral vectors encoding human mutant ataxin-3 with 72 glutamines (Atx3 MUT) were injected in the striatum of 4weeks old C57/Bl6 mice. (B-C) Immunohistochemical analysis of mice striatal sections. (B) Immunohistochemical staining for ataxin-3-positive inclusions (Ubiquitin). Upper panel with an amplification of 1,5x. Lower panel 20x. (C) Immunohistochemical staining for neuronal nuclei protein NeuN, 1,5x amplification (C) Left graphic, quantification of absolute number of mutant ataxin-3-positive inclusions upon 4 weeks of treatment. Values are expressed as mean ±SEM. (Unpaired Student's t-test) *P<0.05. Right graphic, quantification of NeuN depleated volume (mm^3) upon 4 weeks of treatment. Values are expressed as mean ±SEM. (paired Student's t-test). ***P<0.001. n=4 for each conditions.

3.8 No alteration in the weight of the MJD transgenic mice was observed upon cordycepin treatment

As for the lentiviral mouse model, the weight of the MJD transgenic mice model was evaluated every week and no significant differences were observed between the two groups (treated with cordycepin and non-treated), suggesting that cordycepin treatment is non-toxic to these animals.
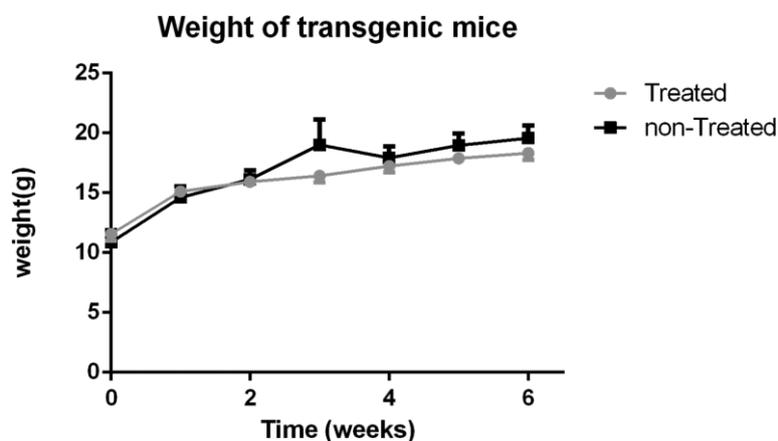
Figure 16 - Cordycepin has no impact in the MJD transgenic mice weight. Upon cordycepin treatment over 6 weeks no differences were observed between the animals injected by daily i.p. with a crescent dose of cordycepin [15, 20 and 25mg/Kg] and the animals injected with a saline solution (NaCl 0,1%). Animal's weight normalized for the initial weight. n = 5, non-treated; n = 10, treated.

# 3.10 Cordycepin rescue behavioural phenotype of a transgenic mouse model of MJD

We next evaluated the therapeutic potential of cordycepin in the promotion of a functional recovery or phenotype improvement in MJD, and for that we use a transgenic mouse model of MJD (Torashima et al., 2008). These animals display an early behaviour impairment, and cerebellar atrophy. We started the cordycepin treatment in animals with 4 weeks of age. A daily 40 µl intraperotonial injection was administrated to the animals during 6 weeks. The cordycepin treated group received an escalated dose of drug starting with 15mg/Kg in the first week, 20mg/Kg in the second and then of 25mg/Kg in the following weeks. Behavioural assessment was performed every two weeks (Fig. 16) and in the beginning of the experiment. The two groups of transgenic animals comprised both genders.

	15mg/kg	20mg/Kg	25mg/Kg	25mg/Kg	25mg/Kg	25mg/Kg
Week 0	Week 1		Week 3		Week 5	Week 6
BA	BA		BA		BA	BA

Beggining of treatment   **Sacrifice of animals**
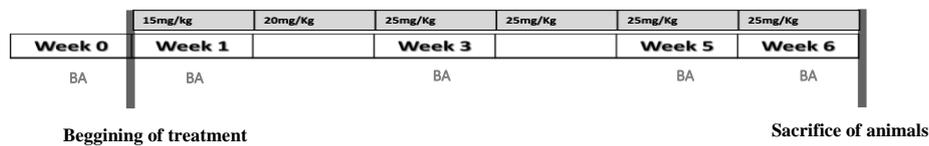
Figure 17 – Schematic representation of experimental design for the MJD transgenic mice treatment and behavioural assessment (BA).

The animals performed a battery of different behaviour tests to assess the functional recovery of MJD mice overtime: rotarod constant and accelerated, footprint and swimming. In the rotarod test they were forced to walk on a rotarod apparatus; the balance and motor coordination deficits were evaluated by constant and accelerated rotarod, as well as by the swimming test. Cordycepin-treated mice presented significant better performances comparing to non-treated mice in both constant, after the 6th week of treatment, and accelerated rotarod after the 5th and 6th weeks (Fig. 18 A1-A2). Additionally, the footprint test was used to evaluate the progression of gait and limb ataxia. We measured the average between the hind and front paws, for both sides, stridelenght (Fig.18 B1), that display a tendency to diminish in the treated group. It was also measured the distance between the left and the right hind and front footprint (hind/frontbase width), that in our model is increased comparing to wild-type mice, as a result of balance defects. With the treatment, mice display an improvement in hindbase width measurements (Fig. 18 B4), as well as a tendency to diminish the overlap distance (Fig.18 B2), when compared to non-treated animals. This suggests that, in accordance to our previous results that the treatment promotes a phenotypic alleviation of motor symptoms upon 6 weeks of treatment. In the swimming test, where mice were placed at one extremity of a recipient filled with water and the time between the moment they were dropped in the water and they reached the platform was registered, we did not detected any significant differences at the end of the experiment, however a significant decrease in the 1st week of treatment was observed (Fig. 18 C).

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A Rotarod test

A1 Constant rotarod

A2 Accelerated rotarod
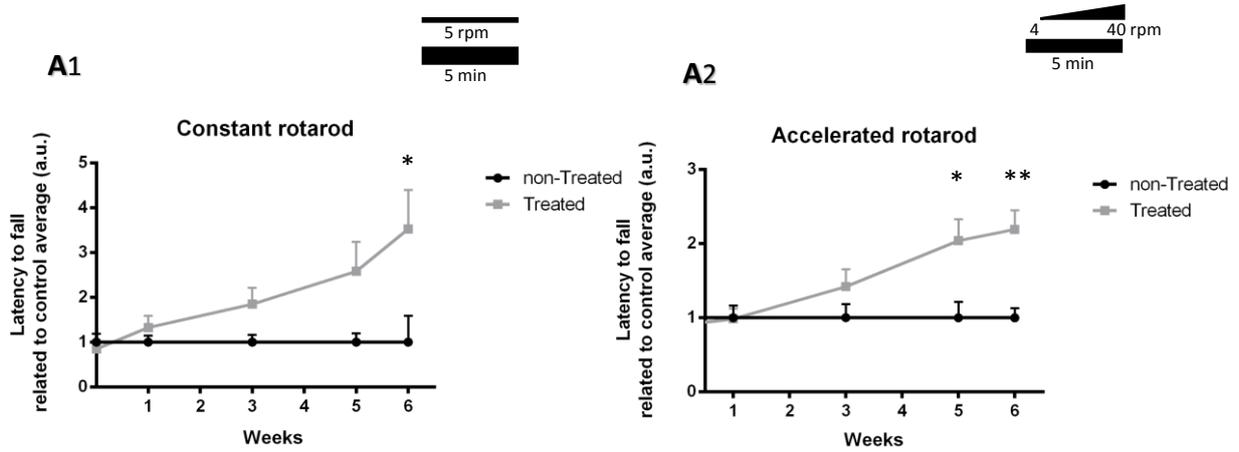
B Footprinting analysis

B1 Stridelenght

B2 Overlap

B3 Frontbase

B4 Hindbase
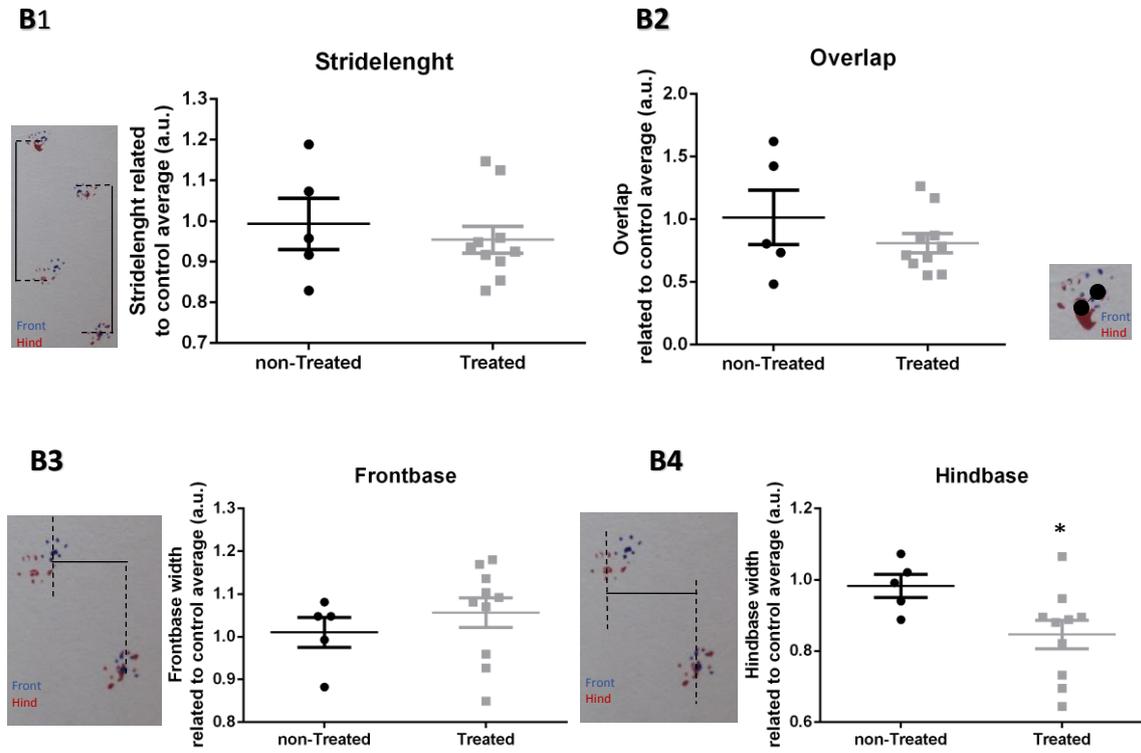
C Swimming test

Swimming
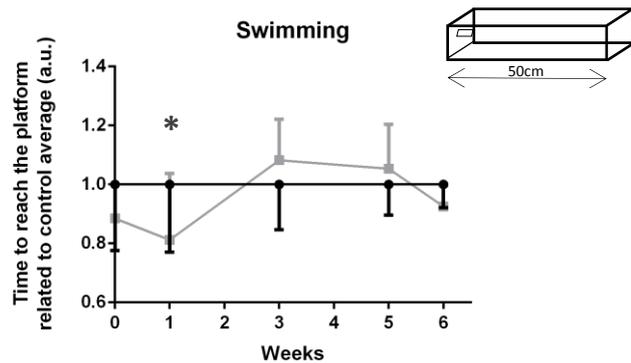
Figure 18 - Cordycepin treatment rescue behavioural deficits in a transgenic mouse model of MJD. (A-C) Behavioral assessment of transgenic mice treated during 6 weeks with growing doses of Cordycepin-NaCl 0,1% (15mg/Kg/day in the first week, 20mg/Kg/day in the second week and then 25mg/Kg/day). (A) Rotarod test. (A1) Constant rotatod. Mice were forced to walk on a constant speed rotarod apparatus at 5rpm (A2) Accelerated rotarod. Mice were forced to walk in an accelerating rotarod apparatus from 4 to 40rpm in 5min. Latency to fall was measured for each time point. The results were normalized to the control average for each time point. All measurements were normalized to controls average for the correspondent time point. Values are expressed as mean ± SEM. *P<0,05, **P<0.01 (Unpaired Student's t-test). (B) Footprint pattern analyses of non-treated and treated animals, at 6 weeks after beginning of the treatment. (B1) Stride length relative to hind paw. (B2) Overlap, distance between front and hind footprint. (B3) Frontbase width, distance between right and left front paw. (B4) Hindbase width, distance between right and left hind paw. All measurements were normalized to controls average for the correspondent time point. Values are expressed as mean ± SEM. *P<0,05 (Unpaired Student's t-test). (C) Swimming test, the time spent to reach the platform on the opposite site of mice placement. Values are presented as mean ± SEM. n=5, non-treated; n=10, treated.

3.9 Effect of cordycepin treatment in aggregates levels of the MJD transgenic mouse model

To further investigate the neuropathological effect of cordycepin we analysed the cerebellum lysates of the transgenic mouse model. For that we used the 1C2 antibody against endogenous human TATA-binding protein, which marks polyglutamine expanded tracts. It is possible to observe that animals treated with cordycepin shows a tendency to reduce the amount of mutant ataxin-3 levels when compared to the non-treated animals (Fig.19).
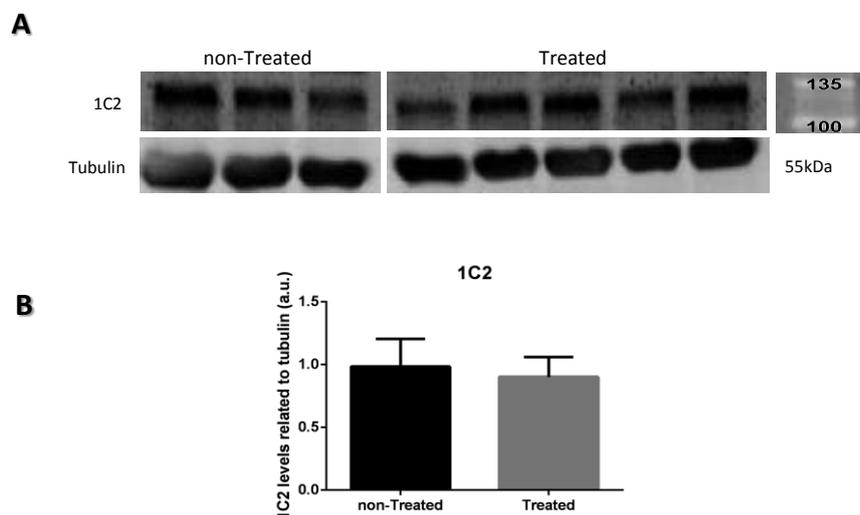
Figure 19 - Cordycepin display a tendency to reduce the levels of ataxin-3 aggregates in transgenic mouse model of Machado-Joseph disease. (A-B) Western blotting analysis of mouse cerebellum from Machado-Joseph disease models treated with growing concentrations of cordycerpin, injected daily for 6 weeks of treatment (Treated) and animals of the same model injected with saline solution (NaCl 0,1%) (non-Treated (NT)) for the same period. (A) Representative Western Blot probed for 1C2. 1C2 stains for expanded polyglutamine tracts. Note the presence 1C2 (MW: 48kDa). (B) Optical densitometry analysis for 1C2. A tendency to decrease the levels of 1C2 in treated mice is observed. Each 1C2 band was normalized according to the tubulin loading control band. Results were expressed as 1C2/tubulin ratio. Values are expressed as mean ± SEM. (n=3 non-Treated; n=5 Treated)

CHAPTER IV- DISCUSSION

In this study we explored a new pharmacological therapeutic approach to treat Machado-Joseph disease. For that we investigated the effect of cordycepin in several MJD models. Cordycepin is described as an anti-cancer (He et al., 2010; Choi et al., 2011; Kadomatsu et al., 2012; Lee et al., 2012, 2013, 2014), anti-bacterial (Rottman and Guarino, 1964a), anti-viral (Wu et al., 1972; White and Dawson, 1979; Xu et al., 2005), imunnodolatory (Zhou et al., 2002, 2008; Kim et al., 2006; Noh et al., 2009; Jeong et al., 2010; Kondrashov et al., 2012a; Ren et al., 2012; Zhang et al., 2014; Peng et al., 2015; Yang et al., 2015), anti-diabetic (Yun, Y., Han, S., Lee, S., Ko., Lee, C., Ha, N., Kim, 2003; Shin et al., 2009) and anti-parasitic drug (Trigg et al., 1971; Vodnala et al., 2009; Dalla Rosa et al., 2015). It display a wide range of actuation: in polyadenilation inhibition (Horowitz, B., Goldfinger, B. A., Marmur, 1976; Müller et al., 1977; Chen et al., 2008a; Holbein et al., 2009; Wong et al., 2010), purine biosynthesis inhibition (Klenow, 1963; Overgaard, 1964; Rottman and Guarino, 1964b), and translation inhibition (Holbein et al., 2009; Wong et al., 2010; Kitamura et al., 2011; Kadomatsu et al., 2012; Ferrari et al., 2013; Tuli et al., 2013).

Taking these effects into account we decided to test it effect in Machado-Joseph disease, and for that we used three different models. Firstly we tested the effect of cordycepin in a wide range of concentrations during different times of exposures to the treatment in N2a cells. We observed a decrease in the amount of mutant protein levels, which was dependent on cordycepin concentration used and the time of exposure to the drug. Accordingly to these results and to previous studies we found a significant decrease in the translation levels upon cordycepin treatment. Nevertheless, our results indicate a smaller decrease in those levels compared to the ones previously reported: ±55% against 95% (Wong et al., 2010). This could be explained due to the use of different cell lines, or even because of the method of translation measure used in both studies. This translation inhibition is also proportional to the cordycepin concentration used and time of treatment, demonstrating a higher decrease at 200µM than that at 20µM. Indeed, a report showed that cordycepin strongly inhibited total protein synthesis in NIH3T3 cells treated with high concentrations (50–200 µM) (Wong et al., 2010). However, a study with in NRK-52E cells, treated with 20 µM cordycepin did not displayed a reduction in general translation (Kitamura et al., 2011). On the other hand, our results showed that this concentration is enough to a decrease of over 40% in the protein translation rates. These differences will need further investigation to determine the exact mechanism of translational suppression by which cordycepin acts. The cell viability was also evaluated and the same pattern was observed, at higher concentrations there was a higher decrease of cell viability, which agrees with other studies already performed (Choi et al., 2011). Although this loss of viability does not seem to be due to cell death, but rather by affecting

proliferation inhibition (Xiong et al., 2013), excluding a toxic effect of cordycepin.

While wild-type ataxin-3 functioning seems important to cell maintenance (Rodrigues et al., 2007a; Schmitt et al., 2007; Alves et al., 2010), in MJD treatment it is thus necessary a specific downregulation of mutant ataxin-3 form (Miller et al., 2003; Rodriguez-Lebron and Paulson, 2005). In this study we demonstrated a certain specificity of cordycepin since the levels of endogenous atx3 *in vitro* and *in vivo* are not altered, whereas the mutant protein and aggregates levels are reduced. Other forms of achieving this specificity could be through RNAi pathway using directed shRNAs or ssRNAs, or even targeting the expanded polyglutamine tract directly through AONs or PNAs (Gaspar et al., 2001; Alves et al., 2008a; Evers et al., 2011; Hu et al., 2011; Liu et al., 2013a, 2013b; Nóbrega et al., 2013). However these strategies may have some problems regarding the safety and delivery methods, which make them unavailable as therapeutic strategies for a near future. In our case, this pharmacological approach with a simple method of administration of an FDA-approved drug could overcome this safety concerns and make it more easy to use in a clinical setting.   Taking into account the results in N2a cells and these favourable features we then tested the cordycepin effect in two *in vivo* MJD models. The lentiviral model is useful to study *in vivo* models of disease, and based in the overexpression the mutant protein involved in the corresponding pathology, in this case mutant form of the ataxin-3 protein in striatum (but also possible to other regions, as cerebellum, substantia nigra or amygdala (de Almeida et al., 2002; Lo Bianco et al., 2002; Arvidsson et al., 2003; Lauwers et al., 2003; Alves et al., 2008b)). This lentiviral-mediated expression of mutant ataxin-3 in mice brains induces neuropathological abnormalities mimicking the ones found in MJD patients. The use of this kind of model is advantageous to elucidate the molecular mechanism of mutant ataxin-3 pathogenesis, like its toxicity for example, and allow the evaluation of the effect of new therapeutic strategies MJD-directed (Alves et al., 2008b, 2010; Nascimento-Ferreira et al., 2011; Simões et al., 2012). In this study WT animals were injected with lentivirus vectors encoding for atx3 mutant in the striatum, and then submitted to a treatment with cordycepin. Mice were daily injected with 20mg/Kg (concentrations also used in other studies (Cheng et al., 2011; Xiong et al., 2013)) for four weeks of treatment. Contrary to what was demonstrated in a previous study claiming that cordycepin may be useful in treating neurodegenerative diseases by an inflammatory mechanism in our model we failed to identify this mechanism for cordycepin action. Nevertheless, we showed that cordycepin decrease the levels of ataxin-3 aggregates and soluble forms, which is also corroborated with a significant reduction in the number of aggregates and decrease of neuronal dysfunction. These data suggest a neuroprotective effect of cordycepin leading to a prevention of the pathological features associated with the disease. Interestingly the levels of endogenous levels of ataxin-3 seems to

be unaffected by cordycepin. This is in agreement to our *in vivo* results, which demonstrated a certain specificity of cordycepin to the mutant ataxin-3 form.

We further analysed the effect of cordycepin in neuropathology and motor behaviour impairments in a transgenic mouse model of MJD (Torashima et al., 2008). This transgenic mouse model is characterized by a Purkinje cell expression of truncated form of human ataxin-3 with 69 glutamine repeats, driven by a Purkinje-cell-specific L7 promoter. Therefore, a functional defect in the Purkinje cells trigger cerebellar ataxia, including balance and gait defects. This allows us to study the rescue effect of a treatment in a setting where the disease is already established. This model present a strong expression of mutant ataxin-3 in the cerebellum, which has an important role in the maintenance of balance and posture, coordination of voluntary movements and motor learning, and in MJD patients is one of the most affected areas (Rosenberg, 1992; Sudarsky and Coutinho, 1995; Dürr et al., 1996; Alves et al., 2008b; Riess et al., 2008). These mice were exposed to a 6 weeks cordycepin treatment and motor behaviour assessment was performed to evaluate the effect of the drug in the phenotypic features. We detected a significant improve in the cordycepin-treated group in the rotarod test comparing to control animals. In the footprint analysis the animals displayed an improvement in the hindbase measurements, and showed a tendency to ameliorate the stride length and footprint overlap measures. In the swimming test the treated group present a significant decrease in the time spend to reach the platform at the first weeks of treatment, which was attenuated along the experimental evaluation. This fact could be due to the fact that the animals start to learn the task and the distance did not display a challenge to this type of model. We also observed a tendency for a reduction in the mutant ataxin-3 levels by western blot analysis. All together, these data suggest that cordycepin could be a good therapeutic strategy for MJD.

Cordycepin was already used in clinical trials, it was already FDA-approved, which facilitates it potential applicability to the clinics. Although more studies need to be performed to evaluate the exact mechanism of actuation and it influence in other proteins or pathways, these results suggest that cordycepin could be an effective therapy for MJD and even for other polyglutamine disorders.

CHAPTER V- CONCLUSIONS AND PERSPECTIVES

In this work it was shown that cordycepin could alleviate motor impairments and neuropathology hallmarks, proving that is a good candidate for a MJD pharmacological treatment. We also demonstrated that cordycepin mediates it effect in the translation levels of certain proteins, which could explained the mechanism of action of the drug. The inhibition of translation observed appears to be specific for some proteins (mainly cytosolic ones). This fact could present an advantageous strategy for the treatment of this type of disease, when the wild-type form of the protein has an important cellular role.

We are currently increasing the number of animals of the transgenic model, and neuropathology analysis of transgenic mice is also on going. Furthermore, plasma and liver of treated animals were also collected to evaluate cordycepin treatment toxicity. Further studies regarding the translational route will also be performed using RT-PCR techniques.

With this study we provided *in vitro* and *in vivo* evidences that cordycepin promotes translation inhibition, reduction of mutant ataxin-3 levels and improvement of MJD associated neuropathology and motor impairments.

Cordycepin is a multicompetent drug that has been tested for the treatment of different diseases. As cordycepin was already tested in humans, it's a step closer to a direct application as a therapeutic strategy for Machado-Joseph disease, as well as other neurodegenerative disorders.

CHAPTER VI — REFERENCES

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