Nélio da Mota Gonçalves

Gene transfer approaches to study the role of adenosine A_{2A} receptors in Machado-Joseph disease

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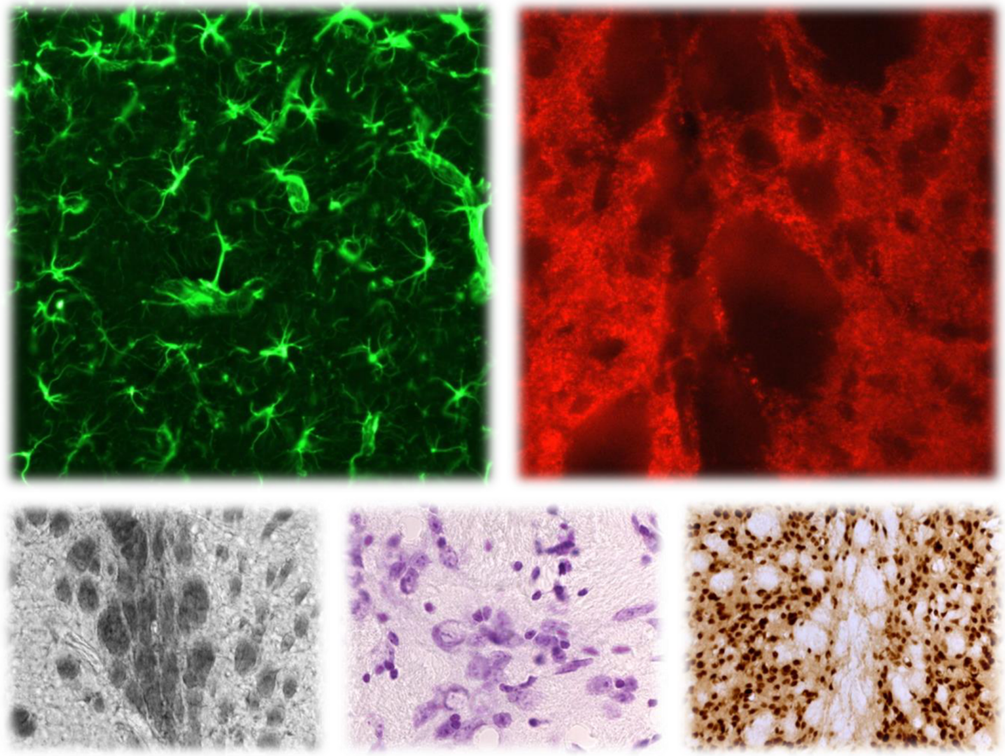

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Tese de doutoramento em Farmácia, na especialidade de Biotecnologia Farmacêutica, orientada pelo Doutor Luís Fernando Morgado Pereira de Almeida e Doutor Rodrigo Pinto Santos Antunes Cunha e apresentada à Faculdade de Farmácia da Universidade de Coimbra

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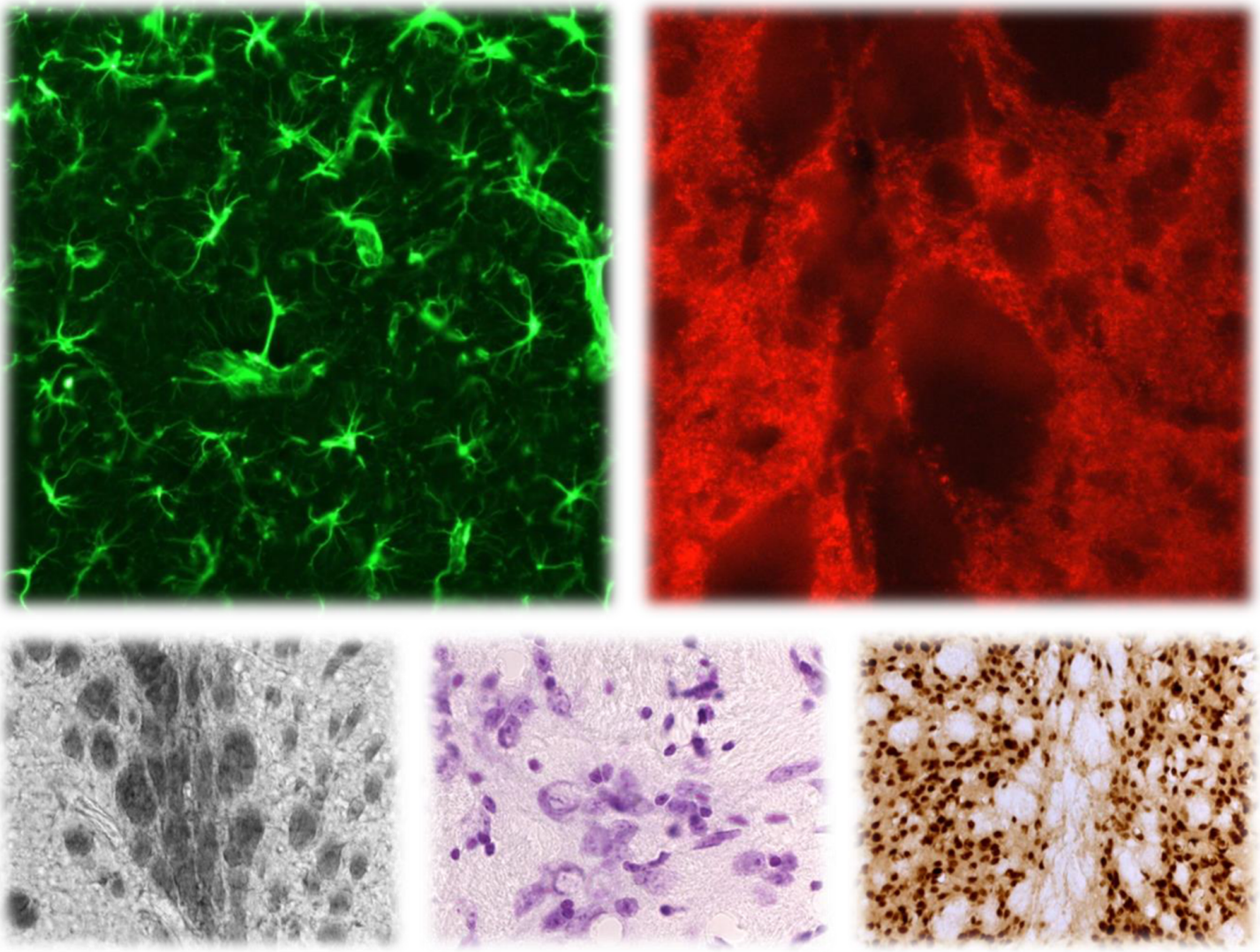

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Nélio da Mota Gonçalves

Thesis for the attribution of the Doctor of Philosophy degree in Pharmaceutical Sciences, in the specialty field of Pharmaceutical Biotechnology, submitted to the Faculty of Pharmacy of the University of Coimbra

Tese apresentada à Faculdade de Farmácia da Universidade de Coimbra, para prestação de provas de doutoramento em Ciências Farmacêuticas, especialidade de Biotecnologia Farmacêutica.

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Gene transfer approaches to study the role of adenosine A_{2A} receptors in Machado Joseph Disease

Nélio da Mota Gonçalves

The research work presented in this thesis was performed at the Center for Neuroscience and Cell Biology (CNC), University of Coimbra, Portugal and at the Faculty of Pharmacy of the University of Coimbra, Portugal, under the supervision of Doctor Luís Pereira de Almeida and Doctor Rodrigo A. Cunha.

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Front cover:

Microscope images of the neurochemical modifications induced by striatal expression of mutant ataxin-3. *Top left*: glial fibrillary acid protein (GFAP); *Top right*: synaptophysin; *Bottom left*: bright field photomicrograph; *Bottom center*: cresyl violet staining; *Bottom right*: neuronal nuclei marker (NeuN).

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Esta tese é-vos dedicada.

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CHAPTER 3

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Abbreviations

AAV Adeno-associated virus
Ab Antibody
AD Alzheimer's disease
ADK Adenosine kinase
ALS Amyotrophic lateral sclerosis
AMPA alpha-amino-3-hydroxyl-5-methyl-4-isoxazole propionic acid
ANOVA Analysis of variance
AR Androgen receptor
ARs Adenosine receptors
Atx3-27Q Wild-type ataxin-3 with 27 glutamines
Atx3-72Q mutant ataxin-3 with 72 glutamines
A_1R Adenosine A_1 receptor
A_{2A}R Adenosine A_{2A} receptor
ATXN3 Ataxin-3 gene
Aβ β-amyloid peptide
BBB Blood-brain barrier
BSA Bovine serum albumin
CAG Trinucleotide cytosine-adenine-guanine coding for glutamine
cAMP Cyclic adenosine 3',5'-monophosphate
CD11b Cluster of differentiation 11 b
cDNA Complementary deoxyribonucleic acid
CNS Central nervous system
cPPT Central polypurine tract
CUG Trinucleotide cytosine-uracil-guanine
DAB 3,3'-diaminobenzidine
DAT Dopamine transporter
DAPI Diamidino-2-phenylindole
DARPP-32 32 kDa dopamine- and cAMP-regulated phosphoprotein
DM1 Myotonic dystrophy type 1
DNA Deoxyribonucleic acid
DRPLA Dentatorubral-pallidoluysian atrophy
DTT Dithiothreitol
EDTA Ethylenediaminetetraacetic acid
EGTA Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetra acetic acid
GFAP Glial fibrillary acidic protein
GFP Green fluorescent protein
GL Granular layer
GPCRs G protein-coupled receptors
HD Huntington's disease
HEK Human embryonic kidney
HEPES hydroxyethyl piperazineethanesulfonic acid
iGluR Ionotropic glutamate receptors
IL-10 Interleukin-10
IL-1Ra Interleukin-1 receptor antagonist
Il-1β Interleukin-1β
IR Immunoreactivity
KO knockout
KW6002 ((E)-1,3-diethyl-8-(3,4-dimethoxystyryl)-7-methyl-3,7-dihydro-1H-purine-2,6--dione)
LTR long terminal repeat
LVs Lentiviral vectors
MAP-2 Microtubule-associated protein 2

miRNA	microRNA
MJD	Machado-Joseph disease
ML	Molecular layer
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MSNs	Medium spiny neurons
MWM	Morris water maze
NeuN	Neuronal nuclei protein
Neuro2a	Neuroblastoma cell line
NGS	Normal goat serum
NII	Neuronal intranuclear inclusions
NMDA	N-methyl-D-aspartate
OD	Optical density
PBS	Phosphate-buffered saline
PCL	Purkinje cell layer
PCR	Polymerase chain reaction
PD	Parkinson's disease
PET	Positron emission tomography
PGK	Phosphoglycerate kinase
PFA	Paraformaldehyde
PMSF	phenylmethylsulfonyl fluoride
PolyQ	Polyglutamine
QA	Quinolinic acid
RFP	Red fluorescent protein
RIPA	Radioimmunoprecipitation assay buffer
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RT	Room temperature
RV-G	ravies virus glycoprotein
SBMA	Spinal bulbar muscular atrophy
SCA	Spinocerebellar ataxia
SCA3	Spinocerebellar ataxia type 3
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SIN	Self-inactivating vectors
shA_{2A}R	Short hairpin against adenosine A_{2A} receptor
shCTR	Short hairpin control
shRNA	Short hairpin RNA
SOD1	Superoxide dismutase 1
TALENs	Transcription Activator-Like Effector Nucleases
TGF-β	Transforming growth factor-β
TgMJD	Transgenic mice for Machado-Joseph disease
TNF-α	Tumor necrosis factor-α
UPS	Ubiquitin proteosome system
VSV-G	Vesicular stomatitis virus G glycoprotein
WPRE	Woodchuck hepatitis B virus regulatory element
WT	Wild-type
ZFNs	Zink Finger Nucleases
3-NP	3-nitropropionic acid

Publications

The results presented in this dissertation are either published in peer-reviewed international scientific journals, or in preparation, as follows:

1. Gonçalves N, Simões AT, Cunha RA, Pereira de Almeida L; "Caffeine and adenosine A_{2A} receptor inactivation decrease striatal neuropathology in a lentiviral-based model of Machado-Joseph disease". Ann Neurol (2013) 73(5):655-666 (Chapter 2)

2. Gonçalves N, Simões AT, Prediger RS, Cunha RA, Pereira de Almeida L; "Caffeine alleviates progressive motor deficits in Machado-Joseph disease transgenic mice"; *in preparation*

3. Gonçalves N, Simões AT, Hockemeyer J*, Muller CE*, Cunha RA, Pereira de Almeida L; "Distinct striatal neuronal compartments of adenosine A_{2A} receptors differently modulate Machado-Joseph disease"; *in preparation*

Summary

Machado-Joseph disease (MJD) is a neurodegenerative disease caused by the expansion of a polyglutamine repeat in the ataxin-3 protein. There is currently no therapy available to prevent or modify disease progression. Since the manipulation of a neuromodulation system operated by adenosine A_{2A} receptors ($A_{2A}R$) demonstrated to be effective controlling and alleviating neurodegeneration in different brain diseases, we now tested its ability to also control MJD-associated neurodegeneration. For that purpose, we have used different mouse models of MJD, namely lentiviral-based and transgenic, and investigated whether $A_{2A}R$ antagonism, molecular and genetic inactivation were able to reduce the associated morphological and behavioral modifications.

Our candidate strategy was the chronic administration of caffeine (*per os*), based on its ability to antagonize the adenosine receptors (ARs), especially of the $A_{2A}R$ (Fredholm et al., 1999), to cross the blood brain barrier, which together with its favorable safety profile suggest it may be a promising prophylactic candidate strategy to interfere with the inexorable evolution of MJD.

In Chapter 2, MJD was modelled by transducing the striatum of male adult C57Bl6 mice with lentiviral vectors encoding mutant ataxin-3 in one hemisphere and wild-type ataxin-3 in the other hemisphere (as internal control). Caffeine (1 g/L) was administered through the drinking water. Neuropathological analysis provided evidence showing that synaptotoxicity and gliosis are precocious events in MJD and that caffeine and $A_{2A}R$ inactivation decrease MJD-associated striatal pathology, which paves the way to consider $A_{2A}R$ as novel therapeutic targets to manage MJD.

In Chapter 3, MJD transgenic mice (TgMJD) were given caffeine (1 g/L, applied through the drinking water) and were tested using a panel of motor and non-motor paradigms. TgMJD animals displayed a constellation of motor and cognitive/emotional alterations, such as loss of gross and fine tuned movements, as well of learning disabilities and mood alterations. We provide evidence showing that caffeine prevents the progressive loss of motor functions, balance and grip strength, and the underlying cerebellar morphology modifications likely through a normalization of an imbalance in the dopaminergic circuitry.

In Chapter 4, expression of mutant ataxin-3 in the striata of male adult C57Bl6 mice: i) was followed by treatment with KW6002, a selective antagonist of $A_{2A}R$, or ii) coupled with silencing or overexpression of $A_{2A}R$ with lentiviral vectors. Mutant ataxin-3 expression in striata caused an extensive loss of DARPP-32 immunoreactivity accompanied by a clear condensation of the internal capsule. KW6002 reduced the loss of DARPP-32 and prevented

striatal loss. On the contrary, $A_{2A}R$ knockdown in striatal GABAergic medium spiny neurons (MSNs) exerted no effect over MJD progression and $A_{2A}R$ over-expression resulted in a tendency for early neuroprotection. Our findings directly implicate $A_{2A}R$ in MJD progression and support a distinct role for $A_{2A}R$ localized at pre and post-synaptic striatal compartments, which should be carefully considered when conceiving therapeutic $A_{2A}R$ antagonistic approaches intended to reduce MJD-associated pathology.

Overall, this thesis provides for the first time evidences that $A_{2A}R$ might be a novel therapeutic target to interfere with MJD evolution.

Resumo

A doença de Machado-Joseph (DMJ) é uma doença neurodegenerativa causada por uma repetição excessiva do trinucleótido CAG no gene *MJD1/ATXN3* que se traduz numa expansão de uma repetição de glutaminas na proteína ataxina-3. Não existe actualmente nenhuma terapêutica que previna ou modifique a progressão da doença. No entanto, como a manipulação do sistema neuromodulador da adenosina, em particular do seu receptor A_{2A}, se tem revelado eficaz no controlo e alívio da neurodegenerescência em diferentes patologias cerebrais, testámos agora a sua capacidade de controlar a neurodegenerescência associada à DMJ. Com este propósito, utilizámos diferentes modelos roedores animais de DMJ, nomeadamente o modelo baseado na introdução intra-cerebral de vectores lentivirais e num modelo transgénico, e averiguámos a eficiência do antagonismo dos receptores A_{2A}, bem como da sua deleção genética e molecular nas modificações neuropatológicas e comportamentais associadas.

A administração crónica de cafeína (por via oral) foi a estratégia escolhida dada a sua capacidade para antagonizar os receptores de adenosina, em particular os receptores A_{2A} (Fredholm et al., 1999), de atravessar a barreira hematoencefálica e, graças ao seu perfil de segurança, de poder tornar-se uma estratégia profilática promissora para modificar a progressão da DMJ.

No capítulo 2, modulámos a DMJ transduzindo o estriado de animais murinos adultos da estirpe C57Bl6 com vectores lentivirais codificando para a ataxina-3 mutante num hemisfério e para a ataxina-3 normal no hemisfério contralateral (como controlo interno), e administrámos cafeína (1 g/L) solubilizando-a na água de beber. Os nossos resultados evidenciam dois fenómenos precoces na DMJ, a sinaptotoxicidade e a gliose, e demonstram que a cafeína e a deleção genética dos receptores A_{2A} diminuem a patologia estriatal associada à doença o que nos leva a considerar os receptores A_{2A} como um potencial novo alvo terapêutico para tratar a DMJ.

No capítulo 3, administrámos cafeína (1 g/L, na água de beber) em murganhos transgénicos da DMJ (TgDMJ) e testámo-los em vários paradigmas motores e não motores. Os animais TgDMJ apresentaram uma série de modificações motoras e cognitivas/ emocionais, tais como défices na movimentação fina, e na capacidade de aprendizagem bem como alterações de humor. Os nossos resultados mostram que a cafeína previne a perda progressiva da função motora, do equilíbrio e da força muscular, acompanhado por uma preservação da morfologia cerebelar e da normalização de um possível desequilíbrio nos circuitos dopaminérgicos.

No capítulo 4, o modelo da DMJ murganho baseado na transdução estriatal de lentivírus codificando para a ataxina-3 mutante foi: i) seguido de tratamento por via oral com o composto KW6002, um antagonista selectivo dos receptores A_{2A}; ou ii) paralelamente co-transduzido com vectores lentivirais que promoveram o silenciamento ou a sobre-expressão dos receptores A_{2A}. A expressão da ataxina-3 mutante no estriado de murganho induziu uma perda extensa de imunoreactividade à proteína DARPP-32 seguida de uma clara condensação da cápsula interna do corpo estriado. O composto KW6002 reduziu a perda de DARPP-32 e preveniu a redução do corpo estriado. Por sua vez, o silenciamento dos receptores A_{2A} nos neurónios GABAérgicos estriatais do tipo espinhosos médios não exerceu qualquer efeito na progressão da DMJ conquanto a sua sobre-expressão resultou numa tendência para a neuroprotecção. Os nossos resultados suportam o envolvimento directo dos receptores A_{2A} na progressão da DMJ e evidenciam a existência de um papel distinto dos receptores A_{2A} localizados nos compartimentos pré- e pós-sináptico estriatais, devendo este facto constituir uma preocupação especial na concepção de terapêuticas baseadas no antagonismo dos receptores A_{2A} direccionadas para reduzir a patologia da doença de Machado-Joseph.

Por fim, esta dissertação apresenta um conjunto de evidências que demonstram pela primeira vez que os receptores A_{2A} constituem um novo alvo terapêutico que permite interferir com a evolução da DMJ.

CHAPTER 1

Introduction

1.1 Neurodegenerative diseases

Neurodegenerative diseases, such as Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS) and polyglutamine diseases that include Huntington's disease (HD) and Machado-Joseph disease (MJD), as well other polyglutamine-associated spinocerebellar ataxias, arise from abnormal protein interactions in the central nervous system. In all of these diseases, there are characteristic deposits of protein aggregates in the brain, such as α-synuclein, amyloid-β (Aβ), mutant SOD1 and polyglutamine containing proteins (DiFiglia et al., 1997; Duyckaerts et al., 1995; Nussbaum and Polymeropoulos, 1997; Paulson et al., 1997b; Stieber et al., 2000), which frequently contain other material besides the disease protein. These abnormal deposits of misfolded proteins produce malfunctioning and degeneration of a distinctive set of neurons.

Although protein aggregates have emerged as common pathological features of neurodegenerative disorders, there has been great controversy about the role of aggregation in the disease process. Actually, despite the many indirect evidences linking aggregation to toxicity (Bucciantini et al., 2002; Desai et al., 2006; Welch and Diamond, 2001), there are reports showing a weak correlation between the inclusion accumulation and the pathogenic process in human patient's brain (Terry et al., 1991; Tompkins and Hill, 1997) and also that the number of such aggregates does not perfectly match with neuronal loss (Kuemmerle et al., 1999). These evidences indicate that protein aggregates might represent a cellular protective response, i.e., an end-stage manifestation of a multistep aggregation process (Arrasate et al., 2004; Saudou et al., 1998; Taylor et al., 2003). In keeping with this observation, increasing evidences have emerged showing that early events before the formation of large inclusion bodies may occur and cause toxicity. Possible culprits include abnormal monomers and small assemblies of the disease proteins (Haacke et al., 2006; Wellington et al., 2000) closely followed by the loss of synaptic markers (Chapman et al., 1999; Li et al., 2001; Sawle et al., 1993) preceding or in parallel with oxidative and endoplasmic reticulum stress, proteosomal and mitochondrial dysfunction (Shastry, 2003) and inflammatory responses that ultimately lead to neuronal death. Thence, the molecular pathogenesis might involve several cellular compartments. Establishing whether a modification is pathogenic or beneficial is a question that has critical therapeutic relevance, especially since these are devastating and untreatable diseases, which slowly progress over years or decades with a reduction in quality of life.

1.1.1 Polyglutamine diseases

Polyglutamine (PolyQ) diseases are a subset of neurodegenerative conditions each deriving from an unstable CAG triplet repeat expansion in a specific gene, which is translated as an expanded tract of repeated glutamines in the codified protein. The first CAG triplet repeat disease was described in 1991: a mutation in the androgen receptor (AR) gene that causes a progressive motor neuron disease named spinal bulbar muscular atrophy (SBMA) (La Spada et al., 1991). So far, several other mutations have been identified associated with this CAG codon becoming abnormally expanded above a certain threshold. There are currently nine dominantly-inherited polyglutamine disorders, namely Huntington's disease (HD), dentatorubral-pallidoluysian atrophy (DRPLA) and spinocerebellar ataxia (SCA) types 1, 2, 3, 6, 7 and 17, in addition to the first described, all commonly sharing a progressive and selective neuronal loss accompanied by a decline in physical and psychological functions (Gatchel and Zoghbi, 2005), although the proteins associated with each different disorder display no homology beyond the polyQ tract, being structurally and functionally unrelated (Table 1).

Table 1. Overview of the current polyQ disorders.

Disease	Protein	Known function	Most affected brain regions
SBMA	Androgen	Steroid-hormone receptor	Anterior horn and bulbar neurons, dorsal root ganglia
HD	Huntingtin	Signaling transport, transcription	Striatum, cerebral cortex
DRPLA	Atrophin 1	Transcription	Cerebellum, cerebral cortex, basal ganglia, Luys body
SCA1	Ataxin-1	Transcription	Cerebellar Purkinje cells, dentate nucleus, brainstem
SCA2	Ataxin-2	RNA metabolism	Cerebellar Purkinje cells, brainstem, frontotemporal lobes
MJD/SCA3	Ataxin-3	Deubiquitination	Cerebellar dentate nucleus, basal ganglia, brainstem, spinal cord
SCA6	P/Q-type calcium-channel α1 subunit	Voltage-sensitive calcium-channel subunit	Cerebellar Purkinje cells, dentate nucleus, inferior olive
SCA7	Ataxin-7	Transcription	Cerebellum, brainstem, macula, visual cortex
SCA17	TATA binding protein	Transcription	Cerebellar Purkinje cells, inferior olive

SBMA, spinal and bulbar muscular atrophy; HD, Huntington's disease; DRPLA, dentatorubral-pallidoluysian atrophy; SCA, spinocerebellar ataxia; MJD, Machado-Joseph disease. Adapted from (Matos et al., 2011; Williams and Paulson, 2008; Zoghbi and Orr, 2000).

Notably, the age of onset inversely correlates with the size of the CAG stretch (Maciel et al., 1995); larger polyQ expansions markedly lead to earlier symptoms and increased severity of the phenotype, a phenomena called anticipation (Fleischer, 1918; Myers et al., 1982). Although not proven definitively, a theoretical molecular model of repeat instability based on replication fork stalling and restart, provided a golden clue for the origin of triplet disorders wherein DNA unwinding or even complete strand separation processes trigger unusual DNA structures formed by expandable repeats in genomic DNA (Mirkin, 2006). This hypothesis was recently confirmed in a human cell model (Liu et al., 2013) whereupon irregular DNA hairpin formation was eliminated to some extent by oligodeoxynucleotides complementarity. These striking reports underpin the core of all processes and allow considering that these unusual DNA structures indeed bewilder the machinery for major genetic transactions, primarily DNA replication but also recombination and repair, ultimately leading to repeat instability, which is a hallmark of polyQ disorders. This consideration implies that all polyQ disorders share a common peculiar genomic profile highly difficult to circumvent.

1.1.1.1 Pathogenesis

The underlying mechanisms that trigger polyQ-associated pathology are yet to be fully elucidated, as well as the selective vulnerability in neurodegeneration. Nevertheless, a wide variety of scientific contributions especially in the last 10 to 15 years have been made fostering a plethora of information, which together allowed greater understanding of these diseases.

1.1.1.1.1 Genetics

The first prominent issue to consider is the propensity of a given expanded CAG, even outside of a particular gene, to induce neurodegeneration itself. Intriguingly, a neurodegenerative phenotype resembling the human translated CAG repeat disorders was observed upon viral delivery of polyQ tracts in the adult rat brain (Senut et al., 2000) and in transgenic mice ectopically expressing a CAG-expansion (Ordway et al., 1997), indicating that polyQ itself is indeed prone to induce neurodegeneration. Secondly, yet an under-explored subject is polyQ RNA toxicity, which may involve defects in the overall RNA functional dynamics with profound implications for cellular physiology (reviewed in (Lukong et al., 2008)). In this regard, there have been episodic reports demonstrating that: i) the expression of an untranslated CAG repeat of pathogenic length conferred neuronal degeneration (Li et al., 2008); ii) CAG expansion constructs express homopolymeric

polyglutamine, polyalanine and polyserine proteins in the absence of an ATG start codon, a mechanism known as repeat-associated non-ATG translation (RAN translation) (Zu et al., 2011); iii) there is an upregulation of a gene implicated in the RNA toxicity of CUG expansion diseases, namely myotonic dystrophy type 1 (DM1) and SCA8 in a MJD/SCA3-induced fly model (Koob et al., 1999; Wells et al., 1998); and iv) a CAG length dependent ribosomal frameshifting occur resulting in translational errors and toxicity mediated by polyalanine-frameshifted peptides (Toulouse et al., 2005); these evidences indicate that there might be actually an RNA-mediated gain-of-function mechanism with a pathogenic role (Wojciechowska and Krzyzosiak, 2011). In accordance with this observation, a recent clinical report showed an interplay between MJD/SCA3-CAG repeats and DM1-CTG repeats in a single patient's phenotype (Miura et al., 2009), which adds further dimension to this topic. And third, the epigenetic status, such as DNA methylation, chromatin remodeling (histone acetylation) and microRNAs (miRNAs) regulators of gene expression (Fig 1.1), still is an under-appreciated issue in polyQ disorders whose modified phenotypic plasticity in parallel with the aging process (reviewed in (Marques et al., 2011) might actually equally contribute to precipitate toxicity and degeneration (Bilen et al., 2006; Laffita-Mesa et al., 2012; Palhan et al., 2005; Ying et al., 2006; Zadori et al., 2009).

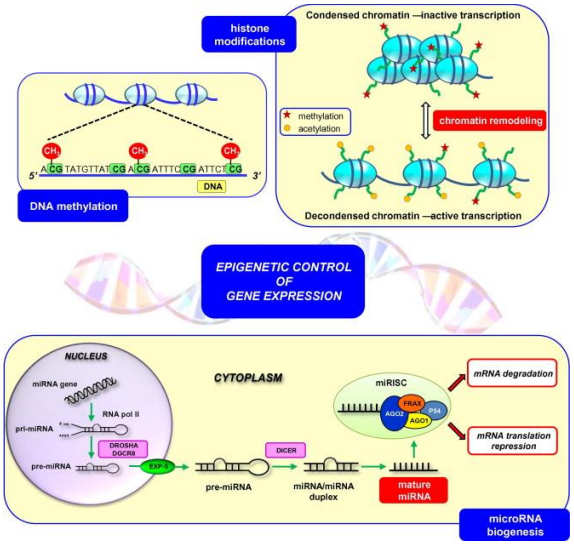


Figure 1.1: Principal epigenetic mechanisms of gene expression regulation. DNA methylation, inhibits gene transcription if occurring in the gene promoter but it may also promote transcription when localized at gene exons sites (*upper left panel*). Epigenetic histone modifications are post-translational changes that influence gene expression by controlling the dynamics of chromatin. Histone tail methylation and acetylation are reported as the main histone epigenetic phenomena as schematically shown in the *upper right panel* of the figure. In the *lower panel* is reported a simplified scheme of microRNAs (small non-coding RNA fragments) biogenesis. The biogenesis of microRNAs occurs across nucleus and cytoplasm of the cell, the latter cell environment being the place where mature microRNAs exert their down-regulating effect on gene transcription by leading to target mRNA degradation or by mRNA translation repression. Adapted from (Udali et al., 2013).

1.1.1.1.2 Alteration of normal protein function

PolyQ disorders might in part derive from perturbation of normal polyglutamine protein function. Accordingly, there are studies implying a loss-of-function of the involved protein in HD (Dragatsis et al., 2000; Leavitt et al., 2001), SCA1 (Bowman et al., 2007) and MJD/SCA3 (Warrick et al., 2005), based on polyQ gene inactivation and over-expression experiments of the respective wild-type form. However, several genetic studies have revealed that the absence of the polyQ-involved proteins (wild-type form) does not cause neurodegeneration both in humans (Ambrose et al., 1994; Davies et al., 1999) and mice (Lastres-Becker et al., 2008; Schmitt et al., 2007), strongly suggesting that the polyQ expanded protein causes disease mainly by a dominant gain-of-function mechanism whereby it confers toxic properties to the host proteins. Therefore, polyQ expanded proteins may aggregate into inclusion bodies (DiFiglia et al., 1997; Holmberg et al., 1998; Paulson et al., 1997b) or undergo proteolytic cleavage (Kim et al., 2001; Li et al., 2007; Simoes et al., 2012; Wellington et al., 2002), and subsequent misfolding may lead to toxic effects in multiple targets (Nagai et al., 2007; Scherzinger et al., 1997; Welch and Diamond, 2001) including the quality control system, energy metabolism, transcriptional machinery and synaptic transmission.

Taken together with genetics (the previous section), these hypotheses may in general represent the triggers of the observed alterations in the regular patterns of cell and brain functioning attributed to expanded polyQ proteins accomplishing ultimately useful information for the understanding of the inexorable progression of polyQ disorders. These issues, incidentally the most studied for polyQ disorders, will be further detailed in section 1.1.1.1.3.

1.1.1.1.3 Induced alterations by expanded polyQ proteins

Figure 1.2 summarizes the key pathogenic mechanisms induced by expanded polyQ proteins.

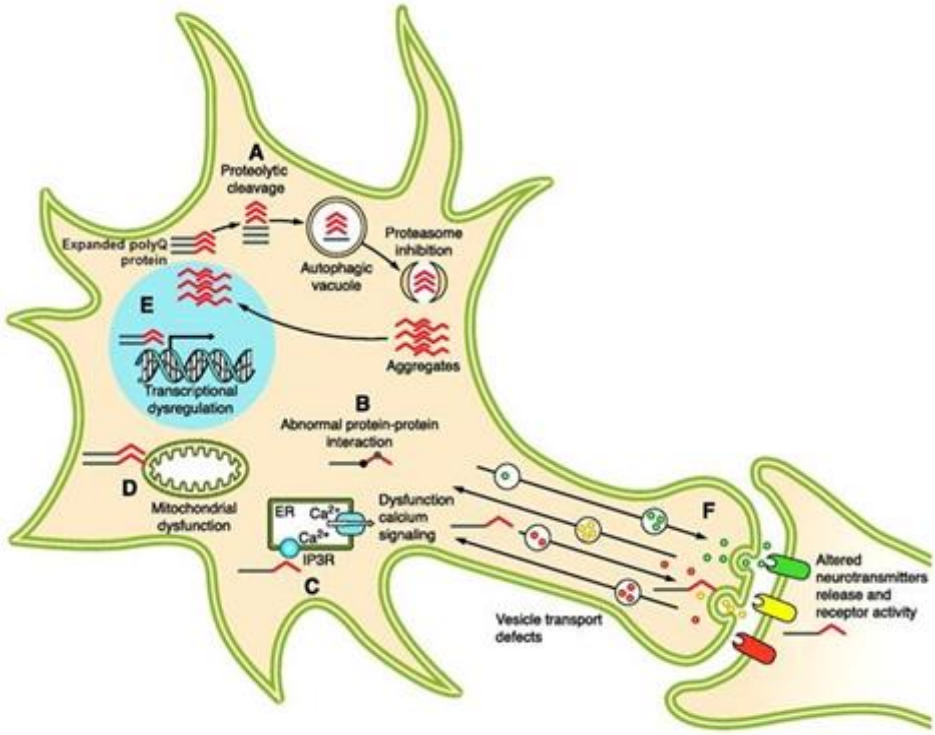


Figure 1.2: Key cellular pathogenic mechanisms induced by expanded-polyQ proteins. Multiple cellular pathways have been implicated in the pathogenesis of polyQ disorders. These mechanisms could be exclusive or, more likely, have a high degree of cross-talk. (A) The polyQ expansion causes a conformational change of the protein that leads to its partial unfolding or abnormal folding, which can be corrected by molecular chaperones. Full-length mutant protein is cleaved by proteases in the cytoplasm. In an attempt to eliminate the toxic polyQ-expanded proteins, fragments are ubiquitinated and targeted to the proteasome for degradation. However, due to the abnormal size of the polyQ fragments, proteasome becomes less efficient. Induction of the proteasome activity as well as of autophagy protects against the toxic insults of mutant proteins by enhancing its clearance. (B) Fragments containing the polyQ stretch accumulate in the cell cytoplasm and interact with several proteins causing impairment of calcium signaling and homeostasis (C) and metabolic deficits through mitochondrial dysfunction (D). (E) Toxic fragments may translocate to the nucleus where they impair gene transcription also forming intranuclear inclusions. (F) The mutation in polyQ proteins alters vesicular transport and recycling, which might modify synaptic transmission. Adapted from (Zuccato et al., 2010).

1.1.1.1.3.1 Quality control system failure

The pathogenic polyQ length predisposes polyQ proteins to misfolding and aggregation (Scherzinger et al., 1997), and the accumulation of misfolded proteins is a common feature of polyQ disorders. As the brain seems uniquely susceptible to protein misfolding, it thus appears to play a key role in pathogenesis. This suggests a critical link between polyQ disorders and protein quality control, a collection of cellular pathways that sense damage to proteins and facilitate their turnover. Consistently, cells are equipped with

systems that quickly attack unfolded proteins by either refolding or degrading them, thus in general the misfolded products are rapidly removed.

The cellular machinery that operates protein refolding is the large family of chaperone proteins, which are distributed in all subcellular compartments even on synapses. The role of chaperones, mostly heat shock proteins, in polyQ diseases has been extensively studied and have shown the ability to be protective in multiple polyQ disease models, namely in SBMA (Adachi et al., 2007), HD (Miller et al., 2005), SCA1 (Al-Ramahi et al., 2006) and SCA3 (Jana et al., 2005). As chaperones display a very competent ability to modulate the toxicity of multiple polyQ disease proteins, they have even been dubbed as polyQ disease modifiers.

If chaperones cannot refold abnormal proteins correctly, they then promote their subsequent ubiquitination, which ultimately directs them to degradation. The degradation system includes the ubiquitin-proteasome pathway (UPS) and the autophagic/ lysosomal pathway, which together are responsible for intracellular protein turnover and recycling of cellular components. Herein, proteasome malfunction has been implicated in several polyQ diseases including SBMA, HD, SCA1, MJD/SCA3 and SCA7 (Bence et al., 2001; Mandrusiak et al., 2003; Park et al., 2005; Schmidt et al., 2002; Wang et al., 2007; Wang et al., 2008) wherein either proteasome subunits are recruited into inclusion bodies (Ciechanover and Brundin, 2003) or proteasome fails to fully digest soluble expanded polyQ proteins (Venkatraman et al., 2004). Additionally, also impaired autophagy has been described in SBMA (Rusmini et al., 2010), HD (Heng et al., 2010; Ravikumar et al., 2004), SCA1 (Vig et al., 2009), MJD/SCA3 (Menzies et al., 2010; Nascimento-Ferreira et al., 2011) and SCA7 (Duncan et al., 2010), whose activation reduces accumulated polyQ aggregates and subsequent cell degeneration.

1.1.1.1.3.2 Metabolism and mitochondria dysfunction

Evidences of metabolic defects correlating with neurological features and functional decline were first described in HD patients (Young et al., 1986). These defects include reduced glucose and mitochondrial oxidative metabolisms assessed by PET (positron emission tomography) and MRS (magnetic resonance spectroscopy) in HD patients (Feigin et al., 2007; Jenkins et al., 1993) as well as a general increase of oxidative stress (Browne et al., 1997; Browne et al., 1999), preceded by early mitochondrial calcium-handling defects (Oliveira et al., 2006; Panov et al., 2002). In addition, as protein quality control system is ATP-dependent, those defects may exacerbate putative difficulties in polyQ proteins refolding and degradation, essential to proteostasis and maintenance of neuronal cell survival (Gines et al., 2003). Such modifications have been also implicated in the

pathogenesis of SBMA, SCA1 and MJD/SCA3 (Beauchemin et al., 2001; Kim et al., 2003; Laco et al., 2012; Lodi et al., 2000; Tsai et al., 2004) thus underlying another common feature shared by polyQ diseases.

1.1.1.1.3.3 Transcriptional dysregulation

It is consensual that there is a general transcriptional disturbance by polyQ disease proteins. Interactions of expanded polyQ proteins with specific transcription factors may perturb and repress gene expression, and thus initiate neurodegeneration. Many of those interactions have been substantially described (Cui et al., 2006; McCampbell et al., 2000; Nucifora et al., 2001) and involve sequestration by polyQ protein monomers (Hoshino et al., 2004) or recruitment into aggregates disrupting the normal regulation of target genes. Nevertheless, with exception for SCA7 whose transcriptional repression might possibly lead to a neuron-specific pathology (La Spada et al., 2001), it still cannot explain cell and brain regional specificity of degeneration for all polyQ disorders.

1.1.1.1.4 Protein sub-cellular localization; susceptibility of neurons and synapses

Inclusion formation happens when protein concentrations rise above critical levels. The situation at the synapse, with its tiny volume, highly crowded environment and harsh chemical conditions could be favorable for moving above this threshold, thus promoting the formation of protein aggregates. Also, synaptic junctions are often located at great distances from the cell soma, which poses some additional limits to the strength and the capacity of the recovery process (Malgaroli et al., 2006). Therefore, large aggregates could potentially disrupt the functional behavior of synapses impeding electrical and chemical signals to propagate acting as a sort of physical barrier, counteracting intracellular trafficking of organelles to and from the synapse (Li et al., 2003a). In fact, Wang and colleagues (Wang et al., 2008) have recently reported that mutant huntingtin decreased synaptic UPS activity in isolated synaptosomes of HD mouse brains, which adds further dimension to this perspective. On the other hand, oligomers have been also implicated as critical players in disrupting synaptic functions in many neurodegenerative diseases acting as pore forming toxins on membranes, thus altering the ion balance of neurons and synapses (Lashuel et al., 2002; Walsh et al., 2002). In keeping with this scenario, the differential susceptibility of various cell types might then depend on the environmental context where polyQ proteins are located, wherein local compensatory mechanisms restoring physiological conditions and different lipid-membrane compositions have a determinative role, beyond the abnormal function and protein-protein interactions exerted by each specific polyQ proteins.

1.1.1.2 Machado-Joseph disease

1.1.1.2.1 Historical overview: from clinical to molecular diagnosis. Epidemiology.

Machado-Joseph disease, also known as spinocerebellar ataxia type 3 (MJD/SCA3), was initially described in Northern American families of Azorean ancestry. In 1972, Nakano and colleagues reported a family descendent from William Machado, a native from São Miguel in Azores, presenting progressive hereditary ataxia (Nakano et al., 1972). Four years later, Rosenberg and collaborators described another Azorean-ancestry family (from Flores) presenting a "new" hereditary ataxia different from the former one (Rosenberg et al., 1976), further called as "Joseph's disease" (Rosenberg et al., 1978). Finally, the Portuguese clinicians and researchers, Paula Coutinho and Corino Andrade, unified the designation of the disease upon studying 15 families from the Azorean Islands proposing that the above mentioned diseases were indeed variations of the same clinical disorder, the "Machado-Joseph disease" (MJD) (Coutinho and Andrade, 1978), although of marked clinical heterogeneity. Progressive cerebellar ataxia and pyramidal signs comprise the major clinical features with an age of onset ranging from the second to the fifth decade; other minor, but more specific, clinical signs such as progressive external ophtalmoplegia, dystonia, dysphagia and facial and lingual fasciculation-like movements were also found in MJD patients (D'Abreu et al., 2010; Lima and Coutinho, 1980) bringing complexity to MJD diagnosis. Importantly, the intellect is preserved in MJD. The preservation of the cognitive function is a key feature of MJD in its differential diagnosis among the large group of spinocerebellar ataxias (Coutinho and Andrade, 1978).

Fifteen years later, a mutated gene showing an expanded CAG repeat was described in MJD patients (Higgins et al., 1996; Lindblad et al., 1996; Maciel et al., 1995; Sequeiros et al., 1994), mapped to chromosome 14q32.1 (Kawaguchi et al., 1994; Takiyama et al., 1993); the number of CAG repeats range from 61 to 87 in disease carriers while it varies between 12 to 44 in healthy population (Maciel et al., 2001). This allowed establishing a molecular diagnostic for MJD, based on the determination of the CAG trinucleotide repeat length in the *ATXN3* gene through targeted mutation analysis. Interestingly, it was also found an intergenerational instability (Igarashi et al., 1996), meaning that the repeat tracts presented different lengths in progenitors and offspring. This dynamic mutation explains the anticipation phenomena observed in MJD families.

MJD is presently considered the most common dominantly inherited ataxia worldwide (Schols et al., 2004). The availability of molecular genetic testing has allowed a thorough identification of cases, changing the initial geographic distribution pattern of MJD, first related with the Portuguese discoveries and currently known to be present in many ethnic

backgrounds, with strong geographic variation (Fig 1.3). Among SCAs, the relative frequency of MJD is higher (above 50%) in countries such as Brazil, Portugal and China; and it is relatively less frequent (21-24%) in Canada and United States (Bettencourt and Lima, 2011). The highest worldwide prevalence occurs in Flores Island, Portugal (1/239) (Bettencourt et al., 2008).

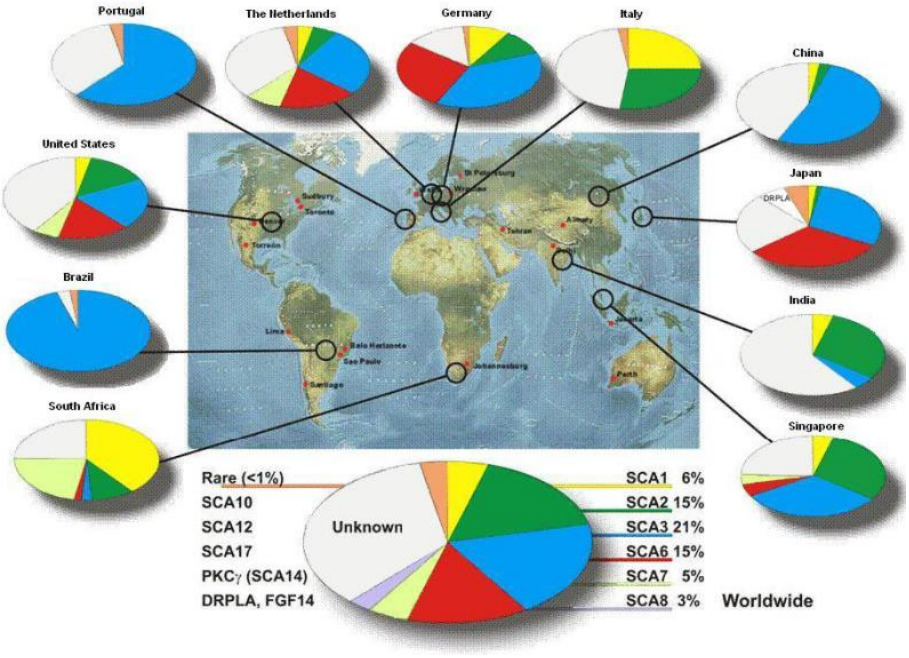


Figure 1.3: Worldwide distribution of SCA subtypes. Figure published courtesy of L Schöls, P Bauer, T Schmidt, T Schulte, O Reiss of University of Tübingen and Ruhr-University Bochum, Germany. Adapted from Hereditary Ataxia Overview. In: Gene Reviews™ [Internet] Pagon RA, Adam MP, Bird TD, et al., editors.

1.1.1.2.2 MJD pathology

ATXN3 mRNA is ubiquitously expressed in neuronal and non-neuronal human tissues (Ichikawa et al., 2001); ataxin-3 protein is present not only in the brain but also throughout the body, either in the cytoplasm or in the nucleus of various cell types, being predominantly cytoplasmic in neurons (Paulson et al., 1997a). The abnormal gene mutation in *ATXN3* locus results in an expanded polyQ tract at the C-terminus of the ataxin-3 protein (Masino et al., 2003) conferring protein propensity for aggregation (Paulson et al., 1997b) (Fig 1.4), an hallmark in the pathological characterization of MJD.

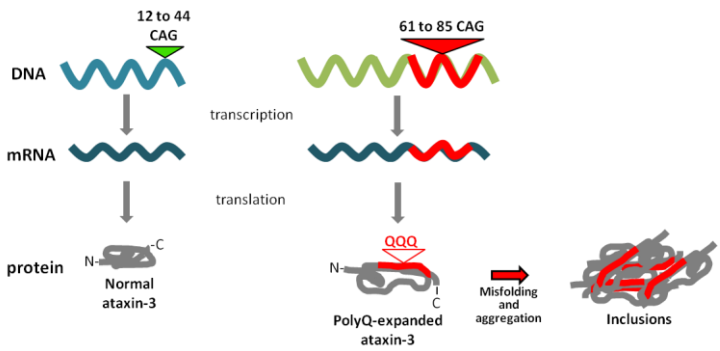


Figure 1.4: Schematic representation of polyQ-expanded ataxin-3 misfolding and aggregation. The expanded polyglutamine tract adopts an incorrect folding conformation subsequently causing toxicity in many ways.

Indeed, the presence of ataxin-3 neuronal intranuclear inclusions (NIIs) has been argued to play a crucial role in MJD neurodegeneration involving neuronal loss in selective brain regions, such as the cerebellum (spinocerebellar pathways and dentate nucleus), brainstem (pons and medulla oblongata), basal ganglia (globus pallidus, caudate and putamen, substantia nigra) and spinal cord (Alves et al., 2008b; Durr et al., 1996; Klockgether et al., 1998; Rub et al., 2008; Sudarsky and Coutinho, 1995; Taniwaki et al., 1997; Wullner et al., 2005), supporting also a sequestration model of pathogenesis where several crucial proteins, such as ubiquitin and proteosomal components, chaperones and transcription factors may be sequestered into aggregates impairing various cellular key pathways (Chai et al., 2002). Nonetheless, there is only a partial correlation between neuronal loss and NIIs (Munoz et al., 2002) which makes hardly to explain selective neuronal degeneration by aggregates in addition to the low mosaicism between regions with and without neuronal loss and the ubiquitous pattern and cellular expression of the disease gene, pointing out the existence of selective cellular vulnerabilities to the genetic defect involved in the restricted neuropathology observed in MJD.

1.1.1.2.3 Neurobiology of MJD. Therapeutic targets

The neurobiology of the disease is not fully elucidated. There have been great efforts in the last few years studying the affected cellular pathways in order to identify putative therapeutic targets to successfully slow or block disease progression. Several not mutually exclusive mechanistic hypotheses have emerged from numerous *in vitro* and *in vivo* studies exploiting cellular toxicity triggered by mutant ataxin-3 and its altered protein interactions. Among all, the follow stand out: i) dysregulation of ataxin-3 proteostasis, namely, ataxin-3 biogenesis (Alves et al., 2008a; Hu et al., 2011), folding (Teixeira-Castro et al., 2011; Warrick et al., 1999), trafficking/localization (Mueller et al., 2009; Pastori et al., 2010) and degradation (Menzies et al., 2010; Nascimento-Ferreira et al., 2011; Simoes et al., 2012); ii) metabolic impairment, such as mitochondrial dysfunction and oxidative stress (Chang et al., 2009; Tsai et al., 2004); iii) transcriptional dysregulation (Chou et al., 2011; Chou et al., 2008); iv) abnormal neuronal signaling (Chen et al., 2008); and v) impairment of axonal transport (Burnett and Pittman, 2005; Rodrigues et al., 2010). These promising developments searching the affected molecular pathways in MJD have been closely followed by successful targeted approaches in different MJD animal models. The most effective and widely applicable therapies are likely to be those designed to eliminate production of the mutant protein upstream of the subsequent deleterious effects, though none has yet been advanced to clinical trials in MJD patients.

In summary, MJD is a slowly progressive chronic disease triggered by a mutation in the *ATXN3* gene, which initiates and extends over decades a disturbance of the normal cellular homeostasis through a cascade of interrelated pathogenic events subsequently leading to a malfunction of the brain circuitries resulting in progressive ataxia, dysfunction of motor coordination and postural instability among several other clinical features (discussed in 1.1.1.2.1). This thesis sought to pursuit the initial cascade of events triggered by polyQ-expanded ataxin-3, in particular, the induced alterations in synaptic and glial functions, which have been under-explored and whose interplay and contribution to the inexorable evolution of the disease are still unknown. From there, this work focused in the effects of blocking the adenosine A_{2A} receptors function whose ability to control synaptic transmission and glial-mediated responses might be beneficial.

1.1.1.2.4 MJD management. Treatment of manifestations.

MJD management remains supportive as no medication has been proven to slow the course of disease (D'Abreu et al., 2010). Accordingly, some strategies have been used in an attempt to reduce symptomatology: i) extrapyramidal syndromes resembling parkinsonism and symptoms of restless legs syndrome may respond to levodopa or dopamine agonists (Buhmann et al., 2003; Nandagopal and Moorthy, 2004; Schols et al., 1998); ii) spasticity, drooling, and sleep problems also respond variably to appropriate agents, namely baclofen, atropine-like drugs, and hypnotic agents (Yun et al., 2005); iii) dystonia has been treated with botulinum toxin (Freeman and Wszolek, 2005); iv) excessive daytime fatigue may also respond to psychostimulants used in narcolepsy ; and v) accompanying depression might be treated with antidepressants (Cecchin et al., 2007). Non-pharmacological approaches, such as physiotherapy, physical aids, such as walkers and wheelchairs, regular speech therapy as well as occupational therapy help patients to cope with disability, dysphagia and dysarthria, and assist in their everyday activities (D'Abreu et al., 2010).

1.2 Adenosine receptors in the context of neurodegenerative diseases

The adenosine receptors (ARs) in the nervous system regulate the release of neurotransmitters and the action of neuromodulators, such as neuropeptides and neurotrophic factors. In fact, ARs are the effectors of adenosine, a ubiquitous homeostatic substance released from most cells including neurons and glia, which has been considered as a fine-tuning modulator of neuronal activity. In keeping with the fact that ARs (A_1R and

$A_{2A}R$) are located in all nervous system cells (neurons and glia) and adenosine is intensively released upon injuries, together they play a key role in the homeostatic coordination of the brain function controlling the efficiency and plasticity-regulated synaptic transmission. Whenever this homeostasis is disrupted, pathology may be installed and selective receptor antagonism or agonism required in a very short time window to handle the outcome of the insult.

1.2.1 Distribution and role of ARs in central nervous system

Neuromodulation by adenosine is mainly exerted through the activation of high-affinity A_1 and A_{2A} receptors, as A_{2B} and A_3 receptors are present at very low densities in the central nervous system (CNS) (Dixon et al., 1996), which are G protein-coupled receptors (GPCRs) belonging to the P1 class of purinoceptors (Burnstock, 1976).

A_1R is widely distributed in the brain, being highly expressed in cortex, cerebellum, hippocampus and dorsal horn of spinal cord (Dixon et al., 1996; Reppert et al., 1991), whereas $A_{2A}R$ is highly expressed in GABAergic medium spiny neurons (MSNs) of the indirect pathway and olfactory bulb (Jarvis and Williams, 1989; Parkinson and Fredholm, 1990) and at lower levels in cerebellum, cortex and hippocampus (Cunha et al., 1995; Svenningsson et al., 1997), thus displaying different relative densities comparable to A_1R in the same brain areas. Both ARs are similarly present in neurons, astrocytes (Biber et al., 1997; Nishizaki et al., 2002) and microglia cells (Gebicke-Haerter et al., 1996; Kust et al., 1999), and also exhibit distinct sub-cellular localizations which may be related to each particular receptor function. A_1R is mostly found at the presynaptic active zone and also abundantly located in the postsynaptic density in rat hippocampus together with N-methyl-D-aspartate (NMDA) receptor subunits and with N- and P/Q-type calcium channels (Rebola et al., 2003) where they inhibit synaptic transmission by reducing calcium transients and the evoked release of glutamate among other neurotransmitters (Dunwiddie and Masino, 2001). In turn, $A_{2A}R$ can be found in pre-synaptic nerve terminals from hippocampus and post-synaptically in MSNs where they exhibit the highest density in the brain; a minority of striatal $A_{2A}R$ is also present pre-synaptically on the cortico-striatal glutamatergic afferents (Rebola et al., 2005a). As $A_{2A}R$ have different sub-cellular localizations, also operate distinct functions: while postsynaptic $A_{2A}R$ seems to control the signaling in MSNs (reviewed in (Fredholm and Svenningsson, 2003), pre-synaptic $A_{2A}R$ mediates facilitation of the release of neurotransmitters, such as glutamate (Lopes et al., 2002), GABA (Cunha and Ribeiro, 2000), acetylcholine (Rebola et al., 2002) and serotonin (Okada et al., 2001). The facilitation of glutamate release may play a role in noxious conditions, as several $A_{2A}R$ antagonists have

been shown to be protective by this mechanism (Melani et al., 2003; Orru et al., 2011; Popoli et al., 2002).

The overall neuromodulatory role of adenosine in the CNS is thus a balance between A_1R and $A_{2A}R$ functions as they can be located at the same synapse (Rebola et al., 2005c). Together they can modify cellular responses to conventional neurotransmitters or receptor agonists. A_1R tend to suppress neuronal activity by a predominant pre-synaptic action, while $A_{2A}R$ are more likely to promote transmitter release and postsynaptic depolarization. Furthermore, adenosine receptors can also control astrogliosis (Brambilla et al., 2003), the release of neuroactive substances (Hindley et al., 1994) and inflammation (Minghetti et al., 2007; Ohta and Sitkovsky, 2001).

1.2.2 Role of ARs in neurodegenerative diseases

Adenosine receptors modulate neuronal function and synaptic plasticity in a range of ways that places them in a central position within the occurrence, development and putative treatment of various neuropathological conditions. Based on their distribution and distinct roles, A_1R and $A_{2A}R$ also oppositely contribute to cell damage in different brain areas in a wide range of degenerative paradigms, where they can also suffer an adaptational change in receptor number or properties. In parallel, several ARs ligands, either agonists or antagonists, have been used, acutely and chronically, as modifiers of brain damage intriguingly demonstrating significantly different and diverse effects on damage outcomes in diverse neurodegenerative brain disorders, namely Alzheimer's, Parkinson's and Huntington's diseases, probably resulting from the nature of each pathogenesis at the corresponding brain areas. Furthermore, whether protection against damage mediated by adenosine itself and AR ligands depends mainly on controlling neurotransmitter release or activity than on modulation of the immune system, as neuroinflammation is a common event in neurodegenerative diseases, is still a matter of debate. Microglial activation is a faithful sensor of pathologic events in the brain (Kreutzberg, 1996) and a decrease in the extent of neuroinflammation is associated with a better prognosis in the progression of neurodegenerative diseases (Marchetti and Abbracchio, 2005).

1.2.2.1 Glutamate excitotoxicity

Glutamate is the major excitatory neurotransmitter of the CNS (for review see (Platt, 2007) and also displays important roles in non-excitable cells within CNS, namely astrocytes (reviewed in (Nedergaard et al., 2002). Acting as a neurotransmitter, released glutamate activates a family of ligand-gated ion channels, designated ionotropic glutamate receptors

(iGluR), which includes NMDA, α-amino-3-hydroxyl-5-methyl-4-isoxazole propionic acid (AMPA) and kainate (Traynelis et al., 2010). Under pathophysiological conditions, where glutamate reaches unusual higher levels in the synaptic cleft, brain damage might occur through over-activation of glutamate receptors, a phenomenon known as “excitotoxicity”. In fact, glutamate-mediated excitotoxicity has been implicated in acute injury to the CNS and in diverse chronic neurodegenerative disorders, namely AD (Gray and Patel, 1995), PD (Loschmann et al., 1994), ALS (Shaw and Ince, 1997) and HD (Sun et al., 2001). In AD, in addition to the Aβ and tau proteins, hallmarks of the disease, NMDA over-activation by glutamate is also a critical mediator of neuronal damage (Butterfield and Pocernich, 2003). Similarly, in PD, loss of nigro-striatal dopaminergic inputs disinhibit glutamatergic neurotransmission which, in turn, allows initiation of striatal glutamatergic over-excitation (Garcia et al., 2010; Vaarmann et al., 2013). In sporadic ALS, which accounts for > 90% of all cases, motor neurons in the spinal cord are unduly susceptible to calcium-mediated toxic events following glutamate receptor activation, as they present low expression of calcium binding proteins and GluR2 AMPA receptor subunit (Corona et al., 2007; Shaw and Ince, 1997). Also in HD, cortico-striatal release of glutamate has been reported to induce apoptosis via NMDA receptors in the particularly vulnerable striatal cells (Fernandes et al., 2007).

In the last few years, episodic reports identified $A_{2A}R$ as key modulators of glutamate release based on the neuroprotective effects afforded by $A_{2A}R$ blockade over glutamate over-excitation upon noxious situations (Melani et al., 2003; Orru et al., 2011; Popoli et al., 2002). In addition, $A_{2A}R$ can also control glutamate release and clearance from astrocytes (reviewed in (Cunha, 2005). Thus, $A_{2A}R$ blockade appears as a promising neuroprotective strategy by preventing glutamate excitotoxicity that is present in diverse neurodegenerative diseases.

1.2.2.2 Neuroinflammation

Neuroinflammation is a double-edged sword as it is present in different conditions of brain damage contributing either for damage or repair and regeneration of brain tissue (Elward and Gasque, 2003; Marchetti and Abbracchio, 2005; Schwartz, 2003; Weiner and Selkoe, 2002). Microglia cells within the CNS are rapidly up-regulated in response to infection or tissue injury (Kreutzberg, 1996; Streit et al., 1999); once activated they release pro-inflammatory cytokines, such as Il-1β and TNF-α, and anti-inflammatory cytokines, namely IL-10, TGF-β and IL-1Ra, which will contribute to initiate, propagate and regulate the inflammatory reactions in CNS, depending on their final balance and effects in the immune system (Benveniste, 1998; Chavarria and Alcocer-Varela, 2004; Dinarello, 2011).

Adenosine senses tissue damage and inflammation triggering a paradoxical modulation of peripheral inflammation and neuroinflammation by $A_{2A}R$ (Sitkovsky, 2003). The activation of $A_{2A}R$ prevents peripheral inflammation acting as an 'OFF' signal of immune responses upon tissue damage (Sitkovsky and Ohta, 2005), which is consistent with reports showing the exacerbation of peripheral tissue damage upon $A_{2A}R$ blockade (reviewed in (Sitkovsky et al., 2004). Conversely, $A_{2A}R$ blockade in CNS mediates neuroprotection by controlling neuroinflammation (Cunha et al., 2007). This has been shown in AD, PD, HD, epilepsy and ischemia models, among others (reviewed in (Gomes et al., 2011).

This contradictory modulation by $A_{2A}R$ reflects the complexity of $A_{2A}R$ actions on neuronal, glial and vascular components, which may exert distinct effects in brain injury (Chen et al., 2007; Cunha et al., 2007).

1.2.2.3 ARs in polyQ disorders. The case of Huntington's disease

Several reports support a cortico-striatal glutamatergic dysregulation in HD pathogeny. Indeed, mutated huntingtin induces glutamatergic dysfunctions through the increase of glutamate release (Guidetti et al., 2004) and decrease of astrocytic glutamate clearance (Behrens et al., 2002), and also by increasing expression and activation of NMDA receptors (revised in (Fan and Raymond, 2007)), among others. As stated above, A_1R activation suppresses glutamate release from neurons. In line with this glutamate-induced excitotoxicity, Blum and colleagues (2002) have reported that an A_1R agonist was able to prevent the neuronal degeneration and motor sequelae of mitochondrial toxin 3-nitropropionic acid (3-NP) administration to mice (Blum et al., 2002). However, since there was no apparent protection in cell cultures, this was presumed as a pre-synaptic action where the release of glutamate could be inhibited. In addition to these observations, neuroprotective effects were found in different animals models of HD, namely 3-NP, quinolinic acid (QA) and R6/2 transgenic mice, attributable to $A_{2A}R$ antagonists and $A_{2A}R$ genetic inactivation (Domenici et al., 2007; Fink et al., 2004; Popoli et al., 2002), which is in accordance with the pre-synaptic localization of $A_{2A}R$ where they may operate a decrease of glutamate levels (Pintor et al., 2001) by prevention of its release (Popoli et al., 2002; Tebano et al., 2004) or decreasing its release and enhancing its uptake by glial cells (Nishizaki, 2004; Pintor et al., 2004). However, $A_{2A}R$ are mainly located post-synaptically in MSNs of glutamatergic synapses (Lei et al., 2004) where they also control the activation/expression of NMDA receptors (Ferrante et al., 2010; Wirkner et al., 2000) and subsequent plastic changes in cortical glutamatergic inputs (Schiffmann et al., 2007); and also present in non-neuronal compartments, such as endothelial and glial cells, where $A_{2A}R$ may control the blood-brain

barrier (BBB) structure and function, and glial responses to injury and inflammation (Coney and Marshall, 1998; Fields and Burnstock, 2006; Ngai et al., 2001). Together, the post-synaptic and extra-synaptic effects of $A_{2A}R$ blockade have been speculative and most studies favor $A_{2A}R$ agonists rather than antagonists as protective agents in the particular case of the degeneration of MSNs, which is attributable to the ability of $A_{2A}R$ agonists to reduce NMDA currents in striatal MSNs whereas $A_{2A}R$ antagonists potentiated NMDA-mediated toxicity (Norenberg et al., 1997; Popoli et al., 2007; Popoli et al., 2002; Tebano et al., 2004; Wirkner et al., 2000).

In conclusion, the available data regarding the exploitation of $A_{2A}R$ ligands to treat HD, the most studied among polyQ disorders, still is controversial reflecting the complexity of $A_{2A}R$ regulation in this disease. Besides both $A_{2A}R$ agonists and antagonists have been shown to provide protection in animal models of HD, it remains to be defined whether it is the activation or the blockade of $A_{2A}R$ that can bring clinical benefits. The complexity of functions operated by AR, particularly by $A_{2A}R$ in specific cellular and regional locations within striatum, mediate detrimental or beneficial effects depending on the time-frame of the disease, and suggest that neither stimulation nor blockade are beneficial or that both can be advantageous.

1.2.3 Neuroprotective mechanisms through central adenosinergic system

Adenosine is released upon stressful situations (Fredholm et al., 2005). Thus, one candidate strategy to mediate neuroprotection might be the control of the adenosine levels. This can be achieved by manipulating the enzyme adenosine kinase (ADK) activity, a key sensor and regulator of adenosine, which plays a prominent role in determining the brain's susceptibility to injury by integrating and fine-tuning glutamatergic and dopaminergic neurotransmission (Boison, 2008). In fact, several reports showed that increasing endogenous levels of adenosine, based on the inhibition of the ADK, effectively conferred neuroprotection in diverse conditions, such as epilepsy, ischemia and schizophrenia (Gouder et al., 2004; Lara et al., 2006; Pignataro et al., 2007), among others. Yet, others have demonstrated that this was not a suitable therapeutic target for successful clinical development as pharmacological manipulation of the adenosine kinase activity may cause severe side effects (Gouder et al., 2004; Ugarkar et al., 2000). Regardless, adenosine-releasing cell transplants have recently emerged and may circumvent that situation (Boison, 2007).

Another possibility to control the adenosine effects might be the manipulation of the adenosine receptors owing to its known role in pathological situations. Indeed, A_1Rs activation play a role in neuroprotection by decreasing the metabolic rate, an attempt to hamper the detrimental effects caused by noxious stimulus, such as excitotoxicity induced by kainate and quinolinic acid or dopaminergic neurotoxicity (Delle Donne and Sonsalla, 1994; MacGregor et al., 1997). Additionally, it also mediates a decrease of glutamate release and hyperpolarizes neurons (Cunha, 2005). Yet, this neuroprotection conferred by A_1R activation is limited in time due to desensitization that occurs in time frames of 12-24h upon chronic noxious brain conditions (Coelho et al., 2006; Cunha, 2005); the poor brain permeability of A_1R agonists and the occurrence of prominent cardiovascular effects (Shryock and Belardinelli, 1997) are also disadvantages to add to the very short “window of opportunity” for their successful use as a neuroprotective strategy.

On the other hand, chronic noxious brain conditions exhibit an A_1R down-regulation in parallel with an A_{2A}R up-regulation, typified by an increase in expression and density of A_{2A}R found in diverse animal models, namely Parkinson’s disease (PD), epilepsy and restraint stress (Cunha et al., 2006; Pinna et al., 2002; Rebola et al., 2005b; Tomiyama et al., 2004). This prompts considering the manipulation of A_{2A}R as a promising therapeutic target to manage adenosine signaling. In fact, there is now greater effort to dissect the putative role of global and regional and cell-type specific A_{2A}R upon brain noxious stimulus. Accordingly, most of A_{2A}R antagonists confer neuroprotection in several pathological conditions in adult animals, either upon ischemia (Chen et al., 1999; Gao and Phillis, 1994; Monopoli et al., 1998), or excitotoxicity (Behan and Stone, 2002; Jones et al., 1998). Additionally, A_{2A}R antagonists provided functional protection against dopaminergic neurotoxicity also displaying a reduction in degeneration of the dopaminergic system in the MPTP model of PD (Chen et al., 2001; Xu et al., 2002). Using forebrain neuronal-specific A_{2A}R knockout mice, Chen and colleagues also reported that A_{2A}R activity in forebrain neurons was critical for control of psychomotor activity, but not for neuroprotection against brain injury, which highlighted a putative role for glial A_{2A}R (Yu et al., 2008). In line with this suggestion, selective A_{2A}R antagonists were also shown to abrogate fibroblast growth factor (FGF)-induced formation of reactive astrocytes (Brambilla et al., 2003), a common feature among neurodegenerative diseases. Together, these data highlighted a novel compartment where the exploitation of the A_{2A}R role should be performed to elucidate the neuroprotective effects mediated by A_{2A}R antagonists upon diverse brain insults.

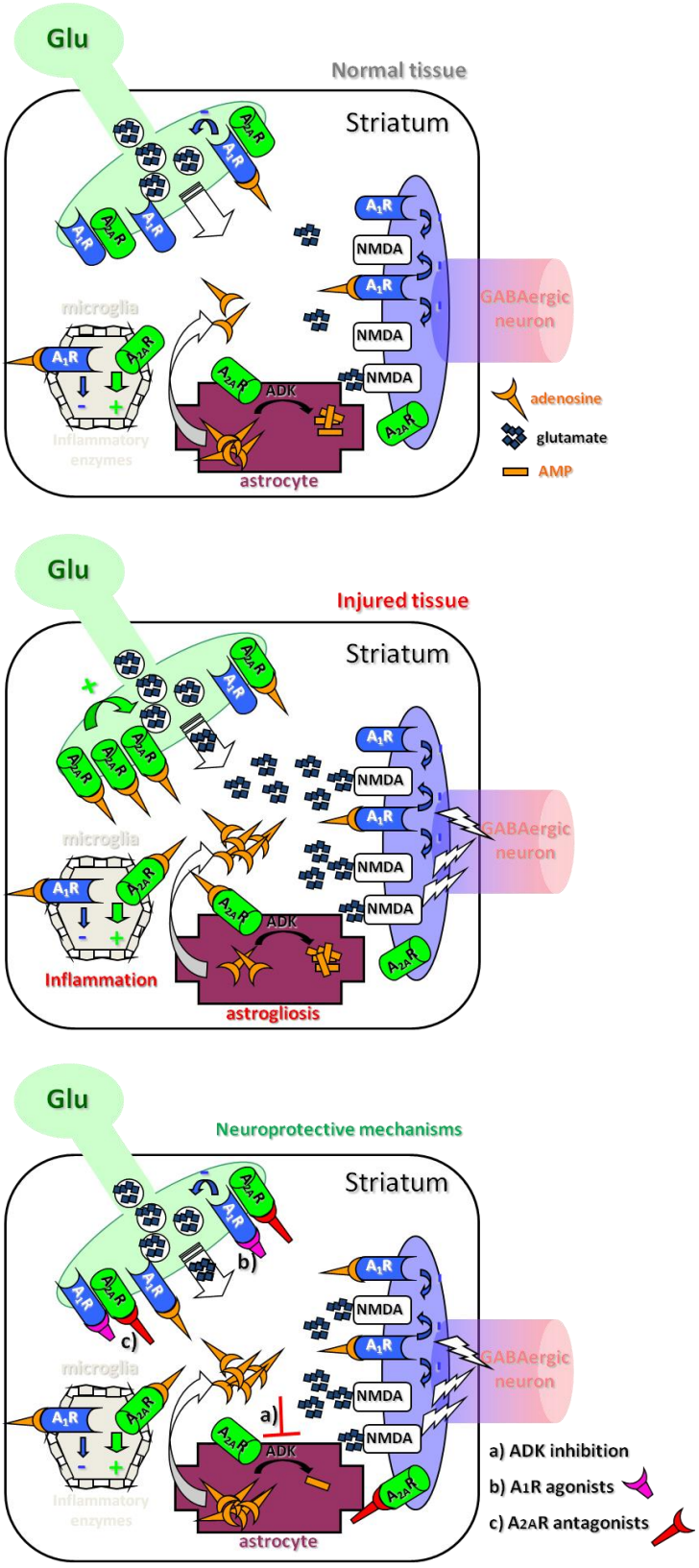


Figure 1.5 summarizes the aforementioned neuroprotective strategies based on adenosine neuromodulation.

In conclusion, to date there are two leading not mutually exclusive mechanistic hypotheses through which $A_{2A}R$ blockade is argued to exert robust neuroprotection: the control of glutamate excitotoxicity and the control of neuroinflammation (discussed in (Cunha, 2005). Nevertheless, it is still critical to distinguish the contribution of $A_{2A}Rs$ in distinct cell-types in modulating brain damage in noxious situations.

Figure 1.5: All cell types and sub-cellular compartments in the brain are endowed with adenosine A_1 and A_{2A} receptors (A_1R, $A_{2A}R$) that fulfil different roles according to their localization. In physiological conditions, extracellular adenosine activates inhibitory A_1R (*upper panel*). Upon injury, extracellular adenosine rises in the synaptic cleft and preferentially activates facilitatory $A_{2A}R$ resulting in glutamatergic over-excitation, neuroinflammation and astrogliosis (*center panel*). Efficient neuroprotective strategies based on adenosine neuromodulation may rise from inhibition of adenosine kinase (ADK) (to burst A_1R activation) together with A_1R agonists and $A_{2A}R$ antagonists.

1.2.3.1 Caffeine and neuroprotection in neurodegenerative disorders

Xanthines and their impact on biomedical research have been extensively studied, in particular the effects of caffeine, which is widely consumed by humans all over the world. The effects of caffeine, which is taken at low doses associated with common beverages during normal human consumption, are mostly due to AR antagonism, especially of the $A_{2A}R$ (Fredholm et al., 1999). Due to its ability to antagonize ARs, to cross the BBB, and also due to low risk of intake, caffeine has therapeutic potential in CNS dysfunctions (e.g., AD, PD). In fact, chronic caffeine intake, which increases plasma concentrations of adenosine (Conlay et al., 1997), modulates the relative levels of A_1R and $A_{2A}R$, and of receptors for neurotransmitters (Jacobson et al., 1996) promoting decreased neuronal excitability and has been shown to exert neuroprotective action in diverse experimental animal models mimicking AD (Arendash et al., 2006; Dall'Igna et al., 2007; Espinosa et al., 2013) and PD (Aguiar et al., 2006; Chen et al., 2001; Sonsalla et al., 2012), and clinically by the observation of an inverted correlation between caffeine intake and the risk of developing cognitive and motor impairments in humans (Ritchie et al., 2007; Ross et al., 2000). However, special attention should accompanied the generalized use of caffeine since its consumption in large amounts, and in particular over extended periods of time, can cause a dependency called caffeinism, which combines caffeine dependency with a wide range of unpleasant physical and mental conditions including nervousness, irritability, anxiety, muscle twitching, hyperreflexia, insomnia and headaches, among others (Daly, 2007; Fredholm et al., 1999).

In conclusion, increasing body of human and experimental studies revealed encouraging evidence that regular human consumption of caffeine in fact may have several beneficial effects on neurodegenerative disorders, namely, motor stimulation/improvement, cognitive enhancement and neuroprotection. The demonstration of a broad spectrum of neuroprotection by chronic treatment of caffeine and adenosine receptor ligands in animal models of neurodegenerative disorders also encouraged the development of several A_{2A} receptor selective antagonists, which avoid caffeine undesirable effects due to its other biological actions (Daly, 2007), and are actually now in advanced clinical phase III trials for Parkinson’s disease.

This thesis explores: i) the putative beneficial effects of chronic administration of caffeine on morphological and behavioral abnormalities displayed by two distinct genetic animal models of MJD, namely, lentiviral-based and transgenic mice; and ii) whether the effects are operated by $A_{2A}R$ blockade through selective $A_{2A}R$ antagonism and $A_{2A}R$ gene deleted animals.

1.3 Lentiviral-mediated gene transfer to CNS: disease modeling and therapeutic and research applications

Neurodegeneration in CNS can be modeled in any mammalian species (rodents and primates) through stereotactic delivery of viral vectors into selected brain regions (Deglon and Hantraye, 2005), which offers several unique advantages over transgenic animal models. Lentiviral vectors (LVs) are one such tool allowing to generate diverse disease models of CNS pathologies through targeting specific brain regions known to be affected, and in a relatively short period of time compatible to the lifespan of rodents due to enabled high expression levels of the disease-causing proteins (Alves et al., 2008b; de Almeida et al., 2002; Senut et al., 2000). Indeed, several morphological and behavioral features of motor system disorders have been replicated so far, namely, Huntington's disease (de Almeida et al., 2002; Palfi et al., 2007), Machado-Joseph disease (Alves et al., 2008b; Nobrega et al., 2012) and Parkinson's disease (Lauwers et al., 2003; Lo Bianco et al., 2002). Lentiviral-based approaches are also advantageous as they allow establishing the onset and follow-up of the time-course progression of degeneration.

Beyond their successful application to replicate neurodegenerative diseases, LVs have proved promising to exploit the role and subsequent therapeutic potential of disease-related and -modifier genes, for example through the manipulation of neurotrophic or antiapoptotic factors (Blomer et al., 1998; de Almeida et al., 2001; Kordower et al., 2000) and several key components of the cellular machinery known to be abnormally reduced or oppositively hyper-functioning (Nascimento-Ferreira et al., 2011; Simoes et al., 2012). Actually, one of the most promising applications of LVs which led to its current widespread use is the capability of mediating RNA interference (RNAi) (discussed in (Couto and High, 2010) (Fig 1.6), i.e., the shutting-down of gene expression with high efficiency and specificity, a breakthrough methodology (Fire et al., 1998) that has proved to successfully delay the onset and development of autosomal dominant disorders, such as Huntington's disease (DiFiglia et al., 2007; Franich et al., 2008; Harper et al., 2005), SCA1 (Xia et al., 2004), and SCA3/MJD (Alves et al., 2008a; Nobrega et al., 2013), which may reduce the preclinical development time if indicated as gene therapy candidate approaches. In the aforementioned studies, the use of short hairpin RNAs (shRNAs) reduced neuropathological abnormalities such as the size and number of neuronal inclusions, improved the behavioral phenotype and delayed the onset and progression of disease.

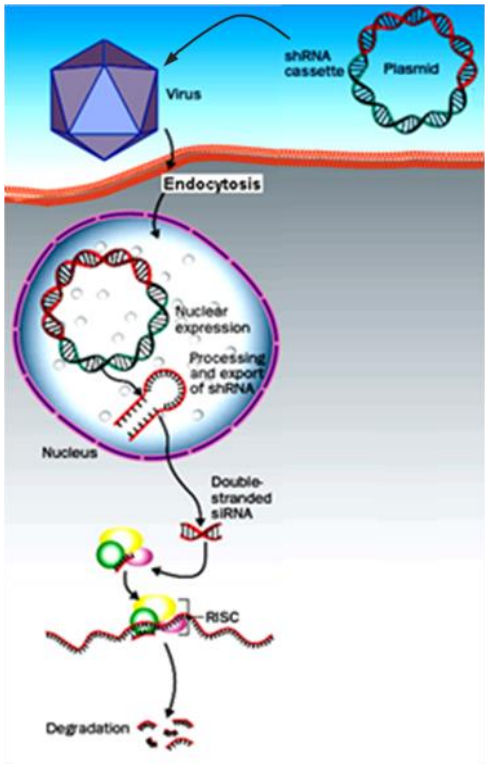


Figure 1.6: Viral-mediated introduction of RNAi inhibitory sequences into target cells. A viral vector encoding a shRNA infects the target cell, via a receptor-mediated endocytosis, releasing the viral genome that after reverse transcription enters the nucleus to be transcribed into a shRNA. After processing, the shRNA is transported to the nucleus, mediating gene silencing. Adapted from (Davidson and Paulson, 2004).

Brain delivery of silencing constructs allows not only to develop therapeutic strategies, but also to create new genetic disease models (Hommel et al., 2003) and adds further dimension in functional genomics by probing for specific roles of proteins in different brain regional compartments deepening our knowledge in the neurobiology of a wide range of pathologies (Johnson and Kenny, 2010; Lazarus et al., 2011).

Together, the combined research applications of lentiviral vectors and RNAi allowed major advances in our understanding of the nervous system in health and disease, though novel powerful tools, such as the engineered nucleases for genome editing, namely, Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs), have emerged as particularly attractive candidates to become effective therapies for polyQ diseases, which might completely change our current therapeutic perspectives once the drawbacks and technical difficulties of designing, engineering and delivering are successfully exceeded.

1.4 Objectives

Our main goal was to investigate the potential of manipulating the adenosine neuromodulation system by blocking the adenosine A_{2A} receptors as a disease modifying strategy in MJD.

The specific aims of the project are as follows:

- to investigate whether synaptotoxicity and neuroinflammation are early features of MJD, by studying the morphological modifications triggered by expressing pathogenic ataxin-3 in mice injected with viral vectors encoding mutant ataxin-3, (Chapter 2);

- to investigate whether A_{2A} receptors antagonism and genetic elimination (knockout) in the lentiviral mouse model of MJD prevents mutant ataxin-3-induced neuronal dysfunction and degeneration, upon a) chronic caffeine consumption, b) A_{2A}R knockout mice (Chapter 2), and c) administration of an A_{2A}R selective antagonist (Chapter 4);

- to study if chronic caffeine consumption alleviates the behavioral deficits of a transgenic mouse model of MJD (Chapter 3);

- to investigate whether gene silencing of A_{2A} receptors by lentiviral-mediated cell-specific expression of short hairpin RNAs prevents mutant ataxin-3-induced neurodegeneration (Chapter 4).

CHAPTER 2

Caffeine and adenosine A_{2A} receptor inactivation decrease striatal neuropathology in a lentiviral-based model of Machado-Joseph disease

2.1 Abstract

Machado-Joseph disease (MJD) is a neurodegenerative disorder associated with an abnormal CAG expansion, which translates into an expanded polyglutamine tract within ataxin-3. There is no therapy to prevent or modify disease progression. Since caffeine (a non-selective adenosine receptor antagonist) and selective adenosine A_{2A} receptor ($A_{2A}R$) blockade alleviate neurodegeneration in different brain diseases, namely at early stages of another polyglutamine-related disorders such as Huntington's disease, we now tested their ability to control MJD-associated neurodegeneration.

MJD was modelled by transducing the striatum of male adult C57Bl6 mice with lentiviral vectors encoding mutant ataxin-3 in one hemisphere and wild-type ataxin-3 in the other hemisphere (as internal control). Caffeine (1 g/L) was applied through the drinking water. Mice were killed at different time points (from 2-12 weeks) to probe for the appearance of different morphological changes using immunohistochemical analysis.

Mutant ataxin-3 caused an evolving neuronal dysfunction (loss of DARPP-32 staining) leading to neurodegeneration (Cresyl violet and NeuN staining) associated with increased number of mutant ataxin-3 inclusions in the basal ganglia. Notably, mutant ataxin-3 triggered early synaptotoxicity (decreased synaptophysin and MAP-2 staining) and reactive gliosis (GFAP and CD11b staining), which predated neuronal dysfunction and damage. Caffeine reduced the appearance of all these morphological modifications, which were also abrogated in mice with a global $A_{2A}R$ inactivation (knockout).

Our findings provide a demonstration that synaptotoxicity and gliosis are precocious events in MJD and that caffeine and $A_{2A}R$ inactivation decrease MJD-associated striatal pathology, which paves the way to consider $A_{2A}R$ as novel therapeutic targets to manage MJD.

2.2 Introduction

Various inherited neurodegenerative diseases result from an increase in the number of CAG codon repeats within the open reading frame of the responsible gene (Koshy and Zoghbi, 1997). Machado-Joseph disease (MJD) or spinocerebellar ataxia type 3, is one such dominantly-inherited polyglutamine neurodegenerative disease and the most common among ataxias (Ranum et al., 1995). It is characterised by an adult age of onset causing premature death associated with unstable expansion of a CAG stretch (over 55 repeats) in the *ATXN3* gene that encodes a polyglutamine repeat in the corresponding ataxin-3 protein (Durr et al., 1996; Kawaguchi et al., 1994). The clinical hallmarks of MJD include progressive ataxia, dysfunction of motor coordination, postural instability and Parkinsonism among other symptoms (Gwinn-Hardy et al., 2001; Taroni and DiDonato, 2004). The neuropathology of MJD involves multiple systems such as cerebellar systems, substantia nigra and cranial nerve motor nuclei (Durr et al., 1996; Sudarsky and Coutinho, 1995), as well as the striatum (Alves et al., 2008b; Klockgether et al., 1998; Taniwaki et al., 1997; Wullner et al., 2005). Degeneration and loss of neuronal cells in MJD is accompanied by the presence of protein aggregates (Paulson et al., 1997b), designated as neuronal intranuclear inclusion bodies (NIIs). Although transgenic animal models closely mimicking the human pathology (Alves et al., 2008b; Bichelmeier et al., 2007; Cemal et al., 2002; Goti et al., 2004), have bolstered our understanding of MJD, the mechanisms accounting for neuronal degeneration are still largely unknown. Albeit not yet explored in MJD, studies in other polyQ disorders suggest that neuronal dysfunction and synaptotoxicity may precede degeneration and appearance of clinical symptoms (Andrews et al., 1999; Li et al., 2001), and that neuroinflammation (Bantubungi et al., 2005), may function as an amplificatory loop exacerbating neuronal damage (Aktas et al., 2005; Gao et al., 2008; Lee et al., 2008).

There is currently no therapy to manage MJD. We posed that chronic caffeine consumption, which affords neuroprotection through the antagonism of adenosine A_{2A} receptors ($A_{2A}R$) (Cunha and Agostinho, 2010), might be a candidate strategy to manage MJD neurodegeneration. Caffeine and $A_{2A}R$ blockade afford robust neuroprotection in different neurodegenerative disorders, in accordance with the key role of $A_{2A}Rs$ controlling synaptic viability, apoptotic neuronal death, astrocytic function and neuroinflammation (Chen et al., 2007; Gomes et al., 2011). In particular, in Huntington's disease, another polyQ disorder, $A_{2A}R$ blockade at the prodrome or early stages of the disease seems to delay the appearance of clinical symptoms, mainly through a normalization of striatal glutamatergic transmission, which impedes the characteristic degeneration of striatal neurons (Popoli et al., 2007). Importantly, it has recently been shown in induced pluripotent stem cells-derived neurons that glutamate overstimulation raises intracellular calcium levels activating the

cysteine proteases calpains and promoting the proteolysis and aggregation of ataxin-3 (Koch et al., 2011). Moreover, calpain-mediated proteolysis of ataxin-3 in a rodent model of MJD mediates translocation of ataxin-3 to the cell nucleus, aggregation and neurodegeneration, which can be prevented by calpain inhibition (Simoes et al., 2012).

In view of this proposed key role of glutamate overstimulation in MJD, we now investigated the time course of neuropathological modifications in a genetic model of MJD and tested the novel hypothesis that the manipulation of $A_{2A}R$ function might also be beneficial in MJD.

2.3 Materials and Methods

2.3.1 Animals

Male C57BL/6 mice (Charles River, Barcelona, Spain) were housed and kept under a conventional 12-h light-dark cycle maintained on a temperature-controlled room with food and water provided *ad libitum* and used at 7 weeks of age. C57Bl/6-background $A_{2A}R$ knockout ($A_{2A}R$ KO) and age-matched wild type control mice were obtained from parallel breeding of our colony of $A_{2A}R$ KO mice, initially obtained from J.F.Chen (Boston University)(Chen et al., 1999). The experiments were carried out in accordance with the European Community directive (86/609/EEC) for the care and use of laboratory animals.

2.3.2 Drug treatment

We chose the dose of caffeine (1 g/L) administered through the drinking water, as a maximally effective and non-toxic dose, which we have previously shown to result in a plasma concentration of 50 µM (Duarte et al., 2012), and similar concentration in the brain parenchyma (Costenla et al., 2010), corresponding to a diary human consumption of circa 5 cups of coffee. Treatment with caffeine was begun 3 weeks before viral delivery onwards, since we have previously reported that a minimum period of 2 weeks is required to allow a metabolic stabilization after beginning the free access to caffeine drinking (Duarte et al., 2012; Duarte et al., 2009).

2.3.3 Viral vectors production

Lentiviral vectors encoding human wild-type ataxin-3 (atx3-27Q) or mutant ataxin-3 (atx3-72Q) (Alves et al., 2008b), were produced in 293T cells with a four-plasmid system, as previously described (de Almeida et al., 2001). The lentiviral particles were resuspended in 1% bovine serum albumin in phosphate-buffered saline (PBS). The viral particle content of batches was determined by assessing HIV-1 p24 antigen levels (RETROtek, Gentaur, Paris, France). Viral stocks were stored at -80ºC until use.

2.3.4 *In vivo* injection into the striatum

Concentrated viral stocks were thawed on ice. After anesthesia of the mice with avertin (12 µL/g, i.p.), lentiviral vectors encoding human wild-type (atx3-27Q) or mutant ataxin-3 (atx3-72Q) were stereotaxically injected into the striatum in the following coordinates: antero-

posterior: +0,6mm; lateral: ±1,8mm; ventral: -3,3mm; tooth bar: 0, which corresponds to the internal capsule, a large fiber tract passing through the middle of the striatum dividing both dorso-ventral and medial-lateral structures. Wild-type and A_{2A}R KO mice received 2 µL injections of lentivirus (200'000 ng of p24/mL) in each hemisphere, administering mutated ataxin-3 (atx3-72Q) in the right hemisphere and control wild type ataxin-3 (atx3-27Q) in the left hemisphere. Different groups of mice were kept in their home cages for different periods ranging from 2, 4, 8 and 12 weeks, before being killed for immunohistochemical analysis of morphological and neurochemical changes in the striatum.

2.3.5 Immunohistochemical procedure

After an overdose of avertin (2.5x 12 µL/g, i.p.), transcardiac perfusion of the mice was performed with PBS followed by fixation with 4% paraformaldehyde. The brains were then removed and post-fixed in 4% paraformaldehyde for 24h and cryoprotected by incubation in 25% sucrose/ phosphate buffer for 48h. The brains were frozen and 25 µm coronal sections were cut using a cryostat (LEICA CM3050 S, Heidelberg, Germany) 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.

Sections were processed overnight at 4ºC with the following primary antibodies: a mouse monoclonal anti-ataxin-3 antibody (1H9; 1:5000; Chemicon, Temecula, CA), a rabbit anti-DARPP-32 antibody (1:1000; Chemicon), or a mouse anti-NeuN antibody (1:1000; Chemicon) followed by 2h incubation at room temperature (RT) with the respective biotinylated secondary antibodies (1:200; Vector Laboratories, Burlingame, CA). Bound antibodies were visualized using the Vectastain ABC kit, with 3,3'-diaminobenzidine tetrahydrochloride (DAB metal concentrate; Pierce, Burlingame, CA) as substrate.

Triple staining for synaptophysin (rabbit polyclonal, 1:300; Chemicon) and MAP2 (mouse monoclonal AP20, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), GFAP (rabbit polyclonal, 1:1000; DAKO, Glostrup, Denmark) and CD11b (rat monoclonal 5C6, 1:500; AbD Serotec, Oxford, UK) together with DAPI (Sigma, St. Louis, MO) were performed. Free-floating sections were kept at RT for 2h in PBS with 0.1% Triton X-100 containing 10% normal goat serum (Gibco-Invitrogen, Barcelona, Spain), then overnight at 4°C in blocking solution with the primary antibodies. Sections were washed three times and incubated for 2h at RT with the corresponding secondary antibodies coupled to fluorophores goat anti-mouse or goat anti-rabbit or goat anti-rat Alexa Fluor 488 or Alexa Fluor 594 (1:200; Molecular

Probes – Invitrogen, Eugene, OR) diluted in the blocking solution. The sections were washed three times and then mounted in mowiol Reagent (Sigma) on microscope slides.

Definition and analysis of protein immunoreactivities were made from the striatal center (site of injection) to the medial-lateral and dorsal-ventral striatal periphery from the needle tract. This disease model is based on the intra-striatal injection of lentivirus, which triggers physiological alterations evolving over time radially. Therefore, comparable striatal sections between animals were defined from the site of injection in both rostral and caudal directions, using the needle tract due to the surgical procedure as reference-point.

Staining was visualized using Zeiss Axioskop 2 plus, Zeiss Axiovert 200 or Zeiss LSM 510 Meta imaging microscopes (Carl Zeiss Microimaging, Germany) equipped with AxioCam HR color digital cameras (Carl Zeiss Microimaging) and 5X, 20X, 40X and 63X Plan-Neofluar or a 63X Plan/Apochromat objectives and using the AxioVision 4.7 software package (Carl Zeiss Microimaging). Quantitative analysis of fluorescence was performed with a semi-automated image-analysis software package (Image J software, NIH, USA).

2.3.6 Cresyl violet staining

Coronal sections were pre-mounted and stained with cresyl violet for 45 sec, differentiated in 70% ethanol, dehydrated by passing twice through 95% ethanol, 100% ethanol and xylene solutions, and mounted onto microscope slides with Eukitt® (Sigma).

2.3.7 Evaluation of DARPP-32

The extent of ataxin-3 lesions in the striatum was analyzed by photographing, with a x1.25 objective, 8 sections stained for DARPP-32 per animal (25 µm thick sections at 200 µm intervals), selected so as to obtain complete rostro-caudal sampling of the striatum, and by quantifying the area of the lesion with a semi-automated image-analysis software package (Image J software). The volume was then estimated with the following formula: volume = $d(a_1+a_2+a_3 \ldots)$, where d is the distance between serial sections (200 µm) and $a_1+a_2+a_3$ are DARPP-32-depleted areas for individual serial sections. The average grey value of all pixels measured in the lesioned area was recorded for each depleted area. Results are expressed as index of immunoreactivity of DARPP-32 considering the unlesioned striatal area as 100% immunoreactivity.

2.3.8 Cell counts of ataxin-3 inclusions

Coronal sections showing complete rostro-caudal sampling (1 of 8 sections) of the striatum were scanned with a x20 objective. The analyzed areas of the striatum encompassed the entire region containing ataxin-3 inclusions, as revealed by staining with the anti-ataxin-3 antibody. All inclusions were manually counted using a semi-automated image-analysis software package (Image J software).

2.3.9 Statistical analysis

Statistical comparisons were performed using either an unpaired Student's *t* test or one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison post hoc test. Results are expressed as mean ± standard error of the mean (SEM). Significance thresholds were set at $p < 0.05$, $p < 0.01$ or $p < 0.001$, as defined in the text.

2.4 Results

2.4.1 Time course

We took advantage of using a lentiviral model of MJD to perform a temporal analysis of the relative appearance of different features from early dysfunction to late neuronal loss. Thus, we carried out a time-course study from 2 to 12 weeks upon lentiviral-mediated expression of wild-type and mutant ataxin-3, and we analysed different neuropathological features such as markers of synaptic loss, neuronal dysfunction, neuronal loss, of astrogliosis and microgliosis as well as ataxin-3 inclusions. Figure 2.1 summarises the temporal evolution of each of these changes, which will be further detailed below.

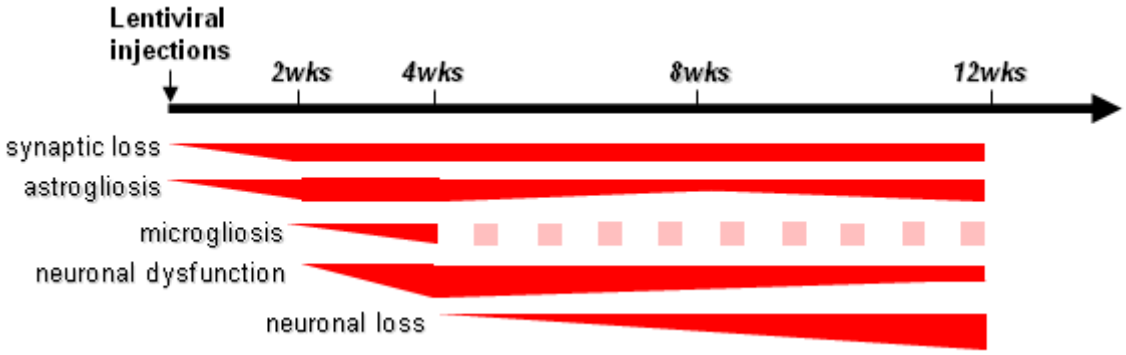


Figure 2.1: Summary of the time-course of appearance of different morphological features in a lentiviral-based model of Machado-Joseph disease. Loss of synaptic markers and astrogliosis were amongst the most precocious morphological alteration, closely followed by microgliosis and neuronal dysfunction as well as increases of the number of ataxin-3 inclusions, whereas overt neuronal damage occurred later in the development of the disease. Microgliosis would probably be found at later time-points, however, that was not evaluated.

2.4.2 Neuronal degeneration and loss

In order to directly test if caffeine and $A_{2A}R$ inactivation indeed mitigate neurodegeneration, we first analyzed photomicrographs under bright field and then upon cresyl violet staining. Lentiviral-mediated expression of mutant ataxin-3 (LV-atx3-72Q) caused a clear condensation of the internal capsule attributable to striatal tissue shrinkage, which was evident in water-drinking mice at 12 weeks, but absent on bright-field sections of both caffeine-drinking animals and global $A_{2A}R$ knockout (KO) mice, as well as in the contralateral striatum challenged with wild-type ataxin-3 (LV-atx3-27Q) (Fig 2.2A). Cresyl violet staining revealed a marked reduction ($p < 0.05$) of the number of degenerated shrunken hyperchromatic nuclei in caffeine-drinking mice even though it remained considerably high; the specificity of the effect over $A_{2A}R$ was confirmed in $A_{2A}R$ KO animals, which reproduced the alleviation of pathology observed in caffeine-treated animals ($p < 0.05$; Fig 2.2B and E).

To further investigate the neuroprotective effects of caffeine at a late time-point in this model of MJD, we evaluated the immunoreactivity of the neuronal nuclear marker NeuN. A clear loss of NeuN-stained neurons could be seen in the water-drinking group at 12 weeks after mutant ataxin-3 transduction, which was not detectable after wild-type ataxin-3 transduction, and was nearly absent in mutant ataxin-3-expressing animals upon caffeine treatment (Fig 2.2C).

These data suggest that there is a progression in the degeneration pattern in MJD-associated striatal pathology leading to loss of neuronal markers, which is reduced by chronic caffeine consumption as well as genetically deleting the $A_{2A}R$.

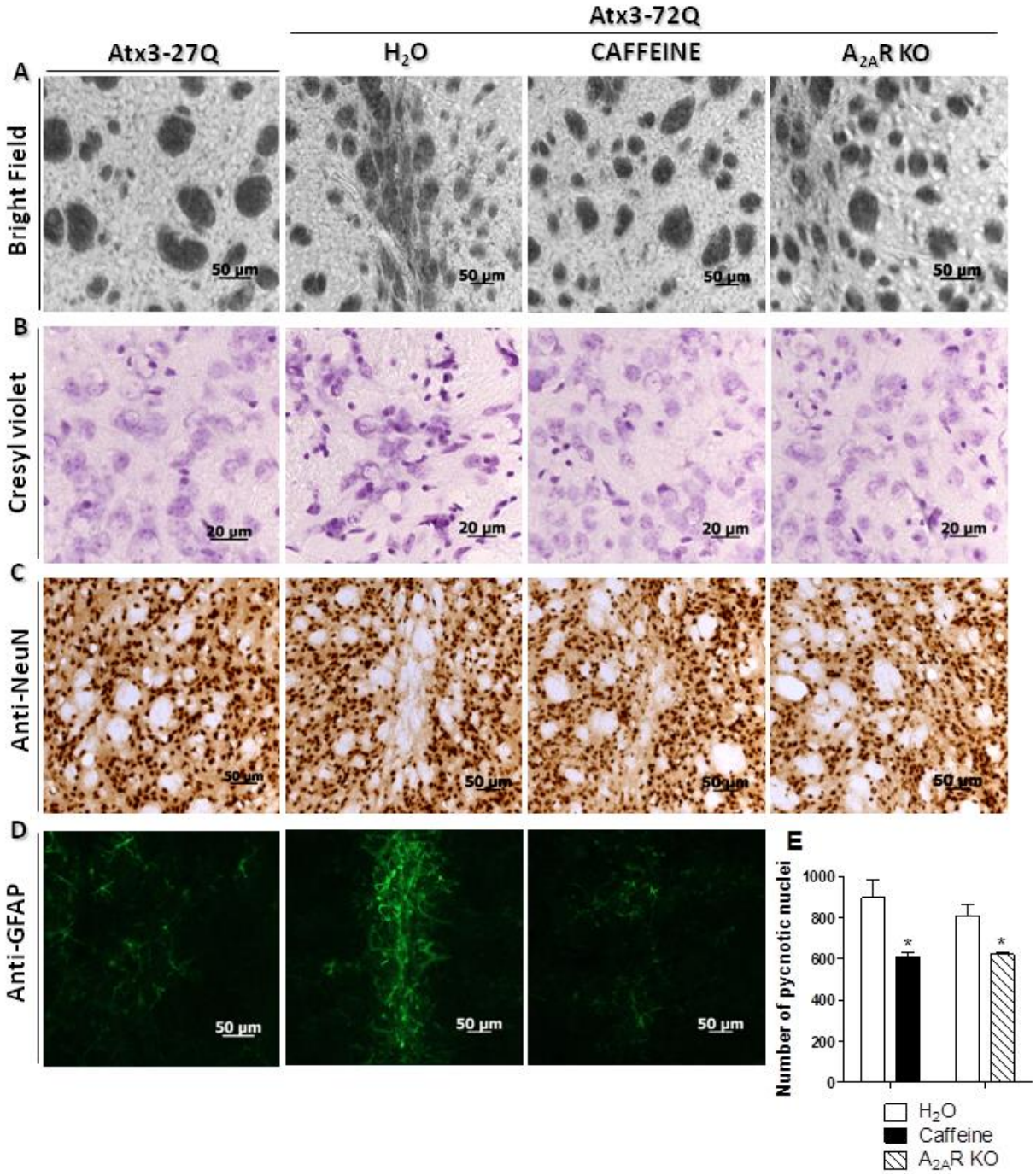


Figure 2.2: Caffeine treatment or $A_{2A}R$ genetic depletion decreased cell injury and striatal degeneration. Representative bright-field photomicrographs and immunohistochemical stainings from around the injection site area at 12 weeks post-injection of the viral vectors encoding wild-type or mutant ataxin-3. (A) Coalescence of the internal capsule of the striatum was neither seen in the caffeine-treated group nor in the $A_{2A}R$ KO animals. (B) Cresyl violet-stained sections showed a significant reduction of the number of striatal condensated nuclei upon mutant ataxin-3 transduction in the caffeine-treated group as well as in the $A_{2A}R$ KO group relative to their respective water-drinking groups, as quantified in *panel E*. (C) A nearly absent loss of neuronal nuclei (NeuN) staining immunoreactivity was seen in the caffeine-treated group as well as in the $A_{2A}R$ KO group. (D) GFAP

immunoreactivity showed an increased accumulation of astrocytes (green) replacing neurons after injection of mutant ataxin-3, which was prevented by caffeine consumption. No morphological modifications were detected upon LV-atx3-27Q injections. Statistical significance was evaluated with Student's *t* test (*$p < 0.05$) comparing both caffeine-drinking and A_{2A}R KO groups with their respective water-drinking wild-type groups upon LV-atx3-72Q injections.

2.4.3 Neuronal functional modifications

Previous reports have indicated that striatal neuronal dysfunction may precede degeneration and appearance of clinical symptoms in MJD (Yen et al., 2002). Additionally, DARPP-32 (dopamine and cAMP-regulated phosphoprotein) was previously shown to be a sensitive marker that allows immunohistochemical detection of this early neuronal dysfunction (Alves et al., 2008b; Cyr et al., 2003; de Almeida et al., 2002; Simoes et al., 2012). Accordingly, DARPP-32 immunohistochemistry revealed a large depleted staining volume of 0.60 ± 0.12 mm^3 (n=5) at 4 weeks post-injection of lentivirus encoding atx3-72Q in the water-drinking group whereas caffeine-treated animals exhibited significantly smaller dysfunctional volume (0.21 ± 0.06 mm^3 (n=6); $p < 0.05$) at this time-point. No loss of DARPP-32 immunoreactivity was detected upon LV-atx3-27Q injections (Fig 2.3A *upper panel* and B).

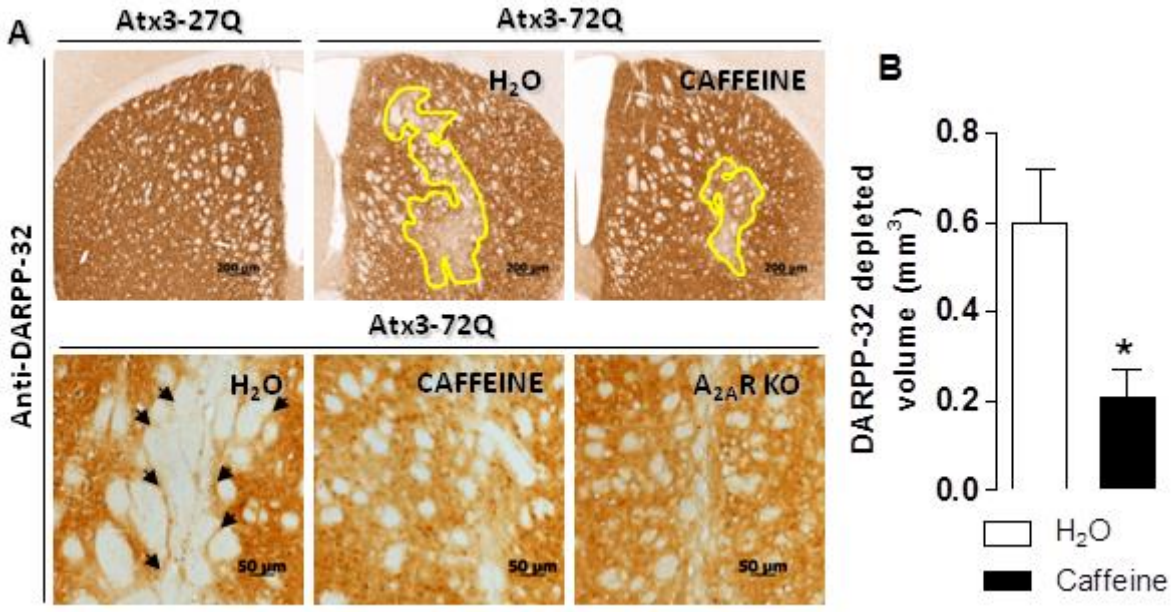


Figure 2.3: Caffeine treatment or A_{2A}R genetic depletion reduced neuronal dysfunction. (A, *upper panel*) A large DARPP-32 depleted volume was observed 4 weeks after injection of the viral vectors encoding mutant ataxin-3 in the water-drinking group whereas caffeine-drinking mice exhibited a much smaller lesion area at this time-point. This is quantified in *panel B* as depleted volume of DARPP-32 staining. No loss of DARPP-32 staining was observed upon LV-atx3-27Q injections. (A, *lower panel*)

At higher magnification, 12 weeks exposure to mutated ataxin-3 revealed a clear condensation of the internal capsule, which joined together fiber patches (arrows). This fiber accumulation was not observed in the caffeine-treated animals or in A_{2A}R KO mice. Statistical significance was evaluated with Student's *t* test (**p* < 0.05) comparing caffeine-treated (n=6) with water-drinking animals (n=5).

At higher magnification, analysis of the striatal DARPP-32-depleted area of water-drinking animals at 12 weeks after injection of lentiviral vectors encoding mutant ataxin-3 revealed that the DARPP-32-stained cell bodies and the corresponding tissue (internal capsule) were no longer present, originating a collapse of the tissue, which joins together fiber patches (see arrows in Fig 2.3A *lower panel*). This fiber accumulation presumably results from neuronal degeneration, which was not observed in the caffeine-treated or in A_{2A}R KO mice challenged with mutant ataxin-3. Density analysis of DARPP-32 immunoreactivity (Table 2.1) showed a significant preservation of this marker in caffeine-treated (*p* < 0.01) and A_{2A}R KO groups (*p* < 0.05) at 12 weeks post-injection of lentivirus encoding atx3-72Q, as compared to the respective water-drinking groups.

Table 2.1: DARPP-32 intensity index

100% IR	**100.0 ± 3.5**	**100% IR**	**100.0 ± 4.1**
H_2O	43.0 ± 4.1	WT	39.1 ± 3.1
Caffeine	70.0 ± 2.2**	A_{2A}R KO	54.5 ± 2.2*

Density analysis of DARPP-32 immunoreactivity 12 weeks after mutant ataxin-3 transduction. Caffeine treatment as well as genetic inactivation of A_{2A}R significantly reduced the loss of this marker (***p* < 0.01; **p* < 0.05, respectively). Data are expressed as indexes of immunoreactivity of the affected striatal regions relative to their corresponding peripheral non-affected striatum (100% IR; mean ± standard error of the mean). IR = immunoreactivity; WT = wild-type; A_{2A}R = Adenosine A_{2A} receptor; KO = knockout.

These data suggest that both chronic caffeine consumption as well as the genetic deletion of A_{2A}R is able to reduce neuronal dysfunction in MJD.

2.4.4 Astrogliosis and microgliosis

Interestingly, the mutant ataxin-3-induced loss of NeuN immunoreactivity was accompanied by a local increase of GFAP immunoreactivity suggestive of astrocytic activation, which was nearly absent in caffeine-treated animals (Fig 2.2D). Since reactive gliosis is widely accepted to contribute to chronic neurodegenerative diseases, we further investigated if the blockade of $A_{2A}R$ was able to prevent the increase of gliosis associated with MJD pathogenesis at an early time-point. The injection of lentiviral vectors encoding mutant ataxin-3 (LV-atx3-72Q) triggered a robust increase of GFAP immunoreactivity at 4 weeks when compared with the contralateral striatum challenged with wild-type ataxin-3 (LV-atx3-27Q) (Fig 2.4A). Notably, this GFAP immunoreactivity triggered by expression of mutated ataxin-3 was observed as early as 2 weeks (Fig 2.4B). Additionally, strong immunoreactivity for the microglial protein, cluster of differentiation molecule B11 (CD11b), was found at 4 weeks revealing microglial recruitment (Fig 2.4C), which was significantly and robustly reduced in caffeine-treated animals. This clearly establishes the presence of reactive gliosis in the striatum in this genetic model of MJD.

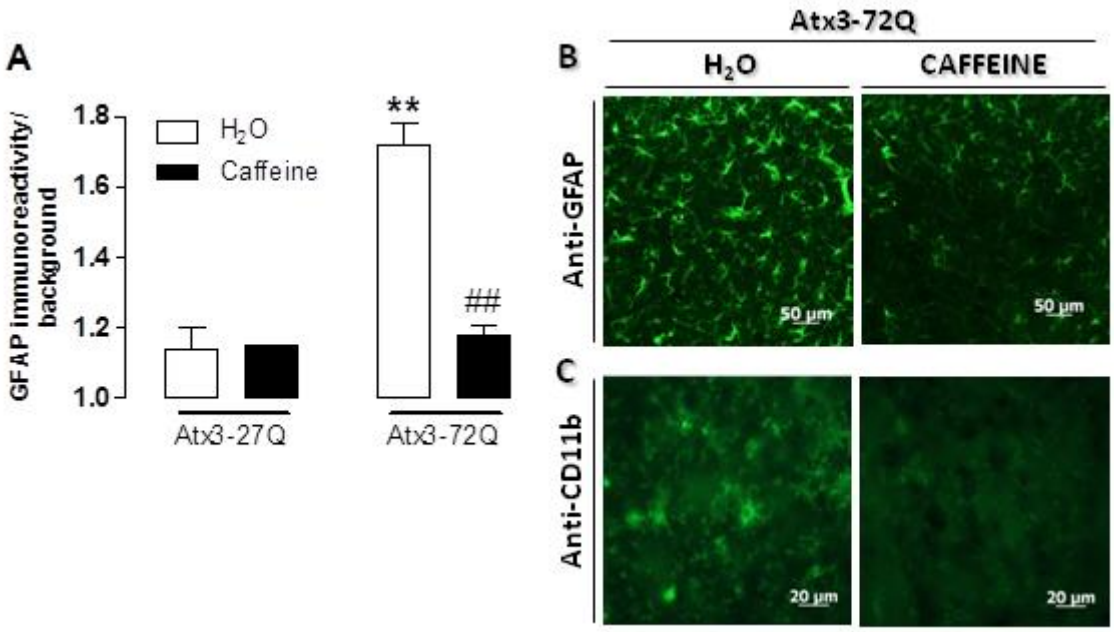


Figure 2.4: Caffeine treatment decreased MJD-associated astroglial activation and prevented microglia recruitment. (A) Quantification analysis of GFAP immunoreactivity at 4 weeks post-injection of the viral vectors encoding wild-type or mutant ataxin-3. Caffeine abrogated astrocytic activation ($^{\#\#}p < 0.01$) to the levels induced by wild-type ataxin-3 (internal control). (B) Caffeine consumption attenuated the mutant ataxin-3-induced activation of astrocytes, gauged by enhanced immunoreactivity of Glial Fibrillary Acidic Protein (GFAP, green) at an earlier time point: 2 weeks. (C) No immunoreactivity for activated microglia, CD11b (Ab 5C6, green), was observed 4 weeks after

insult in the caffeine-treated group whereas it was present in water-drinking mice. Statistical significance was evaluated with Student´s *t* test (***p* < 0.01) comparing mutant ataxin-3 striatal hemisphere with the contralateral wild-type ataxin-3 hemisphere, and caffeine-drinking with water-drinking groups.

Importantly, treatment with caffeine (1 g/L), prevented both the astrogliosis (Fig 2.4A and B) and the putative microgliosis (Fig 2.4C) triggered by mutant ataxin-3 indicating that chronic caffeine consumption can prevent reactive gliosis associated with MJD.

2.4.5 Nuclear inclusions of mutant ataxin-3

Since microglia is well established to play a role in the seeding (through enhanced inflammatory status) and processing (through phagocytosis) of protein aggregates and the presence of ataxin-3 aggregates is one hallmark of MJD, we next tested the impact of caffeine and $A_{2A}R$ inactivation on the aggregation pattern of ataxin-3 by immunohistochemical staining. No aggregation was seen upon LV-atx3-27Q injection (Fig 2.5A) while a significant increase in the total number of mutant ataxin-3 nuclear inclusions was observed at 8 weeks post-injection of lentiviral vectors encoding mutant ataxin-3 in both caffeine-drinking wild type mice ($p < 0.05$) as well as in $A_{2A}R$ KO animals ($p < 0.05$) when compared to the respective water-drinking groups (Fig 2.5B and C). Interestingly, we previously demonstrated that at 8 weeks after lentiviral transduction there were 4 times higher striatal levels of mutant ataxin-3 than those of endogenous ataxin-3 (Alves et al., 2008b) although the levels were similar 4 weeks after lentiviral administration. This observation that caffeine or $A_{2A}R$ inactivation enhance even more the number of ataxin-3 inclusions while decreasing neurodegeneration is in agreement with the scenario that these inclusions may be a way to sequester the soluble and noxious forms of ataxin-3.

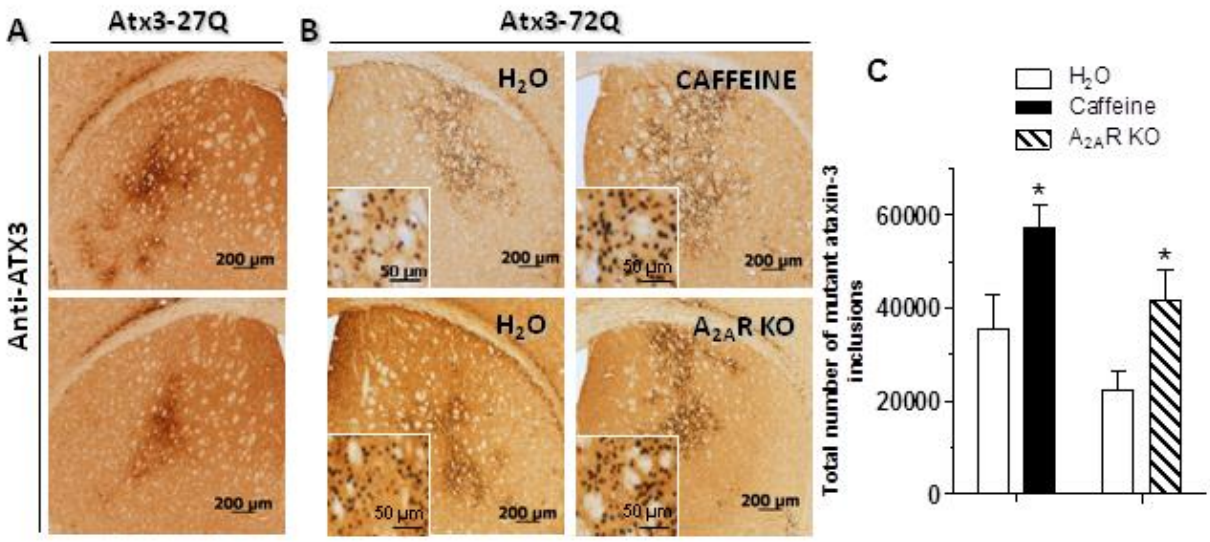


Figure 2.5: Caffeine treatment or $A_{2A}R$ genetic depletion significantly increased the total number of mutant ataxin-3 inclusions. (A) Using anti-ataxin-3 antibody (Ab 1H9), no nuclear inclusions of ataxin-3 were found on both wild-type and $A_{2A}R$ KO animals upon expression of wild-type ataxin-3. (B) A significant increase in the number of mutant ataxin-3 inclusions was observed 8 weeks post-injection of lentiviral vectors encoding mutant ataxin-3 either in caffeine-treated (n=5) or $A_{2A}R$ KO (n=5) mice compared to the respective water-drinking wild-type animals transduced with mutant ataxin-3 *(B,* n=7; *E,* n=4*)*, as quantified in *panel C*. Statistical significance was evaluated with Student's *t* test (**p* < 0.05) comparing caffeine-treated and $A_{2A}R$ inactivated animals with their respective water-drinking control groups.

2.4.6 Synaptotoxicity

There is accumulating evidence that one of the earliest features of neurodegenerative diseases is the dysfunction and loss of synapses (Coleman et al., 2004; Gomes et al., 2011). Also, $A_{2A}R$ are synaptic receptors (Rebola et al., 2005a), and $A_{2A}R$ blockade efficiently normalizes synaptic function and prevents synaptotoxicity in different animal models of brain diseases (Cunha and Agostinho, 2010). Since synaptotoxicity has not been explored in models of MJD, we investigated whether synaptotoxicity was present in our lentiviral model of MJD and if this feature indeed preceded the appearance of ataxin-3 inclusions, neuronal dysfunction and overt neurodegeneration. This was carried out by immunohistochemical evaluation of two synaptic markers, synaptophysin (pre-synaptic protein) and microtubule-associated protein 2 (MAP-2, dendritic protein).

Density analysis of synaptophysin and MAP-2 immunoreactivities at 2 weeks post-injection (Table 2.2 and Fig 2.6) clearly showed a significant loss of both markers ($p < 0.001$) upon mutant ataxin-3 expression. No such early loss of either markers was observed in

caffeine-drinking mice challenged with atx3-72Q, i.e. 2 weeks after insult or upon expression of wild-type ataxin-3 (data not shown). These data provide the first evidence that mutant ataxin-3 induces an early synaptotoxicity in the striatum, which is prevented by chronic caffeine consumption.

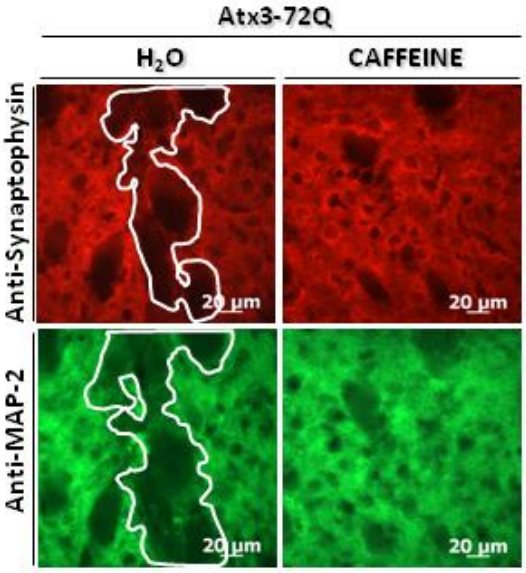


Figure 2.6: Synaptotoxicity precedes the neurodegeneration process of MJD. Detection of nerve terminals with synaptic markers: synaptophysin (red) and microtubule-associated protein 2 – MAP-2 (Ab AP20, green). No loss of synapses is observed in the group that was given caffeine whereas loss of synaptophysin and MAP-2 immunoreactivity was observed in the non-treated group 2 weeks post-injection of the lentiviral vectors encoding mutant ataxin-3.

Table 2.2: Synaptophysin and MAP-2 intensity indexes

	Synaptophysin	**MAP-2**
100% IR	**100.0 ± 3.5**	**100.0 ± 2.6**
H_2O	65.5 ± 4.6***	74.6 ± 3.0***
Caffeine	89.5 ± 0.6	90.1 ± 2.5

Density analysis of synaptophysin and MAP-2 2 weeks after mutant ataxin-3 transduction. A significant loss of both markers was observed in the water-drinking control group (***$p < 0.001$, one-way ANOVA followed by Dunnett's post-hoc test). No loss of either marker was observed in the caffeine-treated group. Data are expressed as indexes of immunoreactivity of the affected striatal regions relative to their corresponding peripheral non-affected striatum (100% IR; mean ± standard error of the mean).

2.5 Discussion

In the present study, we carried out a temporal analysis of different features of brain dysfunction and damage in a genetic model of MJD and tested the impact of caffeine and adenosine $A_{2A}R$ ($A_{2A}R$) blockade thereupon. We provide the first evidence showing that: i) synaptotoxicity and gliosis in the striatum are early events predating neurodegeneration; ii) pharmacological and genetic manipulation of adenosine $A_{2A}R$ can delay MJD-associated striatal pathology.

Consistent with previous reports (Alves et al., 2008b; Bichelmeier et al., 2007; Goti et al., 2004), lentiviral-mediated overexpression of mutant ataxin-3 in the mouse brain induced a clear neuronal dysfunction typified by loss of DARPP-32 immunoreactivity, and overt neurodegeneration, accompanied by an increased number of ataxin-3 inclusions. The present study also revealed two novel morphological features in this model of MJD, namely the loss of synaptic markers (synaptotoxicity) and reactive gliosis. Synaptotoxicity is in line with suggestions of axonal degeneration assessed by MRI (D'Abreu et al., 2009), and loss of dopaminergic terminals assessed by PET in MJD patients (Wullner et al., 2005), and with the presence of axonal inclusions in the human patient's brains (Seidel et al., 2010), as well as with the observed impact of mutant ataxin-3 on the cerebellar mRNA expression of proteins involved in synaptic transmission (Chou et al., 2011). Notably, the present time course study provides direct evidence that this synaptotoxicity might be an early, hitherto unrecognised, feature of MJD. This is in notable agreement with the observations that another polyglutamine-related disease, namely Huntington's disease is characterized by early changes in synapses (DiProspero et al., 2004; Smith et al., 2007), to such an extent that it has been proposed that Huntington's disease might actually be a synaptopathy (Li et al., 2003b). Actually, it is worth noting that synaptotoxicity seems to be an early feature of different other neurodegenerative and neuropsychiatric diseases, strengthening the crucial role of synaptic impairment in the initiation of brain disorders (Coleman et al., 2004; Cunha and Agostinho, 2010; Wishart et al., 2006). Since there is evidence that ataxin-3 is also located in axons and dendrites (Trottier et al., 1998), the present observation that synaptic changes are an early feature of MJD opens a new area of research on the putative role of ataxin-3 in the control of synaptic function and damage. In this context, the recent report that excitatory synaptic transmission can control the aggregation of mutant ataxin-3 adds a further dimension to the relation between MJD and synaptic activity (Koch et al., 2011).

The present time course study of striatal changes in this MJD model also revealed another under-appreciated morphological feature that predated neuronal dysfunction and damage, namely reactive gliosis. There have been episodic reports of astrogliosis and

microgliosis, typified by changes in astrocytes and microglia morphology both in patients (Horimoto et al., 2011), and in transgenic models of MJD (Silva-Fernandes et al., 2010), as well as increased expression of cytokines and proinflammatory chemokines, which are compatible with mutant ataxin 3-induced changes in brain inflammatory mediators (Evert et al., 2001). However, in keeping with the fact that our lentiviral-mouse model resulted in an overexpression of mutant ataxin-3 even in comparable levels to the endogenous form, the present report provides evidence that reactive astrogliosis might be an early feature in MJD, which is particularly relevant in view of the surge of interest in the role of non-neuronal brain cells in the aetiology of neurodegenerative disorders (Lobsiger and Cleveland, 2007). A putative role of glial cells in MJD is further heralded by evidence of the presence of ataxin-3 in glial cells (Paulson et al., 1997b; Wang et al., 1997). Again, this observation should open a novel area of research fostering a better understanding of the role of ataxin-3 in astrocytes and on the consequences of astrocytic adaptation upon accumulation of mutated ataxin-3.

Thus, the present time-course exploration of neuropathological features associated with this genetic model of MJD identifies synaptotoxicity and astrogliosis as precocious modifications followed by microgliosis and neuronal dysfunction, appearance of NIIs and overt neuronal damage. This time course is in general agreement with the recognition that synaptotoxicity and astrocytic-related metabolic imbalance are amongst the most precocious modifications in different neurodegenerative disorders and that neuroinflammation, previously implicated in MJD (Evert et al., 2001), may be a candidate process to mediate the spreading and amplification of damage until overt neuronal dysfunction and damage can be recorded (Coleman et al., 2004; Gomes et al., 2011; Lobsiger and Cleveland, 2007).

The second prominent conclusion of this study is the demonstration that the chronic consumption of a reasonable dose of caffeine compatible to a significant blockade of adenosine effects on A_{2A} (most potent) and A_1 receptors (Fredholm et al., 1999) or the genetic elimination of $A_{2A}R$ mitigated the striatal neuropathological modifications caused by the expression of mutated ataxin-3. This is in agreement with the ability of $A_{2A}R$, mainly targeted by chronic caffeine consumption (Cunha and Agostinho, 2010; Ferre, 2008), to afford neuroprotection against different neurodegenerative disorders, namely Alzheimer's, Parkinson's or Huntington's disease (Cunha and Agostinho, 2010; Popoli et al., 2007; Schwarzschild et al., 2006). Notably, most of the compartments that we now showed to be affected in this genetic model of MJD are effectively normalized by $A_{2A}R$ blockade in chronic brain diseases: thus, $A_{2A}R$ blockade prevents synaptotoxicity (Canas et al., 2009; Silva et al., 2007), in accordance with the enrichment of $A_{2A}R$ in synapses (Rebola et al., 2005a), and also controls astrogliosis (Brambilla et al., 2003; Minghetti et al., 2007; Yu et al., 2008), and microgliosis (Rebola et al., 2011), striatal neurodegeneration (Schiffmann et al., 2007), and

neuronal death (Chen et al., 2007; Cunha, 2005). Accordingly, in our genetic model of MJD, chronic caffeine consumption or genetic deletion of $A_{2A}R$ abrogated the loss of synaptic markers, prevented astrogliosis and microglia activation, reduced cell injury and striatal degeneration rescuing its normal cytoarchitecture. Therefore, this provides the first evidence that the manipulation of a neuromodulation system operated by $A_{2A}R$ is effective in controlling the initial cascade of events triggered by the pathogenic ataxin-3 protein (synaptotoxicity and gliosis) that culminate in a reduced neuronal degeneration.

It is worth noting that this general neuroprotection afforded by caffeine and $A_{2A}R$ blockade is accompanied by an accumulation of ataxin-3 aggregates into intracellular nuclear inclusions. The inverse correlation between the impact of caffeine and $A_{2A}R$ blockade on mutant ataxin-3-induced NIIs and neuropathology strongly suggests that the aggregation of ataxin-3 could correspond to a cellular defensive mechanism against soluble, more toxic species (Arrasate et al., 2004; Saudou et al., 1998; Takahashi et al., 2008b; Taylor et al., 2003), rather than being the main cause of degeneration. Therefore, although it cannot be excluded that this might result from a better neuronal survival in treated animals, we hypothesized that aggregates may be neuroprotective. Nevertheless, the neuroprotection conveyed by caffeine and $A_{2A}R$ blockade might be of limited duration since the number of pycnotic nuclei remained relatively high.

The present exploration of the time course of MJD-associated neuropathological features and its modification by $A_{2A}R$ provides a novel insight into the neuropathology of MJD but does not explore the underlying mechanistic processes. Ataxin-3 is a polyubiquitin-binding protein whose physiological function has been linked to de-ubiquitination (Burnett et al., 2003; Doss-Pepe et al., 2003; Kuhlbrodt et al., 2011; Warrick et al., 2005), and MJD is argued to result from a toxic gain of function of mutant ataxin-3. In keeping with our proposal that synaptotoxicity might be a precocious modification in MJD, several studies highlight the importance of the ubiquitin-proteosome system (UPS) in synapses (Cajigas et al., 2010; DiAntonio and Hicke, 2004), namely in presynaptic terminals (Jiang et al., 2010; Rinetti and Schweizer, 2010), where it is affected in other polyQ neurological diseases (Wang et al., 2008); furthermore, there is preliminary evidence that $A_{2A}Rs$ directly bind to UPS components (Milojevic et al., 2006), and control the UPS activity (Chiang et al., 2009), paving the way for a putative direct control by $A_{2A}R$ of synaptic UPS. The proposal that the ability of $A_{2A}Rs$ to control another polyQ neurological disorder (Huntington's disease) depends on the control of glutamatergic transmission prompts an alternative mechanism by which the $A_{2A}R$-mediated control of the initial event in MJD might be related to the ability of $A_{2A}R$ to control abnormal glutamatergic transmission through direct synaptic effects (Rebola et al., 2008), or indirectly through control of astrocytic glutamate uptake (Matos et al., 2012; Nishizaki et al.,

2002). Thus, albeit the molecular mechanism of $A_{2A}R$-mediated control of MJD remains to be determined, the present study provides new clues for particular compartments where such mechanisms should be explored.

In conclusion, the present study provides a novel insight into the pathology of MJD bringing to the centre stage synaptotoxicity and gliosis as precocious events in MJD. Furthermore, it provides the first realistic and safe promising life style prophylactic strategy to delay the onset of this inherited disorder, based on the consumption of caffeine. Finally, it provides the first suggestion that $A_{2A}R$ might be a novel therapeutic target to interfere with the inexorable evolution of this neurodegenerative disease.

CHAPTER 3

Caffeine alleviates progressive motor deficits in Machado-Joseph disease transgenic mice

3.1 Abstract

Machado-Joseph disease (MJD) is a dominantly-inherited neurodegenerative disorder associated with an expanded polyglutamine tract within ataxin-3 for which there is currently no available therapy. We previously showed that caffeine, a non-selective adenosine receptor antagonist, reduced the neuropathological modifications triggered by lentiviral-mediated over-expression of mutant ataxin-3 in the mouse striatum; we now investigated its ability to also alleviate behavioral deficits in a genetic mouse model of MJD displaying a severe ataxia.

For this purpose, MJD transgenic mice were given caffeine (1 g/L, applied through the drinking water) and were tested using a panel of locomotor behavioral paradigms, namely rotarod, beam balance and walking, pole and water maze cued-platform version tests. Caffeine consumption prevented progressive loss of general and fine-tuned motor functions, balance and grip strength, in parallel with cerebellar morphology preservation. Importantly, caffeine also rescued the putative striatal-dependent executive and cognitive deficiencies affected in this genetic mouse model of MJD.

Our findings provide the first *in vivo* demonstration that caffeine intake alleviates motor disabilities in a severely impaired animal model of MJD.

3.2 Introduction

Machado-Joseph disease (MJD) or spinocerebellar ataxia type 3, is a dominantly-inherited polyglutamine neurodegenerative disease and the most common among ataxias (Ranum et al., 1995). It is characterized by an adult age of onset associated with an unstable expansion of a CAG stretch (over 61 repeats) in the *ATXN3* gene, which encodes a polyglutamine repeat in the corresponding ataxin-3 protein (Durr et al., 1996; Kawaguchi et al., 1994), and causes premature death. The clinical hallmarks of MJD include progressive ataxia, dysfunction of motor coordination, postural instability and Parkinsonism among other symptoms (Gwinn-Hardy et al., 2001; Taroni and DiDonato, 2004). The neuropathology involves multiple systems such as cerebellar systems, substantia nigra and cranial nerve motor nuclei (Durr et al., 1996; Sudarsky and Coutinho, 1995), as well the striatum (Alves et al., 2008b; Klockgether et al., 1998). There is currently no available therapy. Several animal models closely mimicking the human pathology (Alves et al., 2008b; Bichelmeier et al., 2007; Cemal et al., 2002; Goti et al., 2004; Nobrega et al., 2012; Silva-Fernandes et al., 2010) have been widely used to deepen our knowledge on the mechanisms leading to the neuronal degeneration. In fact, taking advantage of those animal models in parallel with human tissue analysis we have recently shown different cellular compartments particularly affected in MJD brains, namely impaired autophagy (Nascimento-Ferreira et al., 2013; Nascimento-Ferreira et al., 2011) and proteolysis (Simoes et al., 2012), preceded by synaptic loss and gliosis (Goncalves et al., 2013) providing new targets for therapy.

As $A_{2A}R$ blockade through chronic caffeine consumption proved protective towards diverse neurodegenerative paradigms, namely Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis (Chen et al., 2001; Cunha and Agostinho, 2010; Popoli et al., 2007; Potenza et al., 2013) as well in striatal-induced MJD neuropathology (Goncalves et al., 2013), we now investigated the ability of chronic caffeine administration to rescue severe sensorimotor behavioral impairments displayed by a MJD transgenic mouse model expressing a truncated form of mutated ataxin-3 in cerebellum (Torashima et al., 2008).

3.3 Materials and Methods

3.3.1 Animals

All experiments were performed with approval of the Coimbra University Authority for Animal Welfare in accordance with the European Community directive (86/609/EEC) for the care and use of laboratory animals. C57Bl/6-background truncated-atx3-69Q/ transgenic MJD (TgMJD) mice were obtained from parallel breeding at CNC, University of Coimbra, of a colony of TgMJD mice initially obtained from Gunma University Graduate School of Medicine (Torashima et al., 2008). Mice were housed and kept under a conventional 12-h light-dark cycle maintained on a temperature-controlled room with food and water provided *ad libitum* and used at 7 weeks of age. Gender- and age-matched TgMJD and wild type littermates (WT) were used in this study.

3.3.2 Drug treatment

We chose the dose of caffeine (1 g/L) administered through the drinking water, as a maximally effective and non-toxic dose, which we have previously shown to result in a plasma concentration of 50 μM (Duarte et al., 2012) and similar concentration in the brain parenchyma (Costenla et al., 2010). Treatment with caffeine was initiated at 7 weeks of age.

3.3.3 Behavioural assessments

All animals were submitted to a battery of locomotor, exploratory and cognitive tests starting at 7 weeks of age. Animals were habituated for 1h to a quite room with controlled temperature and ventilation, dimmed lighting, and handled prior to behavioural testing to overcome the animals' natural fear and anxiety responses, which could have a major effect on performance. All devices were wiped clean with a damp cloth of a 10% ethanol solution and dried before evaluating the next mouse.

3.3.3.1 Rotarod

Motor coordinative abilities and balance was assessed using rotarod apparatus. Mice were tested on an accelerating rotarod (Letica model LE 8200, Panlab, Barcelona, Spain) starting at 4 rpm and accelerating to 40 rpm over a period of 5 min. The time during which mice remain walking in the rotation drum was recorded. Sessions

consisting of 2 trials per day with a 20-min inter-trial interval were carried out and the mean of the trials were averaged. Animals were repeatedly tested every 4 weeks, beginning on the day before treatment.

3.3.3.2 Pen test

In the pen test, a mouse suspended by its tail was slowly lowered from above to a pen (diameter, 9-mm) horizontally fixed approximately 20-cm above the ground. Usually, the mouse grabs to the pen and starts walking on it without difficulties. Any deviation from this normal behaviour was recorded in a 60 s test trial.

3.3.3.3 Beam balance/walking

Motor coordination and balance of mice were assessed by measuring the ability of the mice to traverse a graded series of narrow beams to reach an enclosed safety platform (Carter et al., 1999). The beams consisted of long strips of wood (1-m) with an 18- or 9-mm square wide and a 9- or 6-mm round diameter cross-sections. The beams were placed horizontally, 25-cm above the bench surface, with one end mounted on a narrow support and the other end attached to an enclosed box (20-cm square) into which the mouse could escape. A 60-W desk lamp was positioned above and to one side of the start of the beam to create an aversive stimulus (bright light) to induce mice to cross it. During training, mice were placed at the start of the 9-mm square beam and trained over 3 d (4 trials per day) to traverse the beam to the enclosed box. Once the mice were trained (traversed the 9-mm square beam) they received two consecutive trials on each of the square beams and each of the round beams, in each case progressing from the widest to the narrowest beam. Mice were allowed up to 60 sec to traverse each beam. As mice became progressively impaired, they clung tightly onto the beam to prevent themselves from falling. This increased latency to cross; any animal that did not cross within the full 60-sec trial was allocated a maximum value of 60 sec for analysis.

3.3.3.4 Grip strength

Since motor function can be differentially affected depending on experimental parameters, the mouse limb strength was measured as an indicator of neuromuscular function. The setup consisted of a 300-g metal grid, which was on a scale. The animal was hung with its forepaws to the central position of the grid. Its strength was

determined as the weight pushed (g) from the scale. The grip test was performed 3 times and the mean taken to analysis.

3.3.3.5 Pole test

The pole test was performed as previously described (Matsuura et al., 1997) with minor modifications (Fernagut et al., 2004). The mouse was placed head-upward on the top of a vertical rough-surfaced pole (diameter 1.0-cm; height 52 cm) and the time to orient downward (t-turn) and to reach the floor (t-descend) was recorded; the maximum observation time was 120 s. Animals were submitted to 5 consecutive trials with an inter-trial interval of 60 s. The best 3 scores for each parameter were considered to analyse.

3.3.3.6 Water-maze cued-version test

Tests were performed in a circular swimming pool made of gray acrylic, 140-cm in diameter and 60-cm in height (the animals' hind-paws did not touch the cylinder's bottom). For the tests, the tank was filled with water until 45 cm of height and maintained at 23 ± 2ºC. The target platform (10x10 cm^2) was made of transparent acrylic and it was submerged 1 to 1.5-cm beneath the surface of the water and a yellow rubber ball (5-cm in diameter) was attached to the top of the submerged platform and protruded above the water surface. Mice were submitted to a working memory version of the water maze using a protocol described previously (Prediger et al., 2010). The swimming sessions consisted of 4 training days, four consecutive trials per day, during which the animals were left in the tank facing the wall, then being allowed to swim freely to the submerged platform. If a mouse did not find the platform during a period of 60 s, it was gently guided to it. After each session mice were removed from water, dried with a towel, and placed in a warmed enclosure, and the cylinders were cleaned and refilled. The scores for latency of escape from the starting point to the platform were collected.

3.3.3.7 Object location recognition test

Short-term memory was assessed by using an acrylic made open-field box (30-cm wide × 30-cm deep × 30-cm high). Identical plastic columns (4-cm in height × 5-cm in diameter) were used as objects. This test consisted of an acquisition trial and a test trial with an inter-trial interval of 3 h. On the acquisition trial, each mouse was allowed 5

min to explore two identical objects positioned in two adjacent corners, 7-cm from the walls. On the test trial, one of the objects was moved to a different location while the other retained in the same position as in the acquisition trial, the mouse was reintroduced into the experimental apparatus for 5-min, and its behaviour recorded (Murai et al., 2007). The time spent exploring each object was recorded. Exploration of an object was defined as pointing the nose towards the object at a distance of <1-cm and/or touching it with the nose. Turning around or sitting on an object was not considered exploration. To analyze cognitive performance, a location index was calculated as follows: (Tnovel × 100)/ (Tnovel + Tfamiliar), where Tnovel is the time spent exploring the displaced object and Tfamiliar is the time spent exploring the non-displaced object.

3.3.3.8 Modified Y-maze

Testing was carried out in a Plexiglas apparatus composed of 2 arms connected to a runway, separated by equal angles. The 2 arms (available for exploration) and runway were 30-cm long and 5-cm wide surrounded by black acrylic walls 20-cm high. Each arm met at a central platform equipped with black removable partitions, enabling arms to be opened and closed as desired. The area surrounding the Y-maze did not contain optical cues. The test consisted of an acquisition trial and a test trial with an inter-trial interval of 2 h. On the acquisition trial, each mouse was placed at the end of the runway and was allowed 8 min to access to one of the exploration arms by forced choice (i.e., the other arm was closed). On the test trial, the mouse was allowed to explore both the runway and familiar and the novel unfamiliar exploration arms for a period of 8 min. The time spent in each arm and the number of entries was recorded. To analyze cognitive performance, an index was calculated [Enovel/ (Enovel + Erunway-familiar) x 100], where Enovel is the number of entries on the novel/ unfamiliar arm and Erunway-familiar is the number of entries on both runway and familiar arms.

3.3.3.9 Open field locomotor activity

To assess mice spontaneous explorative behaviour, activity was recorded on an open-field apparatus (Letica model LE 8811, Panlab, Barcelona, Spain). The device contained a 45 x 45-cm arena made of black acrylic and 35-cm high transparent acrylic walls and record ambulatory movements as well as rears. Animals were repeatedly tested every 4 weeks, beginning on the day before treatment. Both vertical (rearing

frequency) and horizontal (distance travelled, in cm) activity on the whole arena, and the maximum reached velocities (in cm/s) were collected using Acti-Track Software (Panlab, Barcelona, Spain). The mice were placed in the center of the arena in a single 15-min session (Boy et al., 2009).

3.3.3.10 Elevated plus maze test

The device consisted of four black acrylic cross-shaped arms of equal dimensions (40-cm x 5-cm) raised 50-cm above the floor, radiating from a central square measuring 6-cm x 6-cm. Two arms were enclosed on three sides by 30-cm high opaque Plexiglas walls and the other two were only surrounded by 0.5-cm high to avoid falls. The enclosed arms and the open arms faced each other on opposite sides. Entries (four-paw criterion) and time spent in enclosed and open arms were measured, together with open/total arm entry and duration ratios [(seconds in each arm/ total time exploring arms) × 100]. An entry occurred whenever the mice crossed from one arm to another with four paws. Mice were gently placed in the central square facing one of the closed arms. It was allowed to explore freely and undisturbed during a single 5-min session.

3.3.3.11 Tail suspension test

The mouse is securely fastened by the distal end of the tail to a flat metallic surface and suspended in a visually isolated area. The presence or absence of immobility, defined as the absence of limb movement, was recorded.

3.3.4 Histological assessments

After an overdose of avertin (2.5x 12 µl/g, i.p.), transcardial perfusion of the mice was performed with phosphate-buffered saline (PBS) followed by fixation with 4% paraformaldehyde (PFA). The brains were then removed and post-fixed in 4% PFA for 24 h and cryoprotected by incubation in 25% sucrose/ phosphate buffer for 48h. The brains were frozen and 25 µm coronal sections were cut using a cryostat (Leica CM3050 S, Heidelberg, Germany) at -21°C. Sagittal slices throughout the brain were collected in anatomical series and stored in 24-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.

Staining for Calbindin (rabbit polyclonal, Chemicon) together with DAPI (Sigma, St. Louis, MO) was performed. Free-floating sections were kept at room temperature (RT) for 2h in PBS with 0.1% Triton X-100 containing 10% normal goat serum (Gibco-Invitrogen, Barcelona, Spain), then twice overnight at 4°C in blocking solution with the primary antibody. Sections were washed three times and incubated for 2h at RT with the corresponding secondary antibody coupled to fluorophore (goat anti-rabbit Alexa Fluor 488, 1:200; Molecular Probes – Invitrogen, Eugene, OR) diluted in the blocking solution. The sections were washed three times and then mounted in *mowiol* reagent (Sigma) on microscope slides.

For cresyl violet staining, coronal sections were premounted and stained with cresyl violet for 45 sec, differentiated in 70% ethanol, dehydrated by passing twice through 95% ethanol, 100% ethanol and xylene solutions, and mounted onto microscope slides with Eukitt® (Sigma). Cresyl violet-stained cross-sectional areas of cerebellar hemisphere were used for volume extrapolation and measures of the molecular layer width.

Staining was visualized using Zeiss Axioskop 2 plus or Zeiss Axiovert 200 imaging microscopes (Carl Zeiss Microimaging, Germany) and equipped with AxioCam HR color digital cameras (Carl Zeiss Microimaging) using 5X, 20X and 40X Plan-Neofluar objectives and the AxioVision 4.8 software package (Carl Zeiss Microimaging). Quantitative analysis was performed with a semi-automated image-analysis software package (Image J software, NIH, USA).

3.3.5 Statistical analysis

Statistical comparisons were performed by unpaired Student's *t* test, and one-way or two-way analysis of variance of multiple experimental groups followed by Dunnett's multiple comparison or Bonferroni comparison post hoc tests, respectively. Results are expressed as mean ± standard deviation (SEM). Significance thresholds were set at $p<0.05$, $p<0.01$ or $p<0.001$, as defined in the text.

3.4 Results

3.4.1 Caffeine prevents loss of balance and progression of gait disturbance

The permanent expression of a truncated form of human ataxin-3, the protein responsible for Machado-Joseph disease (MJD), with 69 glutamine repeats in the mouse cerebella induces a severe ataxic phenotype, associated with cerebellar defects (Torashima et al., 2008). Motor function is mediated by several structures, starting in the cortex, brain stem and spinal cord, and terminating in skeletal muscle. The rotating rod is widely used as a reliable test to study motor function and balance, and it is especially sensitive in detecting cerebellar dysfunction (Caston et al., 1995; Lalonde et al., 1995) allowing quantification of progressive sensorimotor impairments. Therefore, as a first tool for our studies, we scored the performance of wild-type and MJD transgenic mice (TgMJD) on the accelerating rotarod and determined whether caffeine was able to modify their motor function. TgMJD revealed a severe phenotype (Fig 3.1A) as early as 7 weeks of age (n=20, $p < 0.001$) and a progressive decline with age, whereas wild-type (WT) littermate animals (n=18) performed well during all the experiment. Chronic caffeine consumption completely abrogated ($p < 0.05$) the progressive loss of performance of TgMJD animals on the rotating rod, while it did not modify WT littermates' performance.

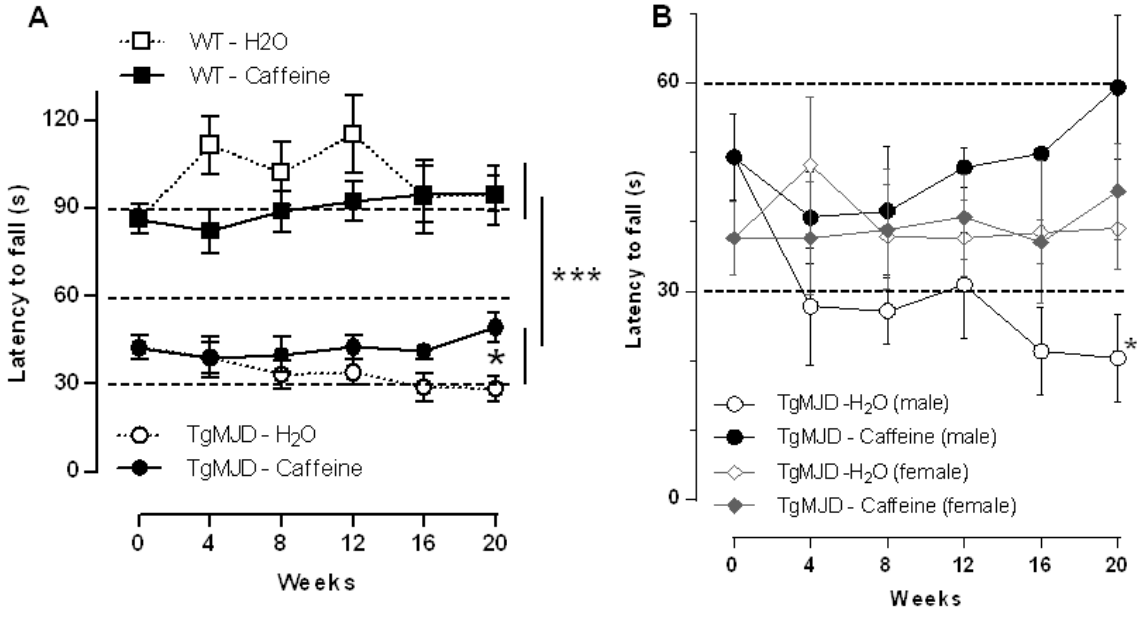


Figure 3.1: Rotarod motor function assessment. (A) Machado-Joseph Disease transgenic (TgMJD) animals performed poorly (***$p < 0.001$, two-way ANOVA) and worsen with age ($p = 0.07$) on an accelerating rotating rod when compared to wild-type littermates (WT), which was

significantly (*p < 0.05, student's t test) prevented by caffeine treatment. (B) Female animals preserved their performance on the accelerated rotarod while male animals worsened with age (*P < 0.05, two-way ANOVA); caffeine significantly ($^{\#}P$ < 0.05, student's t test) prevented male progressive loss of performance.

As caffeine effects may vary between gender due to pharmacokinetics (Fredholm et al., 1999), we separately analyzed the gender effect of TgMJD animals in rotarod performance and therein investigated the effects of chronic caffeine consumption. Interestingly, both male and female TgMJD animals displayed low performance in rotarod task as early as 7 weeks-old, but only male TgMJD worsened with age (Fig 3.1B). Chronic caffeine consumption rescued (p < 0.05) male performance from worsening and did not change female phenotype.

Unexpectedly, TgMJD animals displayed sustained increased spontaneous locomotion with age, both distance traveled (8 weeks, p < 0.01; 20 weeks, p < 0.05) and number of rearings (8 weeks, p < 0.01) when subjected to an open field, although accompanied by a significant decrease in the maximum velocity of running (Time 0, p < 0.001; 8 weeks, p < 0.05) when compared to its WT littermates (Table 3.1). Most of these effects were normalized by 8 weeks of caffeine consumption (number of rearings and maximum velocity, p < 0.05).

Table 3.1: Spontaneous locomotion assessment in an open field apparatus.

		Distance traveled (cm/ 15min)			No. rears			Maximum velocity (cm/ s)		
Group	**Time (wks)**	**0**	**8**	**20**	**0**	**8**	**20**	**0**	**8**	**20**
Wild-type	H_2O	3143.7 ± 134.5	2086.1 ± 150.3	932.0 ± 222.0	165.4 ± 12.2	111.8 ± 19.4	45.4 ± 23.2	15.9 ± 0.5	14.5 ± 0.4	12.7 ± 0.9
	Caffeine		1950.6 ± 297.5	933.5 ± 155.4		91.8 ± 22.2	36.3 ± 8.3		14.7 ± 0.8	13.8 ± 0.7
TgMJD	H_2O	3006.8 ± 184.7	3071.2 ± 230.3**	1773.7 ± 355.6*	181.7 ± 16.6	239.2 ± 40.9**	50.5 ± 20.1	13.0 ± 0.5***	12.7 ± 0.5*	11.2 ± 0.9
	Caffeine		2544.7 ± 555.9	1283.6 ± 622.3		104.0 ± 37.2$^{\#}$	36.5 ± 18.5		14.9 ± 0.8$^{\#}$	11.9 ± 2.3

Analysis of the total distance travelled, number of rears and maximum running velocities during 15 minutes test in an open field. Data are expressed as mean ± standard error of the mean. Statistical evaluation of the genotype was made by two-way ANOVA (*p < 0.05, **p < 0.01, ***p < 0.001). Caffeine effects were evaluated by student's t test ($^{\#}p$ < 0.05).

Moreover, as the phenotype on the rotating rod seemed to differ between genders, we then considered data separately and further detailed: i) the effects of cerebellar expression of truncated ataxin-3 with age; and ii) whether caffeine consumption differently modifies motor performance. In fact, male TgMJD performed even worse than females (n=3-4, $p < 0.05$), which preserved their performance with age (Fig 3.1B). Chronic caffeine consumption displayed a gender-dependent effect as it rescued male TgMJD mice from progressive loss of motor performance in the rotating rod while it did not alter female TgMJD phenotype. No changes in performance were observed in both sexes of WT littermates consuming water or caffeine (data not shown).

Since the rotarod paradigm depends intrinsically on muscle strength, coordination or balance, we further characterized the nature of the rotarod impairment by subjecting mice to the pen test and monitoring the time the animals could withstand on the beam. TgMJD animals displayed increasing difficulties to stay equilibrated over the beam, which were aggravated with age ($p < 0.05$; Fig 3.2). Interestingly, chronic caffeine consumption completely prevented the imbalance ($p < 0.05$) throughout the study, as caffeine-drinking mice performed as well as WT littermates (data not shown).

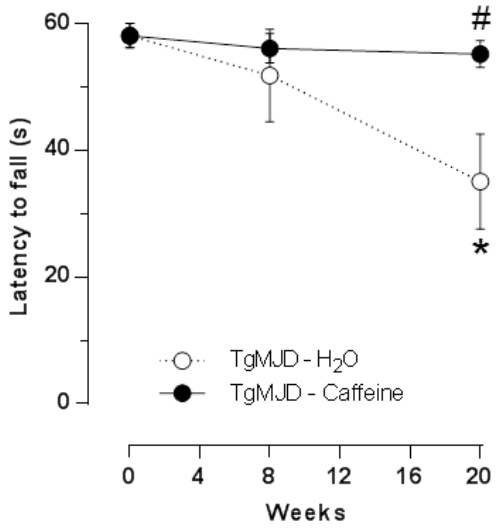


Figure 3.2: Balance assessment TgMJD mice. In a pen test, TgMJD mice displayed increasing (*$p < 0.05$, student's *t* test with Welch's correction) balance impairment with age, which was significantly prevented by caffeine treatment (#$p < 0.05$, student's *t* test with Welch's correction).

3.4.2 Caffeine improves fine-tuned motor function

While the rotarod is useful for determining gross motor deficits in rodents, the detection of more subtle motor effects requires a different approach. Fine motor coordination, can be assessed using a beam walking task, which essentially examines the ability of the animal to remain upright and to walk on an elevated and relatively narrow beam without falling. Therefore, we have now used multiple beams (especially narrow ones) for fine tuned motor characterization of TgMJD animals, and probed for subtle effects that chronic caffeine consumption may operate in their motor function; we evaluated the time animals spent to walk across four progressively difficult beams of square and round cross-section in order to reach an enclosed safety platform. TgMJD mice displayed significantly (*$p < 0.05$) impaired performance on the narrowest square and round beams as early as 7 weeks-old (Fig 3.3A), which significantly worsened with age both in the narrowest and wider square (**$p < 0.01$) and round (***$p < 0.001$) beams. 15 weeks-old TgMJD animals (8 weeks post-initiation of caffeine administration) hardly performed the task on rounded beams (Fig 3.3B and C) and started to exhibit a subtle ($p > 0.05$) impairment on the narrowest square beam when at 27 weeks of age (Fig 3.3D).

Notably, caffeine administration displayed a time-dependent prevention of the inability of TgMJD animals to perform both the widest ($^{\#}p < 0.05$) and the narrowest ($^{\#}p < 0.05$) rounded beams, as the alleviation of the phenotype observed at 15 weeks of age (8 weeks time-point) was no longer observed at 27 weeks of age (20 weeks time-point, Fig 3.3B and C). Additionally, caffeine-treated TgMJD animals also did not exhibit the mild worsening of motor performance on the narrowest square beam at 27 weeks of age (Fig 3.3D). These data indicates that caffeine is capable of preventing loss of MJD transgenic mice performance when executing very challenging and physical demanding motor coordination tests although with a limited duration.

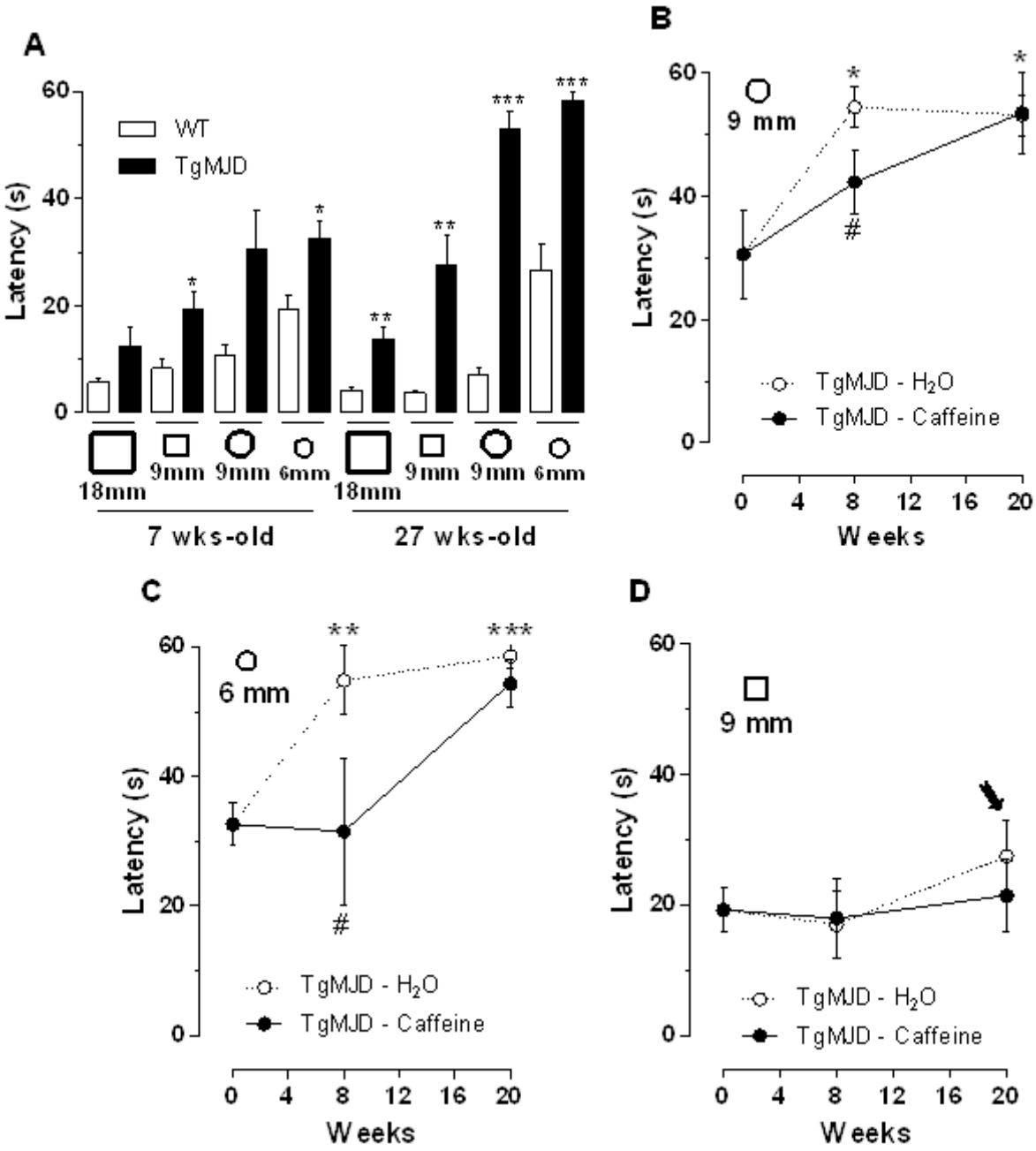


Figure 3.3: Balance and motor coordination on the raised beams. Wild-type (n=22) and TgMJD (n=23) mice were trained to walk across a 9-mm squared beam during 3 consecutive days. In the 4th day, mice were progressively subjected to a series of more difficult beams of square and round cross-section to reach an enclosed safety platform. The latency to cross was recorded on each trial (**A** to **D**). All animals were given two trials on each of the graded square and round beams, in each case progressing from the widest to the narrowest. (**A**) TgMJD mice exhibited a decline in beam-walking ability with increasing beam difficulty and age (*p < 0.05, **p < 0.01, ***p < 0.001; student's t test with Welch's correction). (**B** and **C**) TgMJD animals displayed a progressive beam-walking disability with age (*p < 0.05, **p < 0.01, ***p < 0.001; one-way ANOVA). 8 weeks caffeine treatment significantly (#p < 0.05, student's t test) improved their

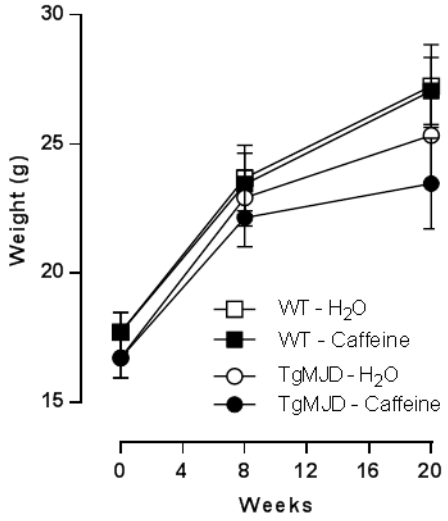
performance in the challenging rounded beams. (**D**) At 27 weeks of age, TgMJD mice demonstrated a subtle impairment in the narrowest square beam, which was not seen in caffeine treated animals ($p > 0.05$, student's *t* test).

Clinical symptoms of peripheral neuropathy have long been recognized in MJD patients as a result of axonal neuropathy of both motor and sensory fibers (C. Franca M et al., 2009; Klockgether et al., 1999). Therefore, in addition to the motor function assessment, the endurance of the animal was also taken into account since it may influence motor performance on several motor tests. We thus subjected mice to a grip strength test to further explore whether a modulation of the neuromuscular transmission was also operated by caffeine. At 7 weeks of age (corresponding to Time 0 in Table 3.2), TgMJD mice displayed equal muscle strength as WT littermates (data not shown), but within the following 8 weeks had a dramatic loss ($p < 0.05$) of grip strength. Importantly, caffeine administration was able to prevent (8 weeks, $p < 0.05$) this effect, although to some extent as long-term treatment (20 weeks) was unable to sustain TgMJD mice initial endurance (Table 3.2).

Table 3.2: Analysis of TgMJD neuromuscular function using a grip strength test.

Grip strength (g)			
Weeks of treatment	**0**	**8**	**20**
H_2O	72.0 ± 3.1	48.5 ± 9.1*	47.9 ± 4.6*
Caffeine		78.3 ± 7.7#	44.2 ± 6.0

Grip strength is expressed as weight pushed (g) from the scale [mean ± standard error of the mean]. *Difference from the initial value ($p < 0.05$; one-way ANOVA). #Difference from control water-drinking group ($p < 0.05$; student's *t* test).



Interestingly, this loss of endurance displayed by TgMJD with age was not correlated with a loss of weight, as those values were similar to WT animals at any time point (Fig 3.4), and also not modified upon caffeine treatment.

Figure 3.4: Mice weight. No significant differences were observed between TgMJD and WT littermate animals ($P > 0.05$, two-way ANOVA).

Taken together, both fine-tuned motor function and early grip force loss are indicative of a putative neuromuscular dysfunction in this animal model of MJD. Caffeine administration prevented those effects to some extent but was unable to prevent aggravation of the phenotype at late stages.

3.4.3 Caffeine rescues striatal-dependent abnormal circuitries

3.4.3.1 Motor system

The present MJD transgenic animals display a robust ataxia associated with the expression of mutant ataxin-3 in cerebella (Torashima et al., 2008). However, other brain circuitries may be altered being partially responsible for this particular phenotype. Therefore, we evaluated the animals in a vertical pole, a task on which decreased performance has been associated with nigrostriatal circuitry impairments in different animal models of striatal degeneration (Fernagut et al., 2004; Gomez-Sintes et al., 2007; Luchtman et al., 2012). Overall, the time required to orient downward (T-turn) was longer in aged TgMJD than in WT littermate animals ($p < 0.01$, Fig 3.5) and the time to descend from the top of the pole to the ground (T-descend) was also longer at both younger ($p < 0.01$) and older ages ($p < 0.05$). Chronic caffeine administration prevented the increased time displayed by TgMJD animals to orient downward ($p < 0.05$), and promoted complete recovery ($p < 0.01$) of their performance to descend, as animals climbed down faster than initially (at younger age) and also similarly to WT animals.

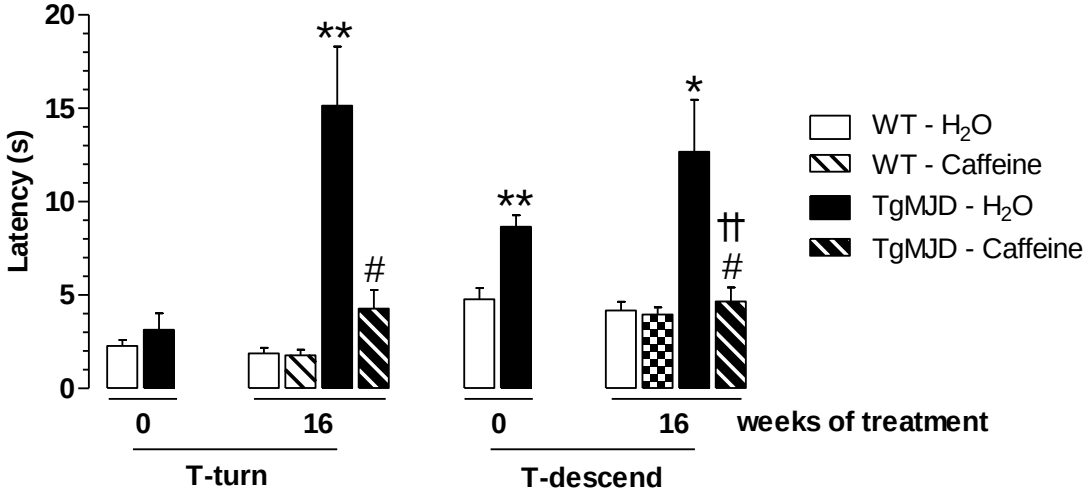


Figure 3.5: Motor performance in a vertical pole. Aged TgMJD mice showed significantly (**$p < 0.01$, student's *t* test with Welch correction) increased time to orient downward when compared to WT animals. This effect was prevented ($^{\#}p < 0.05$, student's *t* test with Welch's correction) by caffeine treatment. TgMJD mice also displayed significantly (*$p < 0.05$, **$p < 0.01$; student's *t*

test with Welch's correction) increased time to descend to the floor, compared to WT animals. Caffeine administration completely reverted ($^{\dagger\dagger}p < 0.01$, student's *t* test relative to TgMJD at 0 weeks of treatment) the time to descend to the floor spent by TgMJD animals, to control WT levels.

This data is highly suggestive of a striatal dopaminergic impaired circuitry affecting motor functions of MJD transgenic animals and occurring right from an early age. Importantly, this impairment is normalized by caffeine treatment.

3.4.3.2 Cognitive system

As the striatum is apparently dysfunctional in this MJD animal model, as shown in other MJD genetic models (Alves et al., 2008b; Simoes et al., 2012) as well in human patients (Alves et al., 2008b; Reetz et al., 2013; Yen et al., 2002), and the striatum targets both motor and cognitive action systems, which positions it as a particularly relevant brain compartment controlling learning of motor skills, we further evaluated whether striatal functions were altered as a whole subjecting TgMJD animals to striatal-dependent procedural learning and memory tasks. Although evaluating motor learning in a motor challenging raised-narrow-beam task during 4 training days would hardly allow ascertaining learning disabilities, we found a mild learning impairment in 15 weeks-old TgMJD animals when performing beam-walking task (8 weeks, day 2, Fig 3.6A), which was prevented ($p < 0.05$) by caffeine treatment; learning impairment in the older TgMJD animals could not be ruled out since animals already displayed motor deficiencies in performing the task.

We also took advantage of the water maze cued-platform acquisition trials, where escape latency was scored. Herein, 13 weeks-old TgMJD mice displayed a starting difficulty ($^{**}p < 0.01$) to reach the safety platform at day 1, the day of first contact with the test (Fig 3.6B), while WT littermates performed well. In the following trial days all the animals performed well albeit motor incoordination of TgMJD animals, which might be explained through the survival instinct inducing rapid search for the safety platform. This fact did not allow us to confirm the putative impairment of striatal based stimulus-response form of learning and memory displayed by TgMJD animals challenged in the beam-walking task (Fig 3.6A). Nonetheless, caffeine administration was again able to prevent ($^{\#\#}p < 0.01$) the increased time to initiate movement such as that observed in the vertical pole (Fig 3.5).

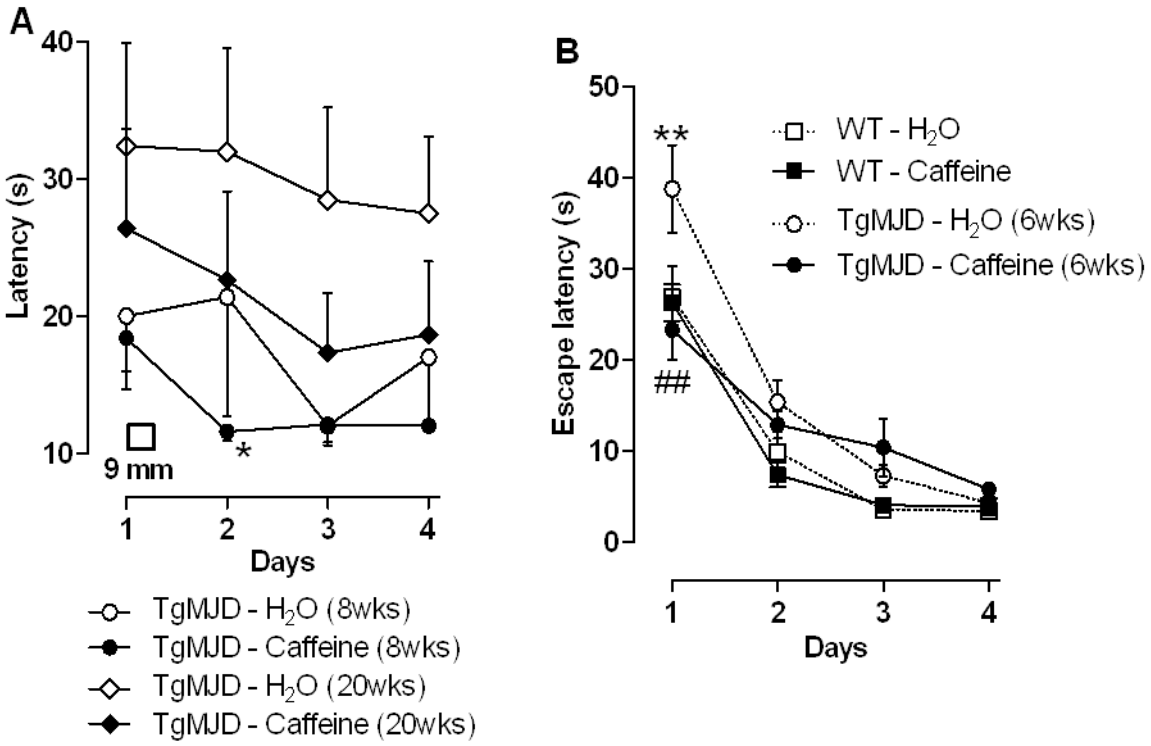


Figure 3.6: Motor skill learning tests. (A) Beam-walking test of 15 weeks-old TgMJD mice (8 weeks of caffeine treatment) revealed learning impairment at day 2 when performing the narrowest 9-mm square beam, which was not observed in caffeine-treated animals (*$p < 0.05$, student's t test). Older TgMJD animals already displayed motor deficiencies, which do not allow learning evaluation. (B) In a freely swimming task using a similar training protocol, 13 weeks-old TgMJD (6 weeks of treatment) animals already demonstrated a significant (**$p < 0.01$, two-way ANOVA) initial akinesia, which was prevented by caffeine treatment ($^{\#\#}p < 0.01$, two-way ANOVA).

We further subjected the animals to another striatal-dependent learning task, namely object location test (OLT), to evaluate whether TgMJD animals displayed impairment in procedural spatial memory. The OLT is based on the spontaneous tendency for rodents to explore novel stimuli. Animals were introduced to two identical objects in the experimental apparatus, and after a 3 h delay, exposed again to the same two objects, one of which had been displaced to a new location (Fig 3.7A). Furthermore, animals that remember the previous exposure spontaneously spend more time exploring the object in the new position (Murai et al., 2007).

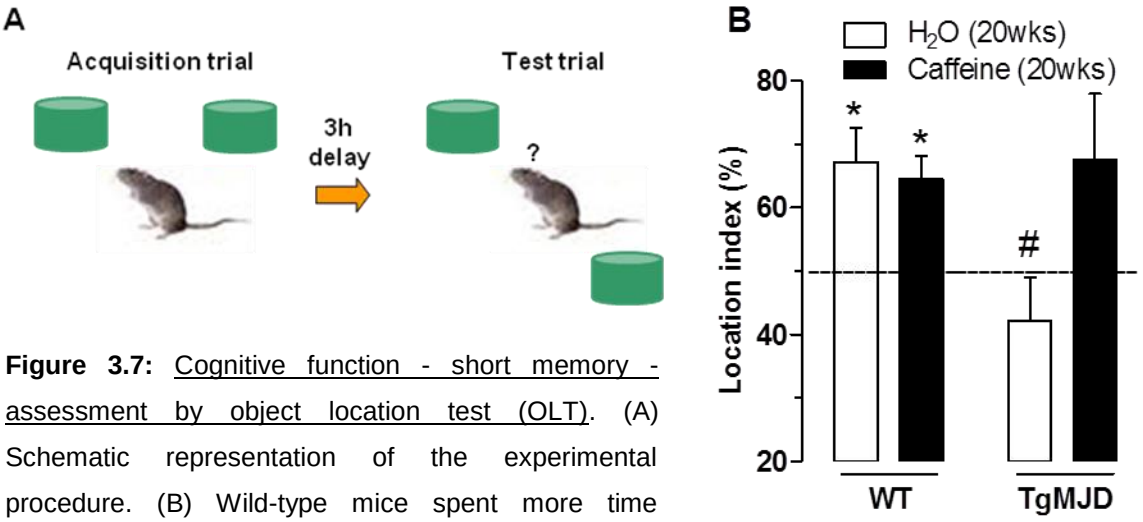


Figure 3.7: Cognitive function - short memory - assessment by object location test (OLT). (A) Schematic representation of the experimental procedure. (B) Wild-type mice spent more time exploring the displaced object from the object placed in the same position in the acquisition trial while TgMJD animals poorly discriminate the displaced object. Caffeine-treatment rescued the ability of TgMJD animals to explore novelty. *$p < 0.05$ vs. chance level (50%) and $^{\#}p < 0.05$ vs. normal condition.

On the acquisition trial, all animals spent an equal amount of time exploiting each of the two identical objects ($p > 0.05$) (Table 3.3). On the test trial, WT animals displayed a location index different from the chance level (*$p < 0.05$, Fig 3.7B) whereas TgMJD mice demonstrated a reduced location index ($^{\#}p < 0.05$) meaning they poorly discriminate the displaced object from the object placed in the same position in the acquisition trial. Additionally, WT animals also decreased their exploitation time in the test trial (Table 3.3), which can be translated by their ability to recognize the environment and the objects while TgMJD animals spent the same time ($p > 0.05$) exploring the objects.

Table 3.3: Total object exploitation time in OLT at both acquisition and test trials.

Animals		N*	Acquisition trial (s)	Test trial (s)
Wild-type	H_2O	8	62.5 ± 8.1	44.3 ± 8.7
	Caffeine	9	56.9 ± 7.2	36.3 ± 7.7
TgMJD	H_2O	7	76.4 ± 10.8	74.1 ± 22.6
	Caffeine	6	55.8 ± 5.3	39.2 ± 11.9

The total time spent exploring the two objects was expressed as mean (seconds) ± standard error of the mean. N*: number of animals.

This data supports the interpretation of TgMJD animals exhibiting an object location recognition difficulty. Importantly, chronic caffeine consumption rescued the inability of TgMJD animals to learn and thus to explore novelty.

Together, these data demonstrates that: i) striatal functions are also affected in this MJD transgenic mouse model; and ii) caffeine normalizes the associated behavioral alterations.

3.4.4 Caffeine abrogates neuropathological deficit progression

To determine whether TgMJD animals' phenotype was directly associated with neuropathological features, we compared TgMJD animals' brains with those of WT littermates in different aspects, such as the size and histological parameters, and further evaluated the effects therein by chronic caffeine consumption. As mutant ataxin-3 was expressed mainly in cerebellum inducing gross morphological defects, namely in Purkinje cells (Torashima et al., 2008), we further evaluated cerebellar hemisphere sizes and dissected folia morphology. TgMJD mice displayed a clearly visible reduction ($p < 0.001$) in cerebellar volume, as shown in mice brain photomicrographs (Fig 3.8A, quantified in B), and also exhibited indistinguishable lateral cerebellar region (the neocerebellum) comprising the hemispheric extensions of lobules VI and VII of the posterior lobe, as early as 7 weeks of age. Cerebellar volume of TgMJD animals continued to decline with age (Fig 3.8C, black bar) exhibiting a general reduction of the anterior lobe (lobules I-V), posterior lobe (lobules VI-IX) and of the flocculonodular lobe (lobule X) (Fig 3.8D, *top*, see arrows). Impressively, chronic caffeine consumption rescued ($p < 0.05$) TgMJD mice cerebella from shrinkage, which might be related to a better neuronal survival.

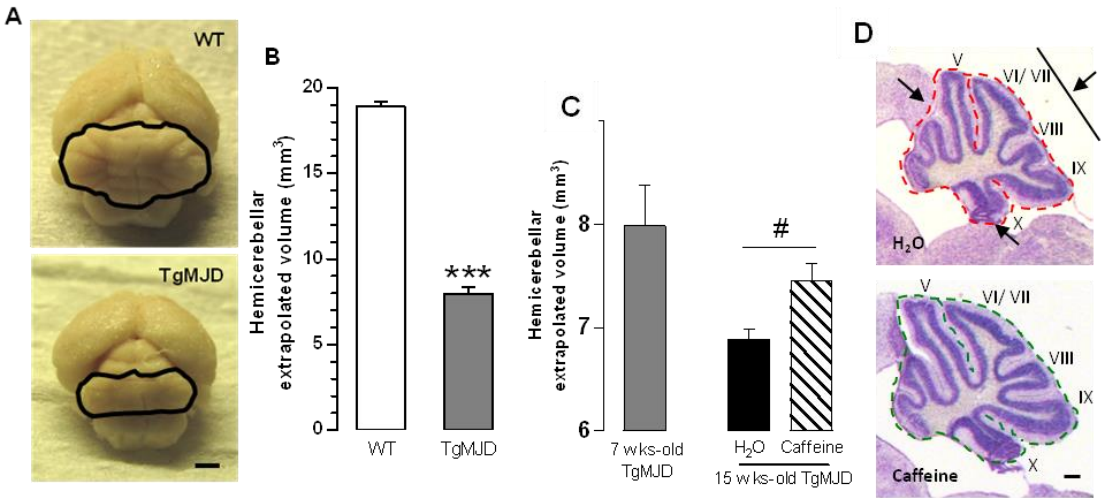


Figure 3.8: Hemicerebellar volume of MJD transgenic and wild-type littermate animals. (A) Representative brains of TgMJD animals and WT littermates. Cerebella anatomical regions are outlined; lobules VI and VII are indistinguishable in TgMJD mice. Scale bar: 1 mm. (B) TgMJD mice showed a robust (***$p < 0.001$, student's *t* test) reduction in the cerebellar volume as early as 7 weeks of age when compared to wild-type animals. (C) Quantification analysis of the extrapolated hemicerebellar volume of young and older water- and caffeine-drinking TgMJD animals. There is a noticeable reduction in the overall cross-sectional cerebellar area of the water-drinking animals, which was rescued ($^{\#}p < 0.01$, student's *t* test) by caffeine treatment.(C) Midsagittal cresyl violet-stained sections from 15 weeks-old water-drinking (top) and caffeine-drinking (bottom) TgMJD animals. Arrows indicate foliation size reduction: decreased anterior, posterior and flocculonodular lobes. Scale bar: 200 µm.

In order to associate the overall cerebellar reduction with morphological defects in specific cellular layers, cresyl violet-stained sections were used to measure the molecular layer thickness. In fact, TgMJD mice cerebella exhibited a significant decrease ($p < 0.001$) of the molecular layer (ML) thickness as early as 7 weeks of age confirmed in different folia (lob-V: 68±6-µm; and lob-IX: 67±5-µm), comparing to WT littermates (lob-V: 137±4-µm; and lob-IX: 155±3-µm) (Fig 3.9A and B). At 27 weeks of age, the molecular layer thickness of TgMJD animals thinned to 51±3-µm and 48±2-µm in lob-V and lob-IX, respectively (Fig 3.9C and D). Additionally, also an evolving disorganization of the Purkinje cell layer (PCL) was observed with age, which is consistent with the molecular layer thinning suggestive of Purkinje cell atrophy. Remarkably, caffeine administration preserved the molecular layer thickness.

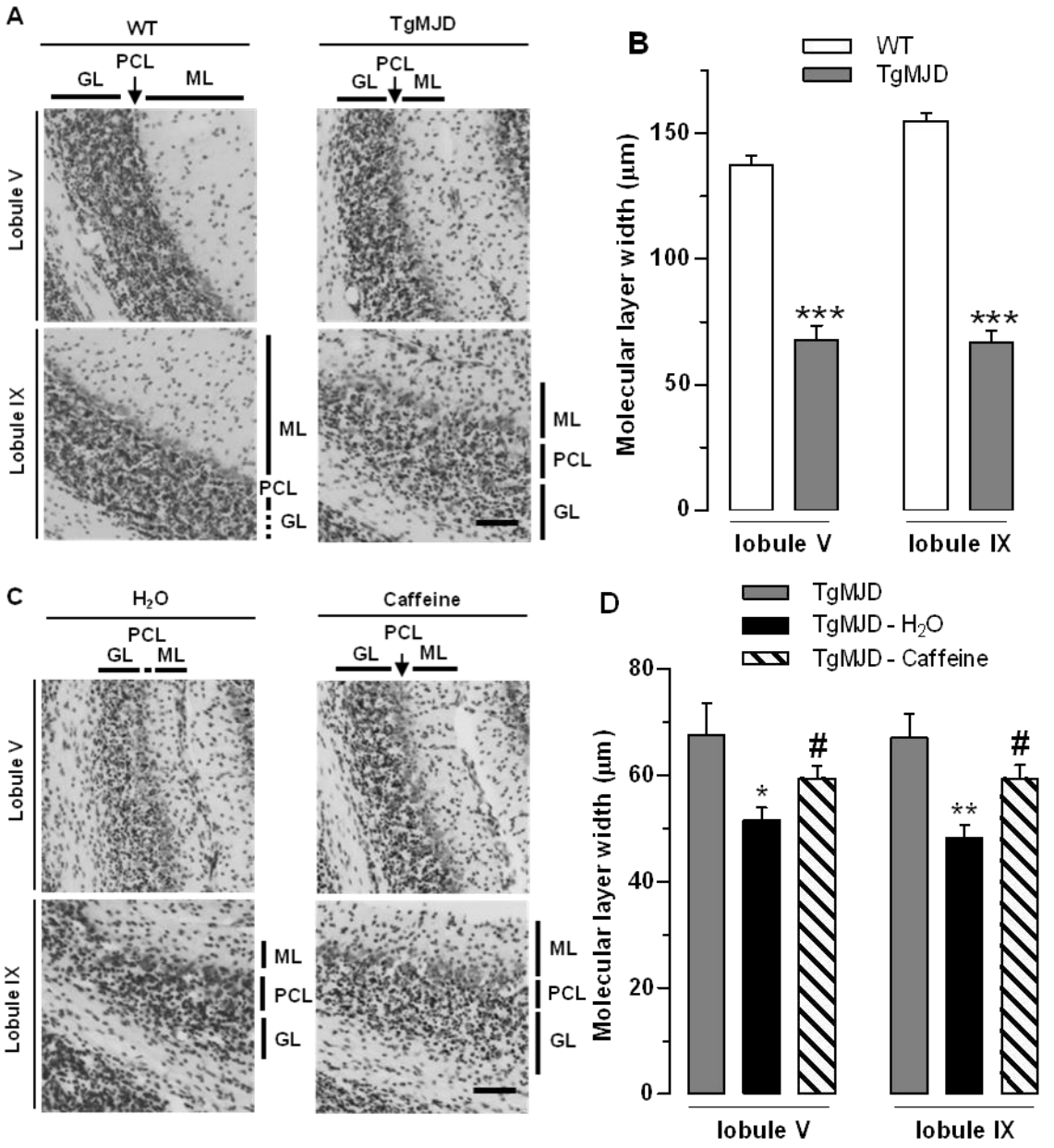


Figure 3.9: Midsagittal sections of both TgMJD animals and wild-type littermates. (**A** and **C**) Cresyl violet immunostaining. Scale bar: 50 µm (**B** and **D**) Quantification analysis of the molecular thickness of lobules V and IX. **A** and **B.** 7 weeks-old TgMJD mice displayed a significantly thinner (***p < 0.001, student's t test) molecular layer (ML) when compared to WT animals, irrespective of lobule. **C** and **D**. A significant progression on ML thickness reduction (lobule V, *p < 0.05; lobule IX, **p < 0.01; student's t test) concurrently with an increase of PC somata (PCL) disorganization were observed with aging. 20 weeks of caffeine administration slowed ($^{\#}P$ < 0.05, student's t test) the progressive reduction of the ML thickness in TgMJD animals.

Furthermore, to correlate the molecular layer loss with the state/ presence of Purkinje cells, we further used calbindin immunofluorescence to visualize and count

Purkinje cells. WT animals showed well defined cerebella cellular layers, namely Purkinje cell and molecular layers, which were immune-positive for calbindin (Fig 3.10A), whereas young adult TgMJD mice displayed a clear disorganization and reduction in the number of Purkinje cells, both in lobules V ($p < 0.001$) and IX ($p < 0.05$) (Fig 3.10B and C, *left*), which progressively worsened with age (lob-V: $p < 0.01$; lob-IX: $p > 0.05$) (Fig 3.10D). Notably, chronic caffeine consumption displayed a remarkable protection of Purkinje cells from degeneration.

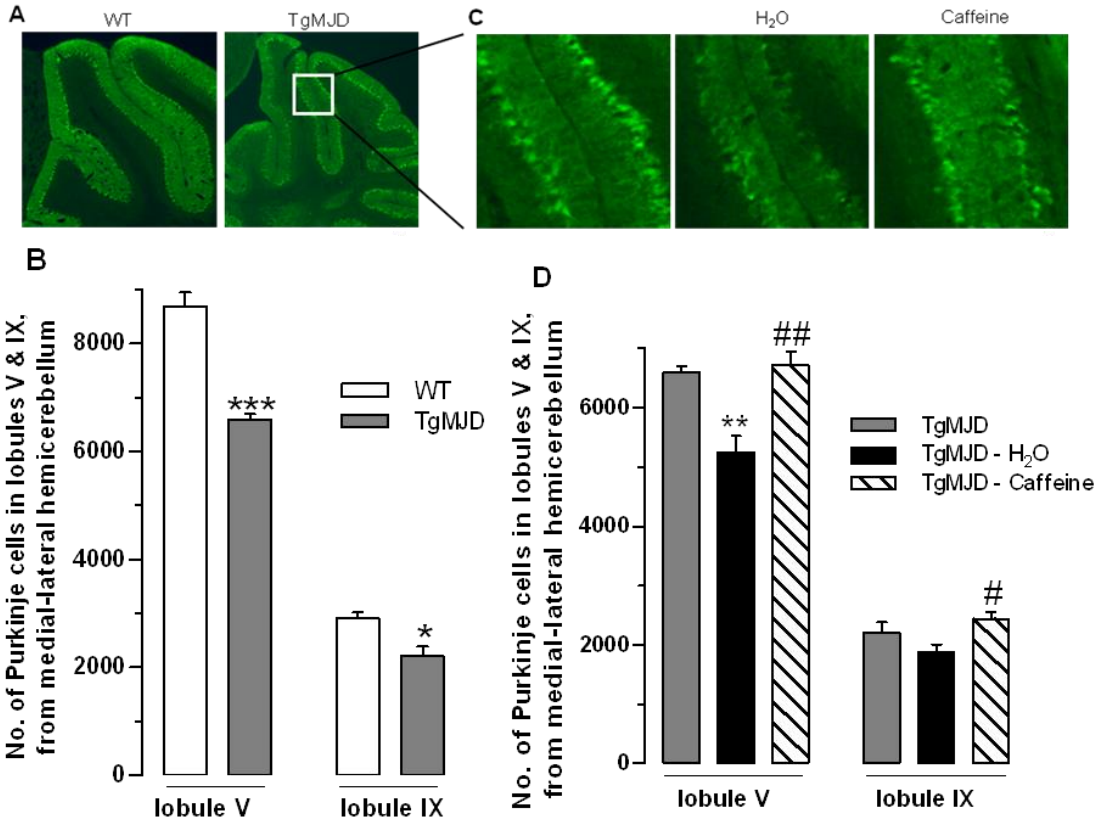


Figure 3.10: Purkinje cell population of both TgMJD and wild-type littermate animals. (A and C) Immunostaining of cerebellar Purkinje cells with an anti-calbindin antibody. (A) Both molecular and Purkinje cell layers appear evenly stained in WT animals and in 7 weeks-old TgMJD mice. Scale bar: 100 µm. (C) An obvious reduction of calbindin immunoreactivity and topographical repartition of the cell loss was seen in 27 weeks-old water-drinking TgMJD animals, which did not happen upon 20 weeks of caffeine treatment. Scale bar: 20 µm. (B) Quantification of the Purkinje cell number of anterior and posterior lobules, V and IX, respectively. TgMJD mice displayed a significant reduction of Purkinje cell number (lobule V, $^{***}p < 0.001$; lobule IX, $^{*}p < 0.05$; student's *t* test). (D) 20 weeks caffeine treatment completely abolished Purkinje cell loss in both lobules V ($^{\#\#}p < 0.01$, student's *t* test) and IX ($^{\#}p < 0.05$, student's *t* test).

Together, these data show a correlation between MJD transgenic phenotype and modified cerebellar features, namely size reduction and disorganized morphology typified by a reduction of the molecular layer and a continuous loss of Purkinje cells. Notably, chronic caffeine administration prevented the worsening of the dramatic phenotype displayed by MJD transgenic mice through the overall preservation of cerebella structure.

3.5 Discussion

In the present study, we investigated the impact of caffeine, a non-selective antagonist of the adenosine A_{2A} receptors ($A_{2A}R$), on a transgenic mouse model of MJD exhibiting pronounced motor and cognitive deficits. In this model, a truncated human form of ataxin-3 with 69 CAG repeats is expressed in cerebella (Torashima et al., 2008), the region widely accepted as the most important contributor for the phenotype presented by MJD patients. Here, we provide the first evidence that pharmacological manipulation of $A_{2A}R$ through caffeine administration rescues the progression of cerebellar morphological damages limiting the worsening of the phenotype.

In the early stages of cerebellar degenerative disorders, the motor dysfunction typified by impaired gait (ataxia) and extremity incoordination (dysmetria), which is consistent with the role of cerebellum in skills requiring coordination and fine motor control (Mishkin and Appenzeller, 1987), is preceded by poor balance and inability to walk in a straight line, and is also commonly accompanied by impaired force of contraction (Schmahmann, 2004). In addition, impairment of the fine motor coordination required for walking is characterized by widened base as the condition progresses; turning is problematic and can result in falls. Subjecting rodents to a beam walking task with graded levels of difficulty, allows to observe aggravation of walking impairment at a given level of task difficulty, and the age at which a deficit is first observed decreases as the level of task difficulty increases (Carter et al., 1999).

The present time-course study of behavioral changes in this genetic model of MJD (see Fig 3.11) revealed a progressive sensorimotor impairment in rotarod and pen tests accompanied by walking difficulties and an early loss of muscle strength, a sign formerly thought to be the pathophysiologic basis of the motor disability, without weight alterations. Additionally, TgMJD mice also displayed hypokinesia (both akinesia and bradykinesia) on a vertical pole. Regarding walking disabilities, TgMJD mice displayed greater difficulties when challenged on the narrowest rounded beams while comparing to the widened squared beams, and aging resulted in emerging difficulties while performing the narrowest square beam. Post-mortem analysis of TgMJD brains also revealed: i) reduced cerebella as early as 7 weeks of age; ii) indistinguishable neocerebellum, a region functionally associated to higher level cognitive/ emotional tasks such as planning, initiation and timing of movements; and iii) age-dependent reduction of anterior and posterior lobes (lobules I-V and VIII-IX) as well of flocculonodular lobe (lobule X) associated with sensorimotor functions connected to the cerebral cortex and spinal cord, therefore related to fine tune body and limb

movements as well as muscle tone, balance and postural stability (Schmahmann, 2004; Stoodley and Schmahmann, 2010). This is in notable agreement with neuroimaging studies showing smaller cerebellums of Attention deficit hyperactivity disorder (ADHD) children (Berquin et al., 1998; Castellanos et al., 1996) and the association of motor impairments with cerebellar dysfunction (Diamond, 2000).

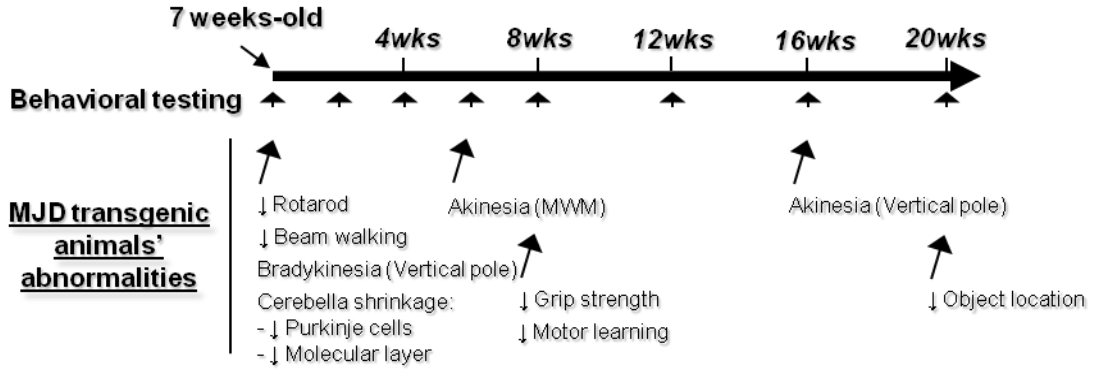


Figure 3.11: Summary of the time-course of behavioral modifications in the transgenic mouse model of MJD expressing a truncated *ATXN3* gene.

Beyond coordination and fine motor control, the cerebellum also plays a role in strategy formation and procedural learning, the memory for motor skills (Mishkin and Appenzeller, 1987; Shiotsuki et al., 2010), which have been recently established to be closely linked to non-motor regions of the cerebral cortex involved in executive functions and cognitive planning, and were reported to be impaired upon cerebellar degeneration, based on clinical neuropsychological tests (Pascual-Leone et al., 1993; Zawacki et al., 2002). Consistently, there have been episodic reports showing cognitive, executive and emotional dysfunctions in MJD patients (Maruff et al., 1996; Roeske et al., 2013; Zawacki et al., 2002). It is worth mentioning that expression of atx3-69Q under the control of the L7 promoter leads to expression in several brain areas (Yoshihara, 2002), which might implicate the overall brain functioning. The exploitation of novel environmental stimuli, which is dependent on the integrity of limbic and non-limbic pathways, and includes basal forebrain, hippocampus, thalamus, prefrontal cortex, and dorsal striatum, as well the vestibular system and cerebellum (Lalonde, 2002) might be likewise impaired in this MJD transgenic model. We therefore investigated whether a dysfunctional frontocorticostriatocerebellar circuitry was also present and played a critical role in the dramatic phenotype presented by TgMJD mice. In fact, TgMJD animals exhibited an early mild learning difficulty on both beam-walking task and Morris water maze acquisition trials, a reduction in object displacement recognition, and also mood alterations, such as impulsivity, aggressive behavior, broad

hyperactivity and disinhibition typified by increased spontaneous locomotion in an open-field, increased open-arm exploitation in an elevated-plus maze, and decreased immobility time in the tail suspension test (unpublished data). This constellation of non-motor behavioral abnormalities presented by TgMJD animals adds further dimension to the putative general dysfunction of frontocorticostriatocerebellar circuitries.

The most prominent conclusion of the present study is the demonstration that the chronic consumption of caffeine rescued motor and cognitive alterations as well of cerebellar morphological defects caused by the expression of mutant ataxin-3 in mice cerebella. Caffeine showed: i) a prevention of the progressive loss of motor performance on both mild (rotarod and pen test) and high-level difficulty tasks (beam walking) as well as a recovery to control levels in a vertical pole; ii) a prevention of the progressive cerebellar retraction and of morphological defects accompanying the degeneration of Purkinje cells; and iii) a rescue of early mild learning impairments. This is in agreement with the ability of $A_{2A}R$, mainly targeted by chronic caffeine consumption (Cunha and Agostinho, 2010; Ferre, 2008; Fredholm et al., 1999), to afford neuroprotection against different neurodegenerative disorders, namely Alzheimer's, Parkinson's or Huntington's disease (Cunha and Agostinho, 2010; Popoli et al., 2007; Schwarzschild et al., 2006). Notably, we now demonstrated another brain compartment, the cerebellum, which is mainly affected in this genetic model of MJD, wherein $A_{2A}R$ blockade effectively rescued neuronal loss and subsequent progression of morphological defects, which is consistent with the widely distribution of $A_{2A}R$ in the brain beyond striatum (Cunha et al., 1996) and the ability of caffeine and $A_{2A}R$ antagonists to confer neuronal protection against induced-toxicity in primary cultures of cerebellar granule neurons (Dall'Igna et al., 2003). This observation is of particular interest at the point we might consider the consumption of caffeine as a prophylactic strategy to rescue cerebella neurodegeneration of other inherited spinocerebellar ataxias displaying considerable atrophy of the cerebellum (reviewed in (Rub et al., 2013)).

The second prominent conclusion of this study is the ability of caffeine to restore procedural learning amongst other peculiar behaviors displayed by this MJD genetic model with confined cerebellar degeneration, such as increased inattention and hyperactivity resembling ADHD, a psychiatric disorder also related to dysfunction of the cerebellum. Altogether, such behaviors are presumed to be linked to dysfunction of frontocorticostriatocerebellar circuits (revised in (Krain and Castellanos, 2006)), strongly suggested to be underlined by dopamine (DA)-dependent frontocorticostriatal plasticity (Kheirbek et al., 2009; Reynolds and Wickens, 2002). Since both adenosine

and dopamine modulation systems are tightly intertwined, and $A_{2A}R$ are located in striatal dopamine-rich region of the brain, revealed by autoradiographic studies (Jarvis and Williams, 1989; Parkinson and Fredholm, 1990), as well in frontocortical nerve terminals (Pandolfo et al., 2013), they can modulate dopamine release (Borycz et al., 2007; Gomes et al., 2009). In fact, long-term administration of caffeine has been shown to elicit changes in tolerance or sensitization of dopamine-mediated responses (Fenu et al., 2000). A recent report has also demonstrated that adenosine receptors functionally antagonize dopaminergic responses, namely through A_{2A}-D_2 receptor heteromers, counteracting D_2R-mediated inhibitory modulation of the effects of NMDA receptor stimulation in the striatopallidal neuron normalizing behavioral responses such as locomotor deficits associated with Parkinson's disease (Schiffmann et al., 2007). Interestingly, we provide data consistent with DA depletion in these circuitries (Matsuura et al., 1997; Pickrell et al., 2011), and we hypothesize that caffeine might have a prominent role increasing the release of DA, which in turn acts on DA receptors normalizing the dysfunctional dopaminergic neurotransmission putatively present in this MJD model. Also consistent with this normalization of dopamine-mediated responses, is the maintenance of female TgMJD animals' performance in rotarod task that might result from estrogen-induced desensitization of serotonin 5-HT_{1A} receptor (Dluzen et al., 1996; Lu and Bethea, 2002), which, in turn, may increase pre-synaptic 5-HT receptor-mediated release of DA (Bantick et al., 2005). This is also in agreement with the observation of serotonergic agonists to effectively improve cerebellar ataxia in MJD patients (Takei et al., 2005; Takei et al., 2002).

In conclusion, these observations strongly support caffeine as an effective treatment to prevent worsening of Machado Joseph disease, a very severe ataxic illness, with both motor and cognitive dysfunctions by rescuing the cerebellum from neurodegeneration and preserving the overall brain functioning through the recovery of the dopaminergic system. Nevertheless, more detailed studies should be performed to elucidate these findings.

CHAPTER 4

Distinct striatal neuronal compartments of adenosine A_{2A} receptors differently modulate Machado-Joseph disease

4.1 Abstract

Machado-Joseph disease (MJD) is a neurodegenerative disease caused by the expansion of a polyglutamine repeat in the ataxin-3 protein. There is currently no therapy available to prevent or modify disease progression. We have recently shown that caffeine, a non-selective adenosine receptor antagonist, and adenosine A_{2A} receptor ($A_{2A}R$) genetic inactivation reduced MJD-associated neuronal dysfunction and consequently degeneration. We now directly targeted $A_{2A}R$ through pharmacological selective antagonism and explored its ability to afford neuroprotection in a lentiviral-based model of MJD. We also determined whether $A_{2A}R$-mediated neuroprotection was centrally mediated by testing lentivirus that allowed regio-specific $A_{2A}R$ manipulation (knockdown and over-expression).

Striatum of male adult C57Bl6 mice were transduced with lentiviral vectors encoding mutant ataxin-3 and were: i) followed by treatment with KW6002, a selective antagonist of $A_{2A}R$, or ii) co-transduced with lentiviral vectors either encoding short hairpin RNAs for $A_{2A}R$ and for red fluorescent protein (as internal control), or carrying the mouse $A_{2A}R$ gene and enhanced green fluorescent protein (as internal control). Mice were killed at different time points (from 2-12 weeks) to probe for the appearance of different morphological changes using immunohistochemical analysis.

Mutant ataxin-3 caused neuronal dysfunction (loss of DARPP-32 staining) which evolved to cell damage and consequently cell loss culminating with later tissue shrinkage (bright field). KW6002 reduced the loss of DARPP-32 and prevented striatal loss. $A_{2A}R$ knockdown in striatal GABAergic medium spiny neurons (MSNs) exerted no effect over MJD progression. Notably, $A_{2A}R$ over-expression in MSNs displayed an apparent early neuroprotection.

Our findings directly implicate $A_{2A}R$ in MJD progression and support a distinct role for $A_{2A}R$ localized at different striatal compartments, which might be carefully considered when conceiving therapeutic $A_{2A}R$ antagonistic approaches intended to reduce MJD-associated pathology.

4.2 Introduction

Machado-Joseph disease (MJD), or spinocerebellar ataxia type 3, is the most common polyglutamine neurodegenerative disorder among ataxias (Ranum et al., 1995) and is characterized by an adult age of onset resulting in premature death. MJD is caused by an abnormal expansion of a polyglutamine repeat within ataxin-3 protein (Kawaguchi et al., 1994) and affects selective brain regions, such as cerebellum, brainstem, substantia nigra (Durr et al., 1996; Sudarsky and Coutinho, 1995) and striatum (Alves et al., 2008b; Klockgether et al., 1998; Reetz et al., 2013) resulting ultimately in diverse symptoms evolving from progressive ataxia to motor uncoordination and postural instability as well as Parkinsonism (Gwinn-Hardy et al., 2001; Taroni and DiDonato, 2004). Unfortunately, there is currently no available therapy.

We have recently demonstrated that caffeine, an adenosine receptor antagonist, effectively controlled the initial cascade of events, namely synaptotoxicity and gliosis, in a genetic mouse model of MJD resulting in a reduction of degeneration (Chapter 2; (Goncalves et al., 2013) and of progressive motor and learning disabilities (chapter 3). Using a global genetic knockout of $A_{2A}R$, in which the receptor is deleted from the entire animal, we demonstrated that the effect of caffeine was operated mainly through the adenosine A_{2A} receptors ($A_{2A}R$). However, it remains to be determined whether a selective blockage of the $A_{2A}R$, such as administering the pharmacological $A_{2A}R$ selective antagonist KW6002, in an animal model of MJD is able to produce such beneficial effects.

Furthermore, $A_{2A}Rs$ are particularly abundant in the striatum displaying a key role in the control of locomotion as well as motivational and learning activities (Schiffmann et al., 2007), and notably exhibited a remarkable cell-type specific-mediated motor and neuroprotective responses when blocked (Yu et al., 2008). Nevertheless, the specific striatal neuronal compartment where $A_{2A}R$ blockade has mainly contributed to the observed neuroprotective effects in MJD has yet to be identified. One candidate strategy to dissect the striatal regional selectivity of $A_{2A}Rs$ controlling MJD is the use of lentiviral vectors as gene delivery tools allowing a site-specific and focal manipulation of $A_{2A}R$ within the striatum of our genetic model of MJD.

Therefore, we designed and validated constructs to over-express or to suppress $A_{2A}R$, aiming at investigating the $A_{2A}R$ role in MJD and also at clarifying whether and which specific striatal $A_{2A}R$ are the major mediators of MJD neuropathology.

4.3 Materials and Methods

4.3.1 Generation of lentiviral vectors

cDNA encoding the mouse A_{2A} receptor (GenBank BC110692) from IRAKp961N06232Q plasmid (imaGenes, Germany) was transferred, with the Gateway BP Clonase and LR Clonase recombination systems, into the lentivector SIN-cPPT-PGK-RFA-WHV.

Four small hairpin RNA were engineered to target the mouse adenosine A_{2A} receptor. A shRNA targeting the Red Fluorescent Protein (RFP) was used as a control. The sequences of the shRNA oligos were as follows:

shA_{2A}R.1: 5'CTAGTTTCCAAAAAGAACAACTGCAGTCAGAAATCTCTTGAATTTCTGACTGCAGTTGTT CGGGGATCTGTGGTCTCATACAGAAC-3';
shA_{2A}R.2: 5'-CTAGTTTCCAAAAACCGTGTGGATCAACAGCAAT CTCTTGAATTGCTGTTGATCCACAC GGGGGGATCTGTGGTCTC ATACAGAAC-3';
shA_{2A}R.3: 5'-CTAGTTTCCAAAAAACGTGGTACCCATGAATTATCTCTTGAATAATTCAT GGGTACCACG TGGGGATCTGTGGTCTCATACAGAAC-3';
shA_{2A}R.4: 5'-CTAGTTTCCAAAAACTATTGCCATCGACAGATATCTCTTGAATATCTGTCGATGGCAATAG GGGGATCTGTGGTCTCATACAGAAC-3';
and shRFP (shCTR): 5'-CTAGTTTCCAAAAATCAAGGAGT TCATGCGCTTTCTCTTGAAAAGCGCATG AACTCCTTGAGGGGATCTGTGGTCTCATACAGAAC-3'.

Each of these oligomers and the H1 forward primer 5'-CACCGAACGCTGACGTCATCAACCCG-3' were used for PCR with the pBC-H1 plasmid (pBC plasmid; Stratagene, Amsterdam, The Netherlands) containing the H1 promoter (GenBank: X16612, nucleotides 146-366) as a template. The silencing H1-shRNA cassettes were then inserted and transferred into the lentivector plasmid SIN-cPPT-PGK-EGFP-WHV-LTR-TRE-RFA, as previously described (Alves et al., 2008a). An EGFP reporter gene was inserted into these constructs to facilitate the identification of transduced neurons.

Lentiviral vectors carrying the mouse A_{2A} receptor (A_{2A}R) gene and shRNAs for A_{2A}R were produced in HEK 293T cells, with a four-plasmid system, as previously described (de Almeida et al., 2001). The lentiviral particles were resuspended in 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). The viral particle content of batches was determined by assessing HIV-1 p24 antigen levels (Gentaur, Spain). Viral stocks were stored at -80ºC until use.

4.3.2 Cell culture and transient transfection

Human Embryonic Kidney 293T and Mouse neuroblastoma-2a (N2a) cell lines were cultured in DMEM-Hi glucose (Gibco-Invitrogen, Alfagene, Carcavelos, Portugal) supplemented with 10% fetal bovine serum (FBS) (Gibco-Invitrogen) and 1% penicillin-streptomycin (PS, 100 U/ml, 100 µg/ml) (Gibco-Invitrogen) at 37ºC in a 5% CO_2/ 95% air atmosphere. 293T cells were plated in 6-well and 12-well tissue culture dishes (Frilabo, Maia, Portugal) at densities of $6x10^5$cells/well and $2x10^5$cells/well for Western blot analysis and immunocytochemistry assays, respectively. Cells were co-transfected 24h after plating by the standard calcium-phosphate method, with the $A_{2A}R$ lentivector plasmid (5 and 10 µg for western blot analysis; 1 µg for immunocytochemistry) or EGFP (enhanced Green Fluorescent Protein) lentivector plasmid (5 µg) as a control. For the silencing experiments, 293T cells were co-transfected with the $A_{2A}R$ (2 µg) and sh$A_{2A}R$ (2, 4 and 10 µg) or shCTR (4 and 10 µg) lentivector plasmids. N2a cells were plated in 6-well dishes at a density of $3x10^5$cells/well, and 24h later lipofectamine-transfected (Alfagene) with the $A_{2A}R$ (2 and 4 µg) or EGFP (2 µg) lentivector plasmids. Both cell lines were harvested for western blot processing or immunocytochemistry analysis 48h after transfection.

4.3.3 Primary cultures of cerebellar granule neurons and infection

Primary cultures of rat cerebellar granule neurons were prepared from P7 post-natal Wistar rat pups. Cerebella were dissected and 15 minutes dissociated at 37ºC with trypsin (0.01%) (Sigma) and DNase (45 µg/mL) (Sigma) in Ca^{2+} - and Mg^{2+} - free Krebs buffer (120 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 13 mM glucose, 15 mM HEPES, 0.3% BSA, pH 7.4). Cerebella were then washed with Krebs buffer containing trypsin inhibitor (0.3 mg/mL) (Sigma), centrifuged at RT for 5 min at 1000 rpm and resuspended in Basal Medium Eagle supplemented with 25 mM KCl (Fluka-Sigma), 30 mM glucose (Sigma), 26 mM $NaHCO_3$ (Sigma), 10% FBS (Gibco-Invitrogen) and 1% penicillin-streptomycin (PS, 100 U/ml, 100 µg/ml) (Gibco-Invitrogen). Cells were plated on 12-well dishes coated with poly-D-lysine (Sigma) at densities of $7x10^5$cells/well and $3x10^5$cells/well for western blot analysis and immunocytochemistry assays, respectively, and maintained in a humid incubator at 37ºC in a 5% CO_2/ 95% air atmosphere.

The cell cultures were infected with lentiviral vectors at ratio of 10 ng of p24 antigen/ 10^5 cells 1 day after plating (1DIV) (Zala et al., 2005). At 2 DIV and then every

three days later, medium was replaced with freshly prepared culture medium. Cultures were kept up to 11 days post-infection (11DIV).

4.3.4 Western blot analysis

Cells were lysed and sonicated in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS, 10 µg/mL DTT, 1 mM PMSF, protease inhibitors cocktail; Roche). Protein concentration was determined with the Bradford protein assay (BioRad, Amadora, Portugal). Equal amounts (30 µg of protein) were resolved on 10% SDS-polyacrylamide gels and transferred onto PVDF membranes. Immunobloting was performed using mouse monoclonal anti-A_{2A}R antibodies (clone 7F6-G5-A2, 1:1000, Santa Cruz Biotechnology, Heidelberg, Germany; 1:2000, Millipore, Porto, Portugal), rabbit polyclonal anti-Erk-1/2 (ab9102, 1:1000, Cell Signaling) and anti-P-Erk-1/2 (clone 197G2, 1:1000, Cell Signaling), and mouse monoclonal anti-β-Tubulin (clone SAP.4G5, 1:15000, Sigma). Membranes were then analysed with VersaDoc 3000 (BioRad) after incubation with ECF (Amersham, Buckinghamshire, UK).

4.3.5 Imunocytochemistry

Either 293T cells transfected with A_{2A}R lentivector plasmid (1 µg) or cerebellar granule neurons infected with A_{2A}R lentiviral vector alone (at a ratio of 10 ng of p24 antigen/ 10^5 cells) or in association with shA_{2A}R or shCTR lentiviral vectors, each at a ratio of 10 ng of p24 antigen/ 10^5 cells, were processed 20 min in 4% paraformaldehyde (PFA) fixation and 5 min in 1% Triton permeabilization, each followed by twice 5 min PBS washing, proceeded by 3% (w/v) BSA (Sigma) blocking and immunostained overnight at 4ºC with the mouse monoclonal anti-A_{2A}R antibody (clone 7F6-G5-A2, 1:200, Millipore) followed by 2h incubation at room temperature (RT) with the corresponding secondary antibodies coupled to fluorophores goat anti-mouse Alexa Fluor 488 or Alexa Fluor 594 (1:200, Molecular Probes – Invitrogen, Eugene, OR) together with DAPI (1:5000, Sigma, St. Louis, MO), diluted in the blocking solution (3% (w/v) BSA in PBS, pH 7.4).

4.3.6 *In vivo* infection and experiments

Concentrated viral stocks were thawed on ice. Mice were anaesthetised with avertin (240 µg/ g, i.p.). To test the *in vivo* capability of infection by the new designed lentiviral vectors, lentivirus encoding $A_{2A}R$ (400'000 ng of p24 antigen/ mL) or sh$A_{2A}R$.3 (286'000 ng of p24 antigen/ mL) were unilaterally injected into the mouse striatum; mice received 1 µL ($A_{2A}R$) or 1.4 µL (sh$A_{2A}R$.3) injections. To test the effect of $A_{2A}R$ over-expression or silencing in the striatal MJD-lentiviral mouse model (Simoes et al., 2012), lentivirus encoding mutated ataxin-3 (atx3-72Q) (1 µL; 400'000 ng of p24 antigen/ mL) were stereotaxically co-injected into the left and right striatal hemispheres either with EGFP and $A_{2A}R$ (1 µL; 400'000 ng of p24 antigen/ mL) or shRFP and sh$A_{2A}R$.3 (1.4 µL; 286'000 ng of p24 antigen/ mL) lentiviral vectors. To test the pharmacological blockade of the A_{2A} receptors in the MJD-striatal lentiviral mouse model, mice received 1 µL injections of lentivirus (400'000 ng of p24 antigen/ mL) in each hemisphere, administering atx3-72Q in the right hemisphere and control wild-type ataxin-3 (atx3-27Q) in the left hemisphere. The viral suspensions were injected at 0.2 µL/ min by means of an automatic injector (Stoelting Co., Wood Dale, USA) in the following coordinates: antero-posterior: +0,6mm; lateral: ±1,8mm; ventral: -3,3mm; tooth bar: 0. Different groups of mice were kept in their home cages for different periods ranging from 2 to 12 weeks, before being killed for immunohistochemical analysis of morphological and neurochemical changes in the striatum.

4.3.7 KW6002 treatment

KW6002 was developed as a selective A_{2A} receptor antagonist to be used in *in vivo* studies (Yang *et al.*, 2007). KW6002 ([(E)-1,3-diethyl-8-(3,4-dimethoxystyryl)-7-methyl-3,7-dihydro-1H-purine-2,6,dione]), kindly provided by Prof. Dr. Christa E. Müller (Bonn University, Germany), was prepared daily in a vehicle solution (0.4% methylcellulose (Sigma) and 0.9% NaCl. For complete colloidal dispersion, the KW6002 sol was subjected to ultra sounds for 20 min. Mice were daily exposed to 3 mg/ kg of compound added to 5 mL of vehicle starting 3 days before surgeries onwards until the corresponding day of sacrifice. After total consumption of the drug, water was given during the rest of the day.

4.3.8 Immunohistochemical procedure

After an overdose of avertin (2.5x 240 µg/ g, i.p.), transcardiac perfusion of the mice was performed with PBS followed by fixation with 4% paraformaldehyde. The brains were then removed and post-fixed in 4% paraformaldehyde for 24h and cryoprotected by incubation in 25% sucrose/ phosphate buffer for 48h. The brains were frozen and 25 µm coronal sections were cut using a cryostat (LEICA CM3050 S, Heidelberg, Germany) 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.

Sections were processed overnight at 4ºC with the following primary antibody: a rabbit anti-DARPP-32 antibody (1:1000; Chemicon) followed by 2h incubation at room temperature (RT) with the respective biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, CA). Bound antibodies were visualized using the Vectastain ABC kit, with 3,3'-diaminobenzidine tetrahydrochloride (DAB metal concentrate; Pierce, Burlingame, CA) as substrate.

Double staining for A_{2A}R (1:200; Millipore) together with DAPI were performed. Free-floating sections were kept at RT for 2h in PBS with 0.1% Triton X-100 containing 10% normal goat serum (Gibco-Invitrogen, Barcelona, Spain), then overnight at 4°C in blocking solution with the primary antibody. Sections were washed three times and incubated for 2h at RT with the corresponding secondary antibody coupled to fluorophores goat anti-mouse Alexa Fluor 488 (1:200; Molecular Probes – Invitrogen) diluted in the blocking solution. The sections were washed three times and then mounted in mowiol Reagent (Sigma) on microscope slides.

Staining was visualized using Zeiss Axioskop 2 plus imaging microscope (Carl Zeiss Microimaging, Germany) equipped with an AxioCam HR color digital camera (Carl Zeiss Microimaging) and 5X, 20X, 40X and 63X Plan-Neofluar objectives and using the AxioVision 4.8 software package (Carl Zeiss Microimaging).

4.3.9 Evaluation of the volume of the DARPP-32 depleted region and of DARPP-32 immunoreactivity indexes

The extent of ataxin-3 lesions in the striatum was analyzed by photographing, with a x1.25 objective, 8 sections stained with DARPP-32 per animal (25 µm thick sections at 200 µm intervals), selected so as to obtain complete rostro-caudal sampling

of the striatum, and by quantifying the area of the lesion with a semi-automated image-analysis software package (Image J software, NIH, USA). The volume was then estimated with the following formula: volume = $d(a_1+a_2+a_3 \ldots)$, where d is the distance between serial sections (200 µm) and $a_1+a_2+a_3$ are DARPP-32-depleted areas for individual serial sections. The immunoreactivity indexes were measured through optic density analysis of the affected striatal regions relative to their corresponding peripheral non-affected striatum (defined as 100% immunoreactivity (IR)).

4.3.10 Statistical analysis

Statistical comparisons were performed using unpaired student's *t* test. Results are expressed as mean ± standard error of the mean (SEM). Significance thresholds were set at $p < 0.05$ and $p < 0.01$, as defined in the text.

4.4 Results

4.4.1 Selective pharmacological blockade of $A_{2A}R$ reduces MJD-associated neuropathology

We have previously demonstrated that caffeine, an adenosine receptor antagonist, reduced MJD neuropathology as well as progressive motor and learning disabilities in two distinct animal models of MJD, namely the MJD lentiviral-based mouse model (Chapter 2; (Gonçalves et al., 2013) and MJD transgenic mice (Chapter 3; Gonçalves et al., *in preparation*). Using a global genetic knockout of $A_{2A}R$, we demonstrated that the effect of caffeine was operated mainly through the adenosine A_{2A} receptors ($A_{2A}R$). However, it has yet to be directly confirmed if $A_{2A}Rs$ are indeed the main effectors of the observed neuroprotection through antagonism. For this purpose, we daily administered KW6002, a selective antagonist of $A_{2A}R$, in our genetic mouse model of MJD (Fig 4.1A) and further analysed the appearance of MJD features, such as brain dysfunction (DARPP-32) and degeneration (bright field), we have previously described (Gonçalves et al., 2013). Accordingly, 12 weeks after lentiviral transduction of mutant ataxin-3 (LV-atx3-72Q), the corresponding LV-atx3-72Q-challenged striatal hemisphere of control animals administered with vehicle (n=5) displayed an extensive loss of DARPP-32 immunoreactivity (Fig 4.1B, *top*; Table 4.1), which was accompanied by a clear condensation of the internal capsule, as observed under bright field (Fig 4.1B, *bottom*), attributable to striatal tissue shrinkage. Notably, animals chronically treated with KW6002 (n=5) displayed a significant (Table 4.1; $p < 0.01$) reduction of DARPP-32 loss as well as no evident striatal tissue shrinkage. Neither DARPP-32 loss nor alterations on bright field pattern were observed upon LV-atx3-27Q transduction (data not shown).

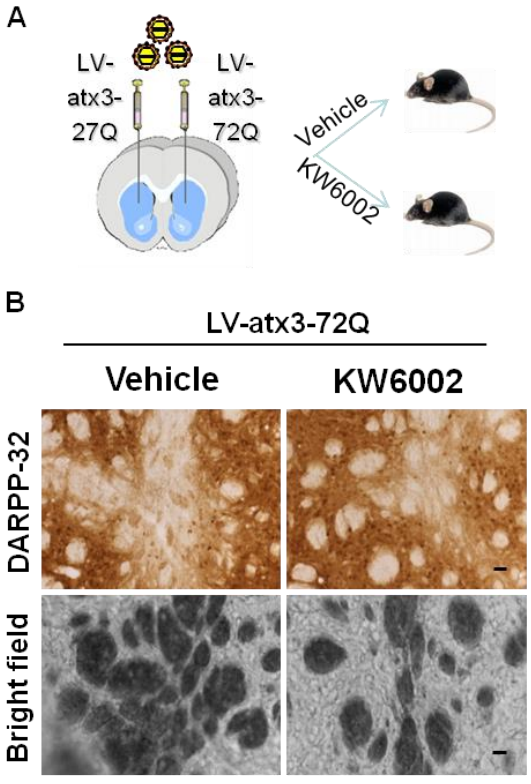


Figure 4.1: Pharmacological $A_{2A}R$ blockade in a MJD-lentiviral based model through consumption of KW6002, a selective antagonist of $A_{2A}R$. (A) Schematic representation of striatal lentivirus delivery, namely lentivirus encoding mutant ataxin-3 (LV-atx3-72Q) in one hemisphere and

wild-type ataxin-3 (LV-atx3-27Q) in the other hemisphere (as internal control). (B) Representative immunohistochemical DARPP-32 staining and bright field photomicrographs from around the injection site area at 12 weeks post-injection of LV-atx3-72Q. Animals treated with vehicle displayed a considerable loss of DARPP-32 immunoreactivity (quantified in Table 1) and coalescence of the internal capsule of the striatum. KW6002 treatment reduced DARPP-32 loss and also displayed a negligible striatal tissue loss. Bar, 20 µm.

Table 4.1: DARPP-32 intensity index.

100% IR	100.0 ± 2.9
Vehicle	42.8 ± 1.7
KW6002	51.2 ± 1.8**

Density analysis of DARPP-32 immunoreactivity 12 weeks after mutant ataxin-3 transduction. KW6002 treatment significantly reduced the loss of DARPP-32 (**$p < 0.01$).

These data show that $A_{2A}R$ selective antagonism is able to reduce dysfunction and degeneration in MJD and also support the crucial role of striatal $A_{2A}R$ for MJD progression.

4.4.2 Strategies used to molecularly manipulate the mouse A_{2A} receptors

We previously demonstrated the usefulness of viral vectors to generate genetic models of Machado-Joseph disease (MJD) (Alves et al., 2008b; Nobrega et al., 2012). Another potential use for viral vectors is gene therapy. Indeed, we have recently shown successful applications for viral vectors as gene therapy tools modulating the putative intracellular mechanisms accounting for MJD neuropathology (Alves et al., 2008a; Nascimento-Ferreira et al., 2011; Simoes et al., 2012).

Since $A_{2A}R$ selective and non-selective blockade mediate neuroprotective effects modifying MJD progression, we now developed lentivectors encoding for four different shRNAs targeting the mouse $A_{2A}R$ ($shA_{2A}R$) (Fig 4.2, *top*) to provide stable *in vivo* $A_{2A}R$ knockdown as a candidate strategy to control MJD. Additionally, a lentivector carrying the mouse $A_{2A}R$ gene (Fig 4.2, *bottom*) was also developed to allow over-expression of $A_{2A}R$ and further provide full comprehension of the $A_{2A}R$ role in MJD. The $A_{2A}R$ silencing constructs were inserted downstream from the human Pol III promoter H1 and target different $A_{2A}R$ nucleotides: $shA_{2A}R.1$ – 416 to 434; $shA_{2A}R.2$ – 79 to 97; $shA_{2A}R.3$ – 493 to 511; $shA_{2A}R.4$ – 280 to 298; the enhanced green fluorescent protein (EGFP) gene was also inserted in the shRNA lentivectors under the control of the internal mouse phosphoglycerate kinase 1 (PGK) promoter, to allow transduced cells to

be identifiable. Lentivectors encoding a shRNA targeting the red fluorescence protein (shCTR) or EGFP only, similar to those encoding $shA_{2A}R$ or $A_{2A}R$ gene, respectively, were designed and used to ensure the use of the appropriate experimental controls.

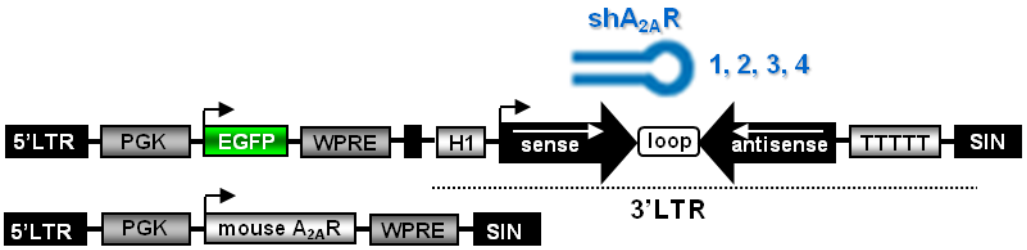


Figure 4.2: Schematic representation of the lentiviral constructs used to down- and up-regulate $A_{2A}R$. Diagram of the shRNAs designed to knockdown $A_{2A}R$: shRNA cassete under control of the H1 promoter (pol III) and a separate cassette containing the enhanced green fluorescent protein (EGFP) reporter gene under control of the phosphoglycerate kinase-1 (PGK) promoter (*top*), and the construct encoding the mouse $A_{2A}R$ to convey over-expression (*bottom*).

To test whether short hairpin $A_{2A}R$ constructs were able to knockdown the $A_{2A}R$ mRNA, we first tested *in vitro* the ability of the construct encoding the $A_{2A}R$ cDNA to increase $A_{2A}R$ density. Both 293T and N2a cell lines were transiently transfected with different concentrations of the expression vector construct encoding the $A_{2A}R$ or EGFP (as a transfection control). No detectable $A_{2A}R$ protein was observed on 293T cells while a small amount was constitutively present on N2a cells (Fig 4.3A). Increasing quantities of transfected-$A_{2A}R$ construct resulted in higher densities of $A_{2A}R$ protein in both cell lines. By means of immunocytochemistry upon 293T transfection with the $A_{2A}R$ construct, we also visualized $A_{2A}R$ over-expression and confirmed their somato-branch localization in positively transfected cells (Fig 4.3B).

Next, we co-transfected 293T cells with the $A_{2A}R$ and $shA_{2A}Rs$ expression vector constructs at different ratios and tested their silencing efficiencies, using the shCTR as a mistargeted non-silencing control. $A_{2A}R$ co-transfection with control shRNA resulted in a robust $A_{2A}R$ protein density, while cells transfected with the four $shA_{2A}R$ constructs exhibited different degrees of $A_{2A}R$ silencing (Fig 4.3C). The $shA_{2A}R.1$ and $shA_{2A}R.3$ constructs mediated the most efficient gene silencing; thenceforth we have only used the $shA_{2A}R.3$ for further experiments.

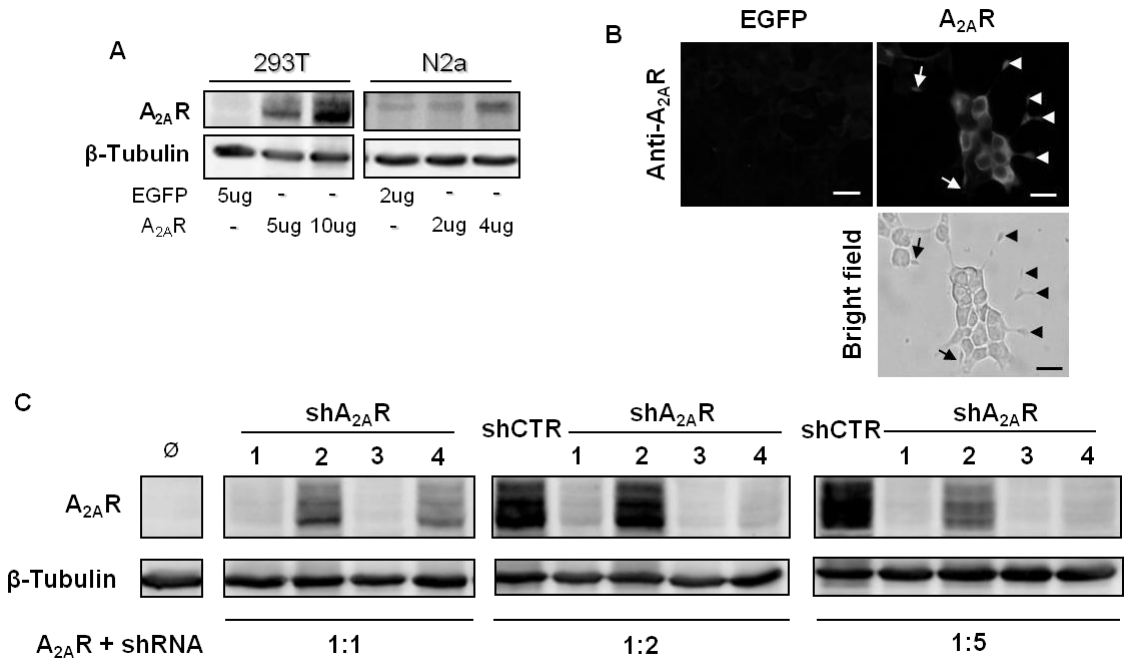


Figure 4.3: *In vitro* up-regulation and suppression of $A_{2A}R$. (A-D) Western-blot and immunocytochemical analysis of 293T and N2a cells 48h post-transfected either with the plasmid construct encoding $A_{2A}R$ alone or in association with shRNAs encoding $A_{2A}R$ ($shA_{2A}R$) or the mistargeted control (shCTR). (A) 293T cells transfected with the control EGFP plasmid displayed no detectable $A_{2A}R$ densities whereas N2a cells presented low levels of $A_{2A}R$. Increasing concentration of transfected $A_{2A}R$ plasmid increased the $A_{2A}R$ densities in both cell lines. (B) Immunocytochemical analysis of 293T cell line transfected with EGFP plasmid exhibited no $A_{2A}R$ immunoreactivity, while it was clearly present upon $A_{2A}R$ plasmid transfection also showing somato-branch localization (arrows) as observed in parallel under bright field microscopy. (C) 293T cells co-transfected with $A_{2A}R$ and shCTR plasmids (1:2 and 1:5 ratios) displayed increased densities of $A_{2A}R$, whereas co-transfection with $A_{2A}R$ and $shA_{2A}R$ plasmids (1:1, 1:2 and 1:5 ratios) resulted, in general, in lower $A_{2A}R$ densities. $shA_{2A}R.1$ and $shA_{2A}R.3$ plasmids displayed the highest silencing efficiencies independently of $A_{2A}R$:$shA_{2A}R$ transfection ratio. β-Tubulin is shown as a loading control. Bar, 20 µm.

To evaluate the transduction efficiencies of the lentiviral vectors encoding the $shA_{2A}R$ (LV-$shA_{2A}R.3$) or the $A_{2A}R$ cDNA (LV-$A_{2A}R$), P7 rat cerebellar granule neurons, which express $A_{2A}R$ (Cunha et al., 1994; Dall'Igna et al., 2003), were cultured and transduced with LV-$A_{2A}R$ alone or in association with LV-shRNA (1:1 ratio). The $A_{2A}R$ density increased in direct proportion to the concentration of lentiviral vectors used to transduce cells (Fig 4.4A). Immunocytochemical analysis of cerebellar primary cultures co-transduced with LV-$A_{2A}R$ and LV-shCTR demonstrated no differences on $A_{2A}R$ immunoreactivity while compared to the LV-$A_{2A}R$ transduction alone (Fig 4.4B) despite the apparent decrease on $A_{2A}R$ density displayed in the western blot (Fig 4.4C), which

might be explained by the double amount of lentivirus used that may have divided the cellular machinery to produce both $A_{2A}R$ mRNA and shRNA. Finally, co-transduction of cerebellar granule neurons with LV-$A_{2A}R$ and LV-sh$A_{2A}R$ resulted in complete silencing of $A_{2A}R$ (Fig 4.4B and C).

Together, these data validate and support a successful application of these two lentiviral vectors to over-express and silence $A_{2A}R$.

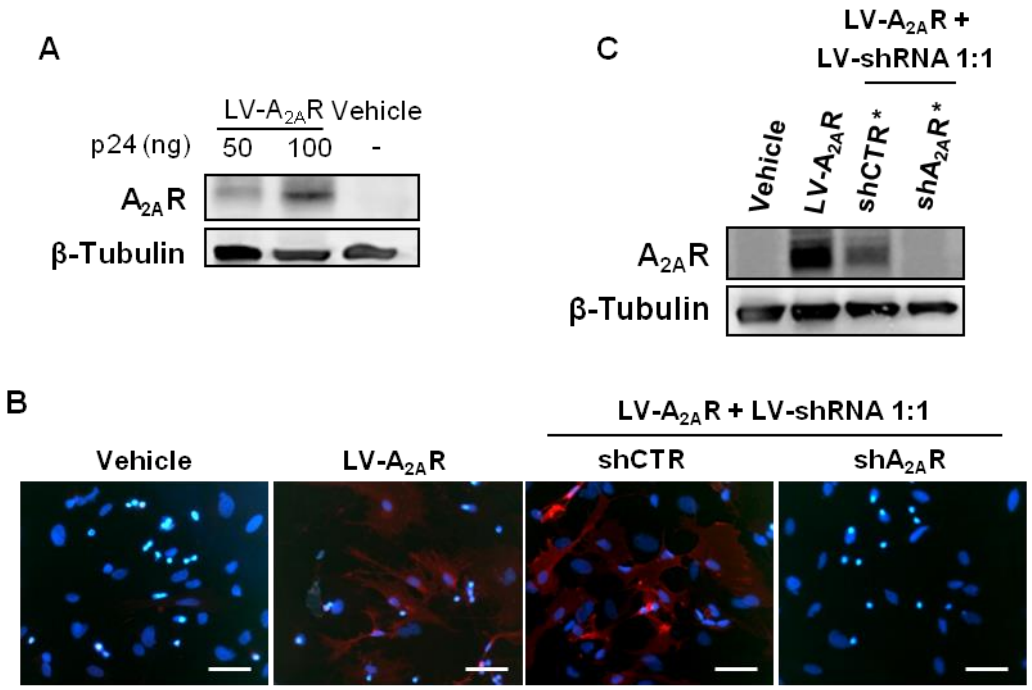


Figure 4.4: Lentiviral-mediated $A_{2A}R$ over-expression and knockdown in cerebellar primary cultures. (A) Infection of cerebellar primary cultures mediated by lentiviral vectors encoding $A_{2A}R$. Control cells treated with vehicle (PBS/BSA 1%) displayed no $A_{2A}R$ density. Transduction with 50 and 100 ng (p24 antigen/ mL) of $A_{2A}R$ plasmid mediated increasing $A_{2A}R$ densities. (B and C) Co-transduction with LV-$A_{2A}R$ and LV-shCTR displayed either no modifications (B) or a minor decrease (C) on $A_{2A}R$ immunoreactivity when compared to LV-$A_{2A}R$ transduction alone, whereas co-transduction with LV-$A_{2A}R$ and LV-sh$A_{2A}R$ showed complete absence of $A_{2A}R$ immunoreactivity. $A_{2A}R$ (red), DAPI staining (blue). β-Tubulin is shown as a loading control. Bar, 40 µm.

4.4.3 *In vivo* lentiviral manipulation of $A_{2A}R$

To foresee the ability of the vectors to manipulate the $A_{2A}R$ density *in vivo*, unilateral striatal injections with LV-sh$A_{2A}R$ or LV-$A_{2A}R$ alone were made on wild-type mice. Similar experiments were performed in global knockout mice for the $A_{2A}R$ (gb-KO-$A_{2A}R$) by injecting LV-$A_{2A}R$ in one striatal hemisphere and the vehicle used to

suspend viral particles (PBS/BSA1%) in another hemisphere (as control). 4 weeks post-injection, striata of wild-type animals challenged with LV-shA_{2A}R displayed a large although not significant decrease in A_{2A}R density (100.0±43.2% *versus* 68.8±12.0% in control, n=3, *p*=0.51); in turn, either wild-type (n=3) or gl-KO-A_{2A}R (n=2) animals transduced with LV-A_{2A}R displayed an increase in A_{2A}R density (100.0±15.0% on WT injected *versus* 109.8±6.1% in control, n=3, *p*=0.58) (Fig 4.5A and B, *left*). Interestingly, LV-A_{2A}R transduction in gl-KO-A_{2A}R animals resulted along to the transduced area in a comparable slightly stronger immunoreactivity as to that observed in non-injected wild-type striatum (Fig 4.5B, *right*).

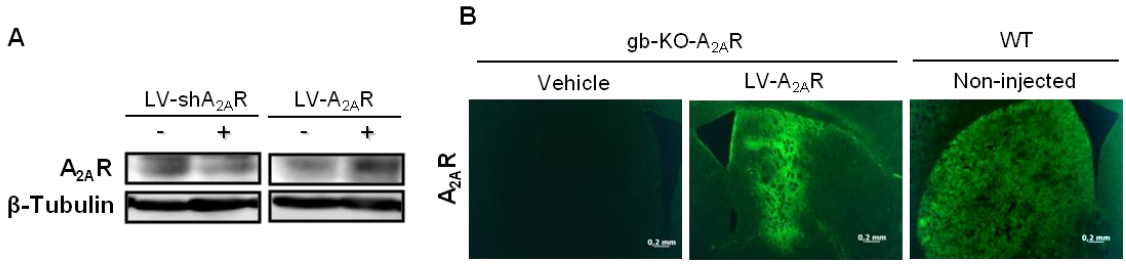


Figure 4.5: *In vivo* lentiviral-mediated A_{2A}R knockdown and over-expression 4 weeks post-injection. (A) Striatal hemisphere injected with LV-shA_{2A}R displayed a reduction on A_{2A}R density (*left*) while challenging striatum with LV-A_{2A}R demonstrated an increase in A_{2A}R density (*right*) when compared to the respective contra-lateral non-injected hemispheres. (B) A_{2A}R immunohistochemical analysis of gl-KO-A_{2A}R mice injected with vehicle or LV-A_{2A}R. No immunoreactivity for A_{2A}R was observed upon vehicle injection. LV-A_{2A}R injection triggered a robust A_{2A}R immunoreactivity along the injection site (*center*), which is higher than the A_{2A}R immunoreactivity displayed by non-injected wild-type animals (*right*).

In conclusion, LV-shA_{2A}R transduction resulted in a considerable reduction in A_{2A}R density under physiological conditions. This effect is expected to be higher in pathophysiological conditions where an up-regulation of A_{2A}R is triggered (Cunha et al., 2006; Rebola et al., 2005b; Tomiyama et al., 2004). Additionally, these data also show that the vector encoding the A_{2A}R ensures its efficient expression.

4.4.4 Effect of lentiviral-mediated A_{2A}R knockdown in MJD mice

We have highlighted A_{2A}R as a novel target to interfere with the progression of MJD (Goncalves et al., 2013). We now tested the ability of long-term knockdown of A_{2A}R as a promising gene therapy tool to manage MJD, and simultaneously defined the sub-regional compartment where A_{2A}R mediated pharmacological blockade exerted MJD neuroprotection. For this purpose, we performed intra-striatal injections of the

lentiviral vectors and co-transduced striatal hemispheres with LV-atx3-72Q either with LV-shA_{2A}R or LV-shCTR (as internal control) (Fig 4.6A). A time-course was established by sacrificing the animals from 2 to 12 weeks post-transduction (n=3-4/ experimental group), which is within the time-frame of appearance of morphological modifications previously described for this genetic model of MJD (Goncalves et al., 2013). Neuropathological analysis involved: i) striatal lentiviral-mediated transduction pattern; ii) dopamine and cAMP-regulated phosphoprotein (DARPP-32) -depleted volume and immunoreactivity indexes; and iii) evaluation of tissue shrinkage and collapsing of internal capsule in bright field photomicrographs.

Both striatal hemispheres injected with lentiviral vectors displayed extensive transduced areas (Fig 4.6B, *top*), as shown by fluorescence of the EGFP reporter carried by LV-shRNA. Immunohistochemical analysis of DARPP-32, 4 weeks after co-injection of LV-atx3-72Q and LV-shCTR, exposed a large depleted staining volume of 0.52 ± 0.12 mm^2 (n=3; Fig 4.6B *center*), as quantified in Figure 4.6C. No evident tissue shrinkage was observed on bright field photomicrographs, at the later stage of 12 weeks post-transduction. Silencing A_{2A}R with LV-shA_{2A}R neither modified DARPP-32-depleted volume 4 weeks post-transduction (0.59 ± 0.13 mm^2, n=3, $p > 0.05$, Fig 4.6C), nor DARPP-32 immunoreactivity indexes 12 weeks post-transduction (Table 4.2), as well as it did not change striatal morphology under bright field photomicrographs (Fig 4.6B, *bottom*).

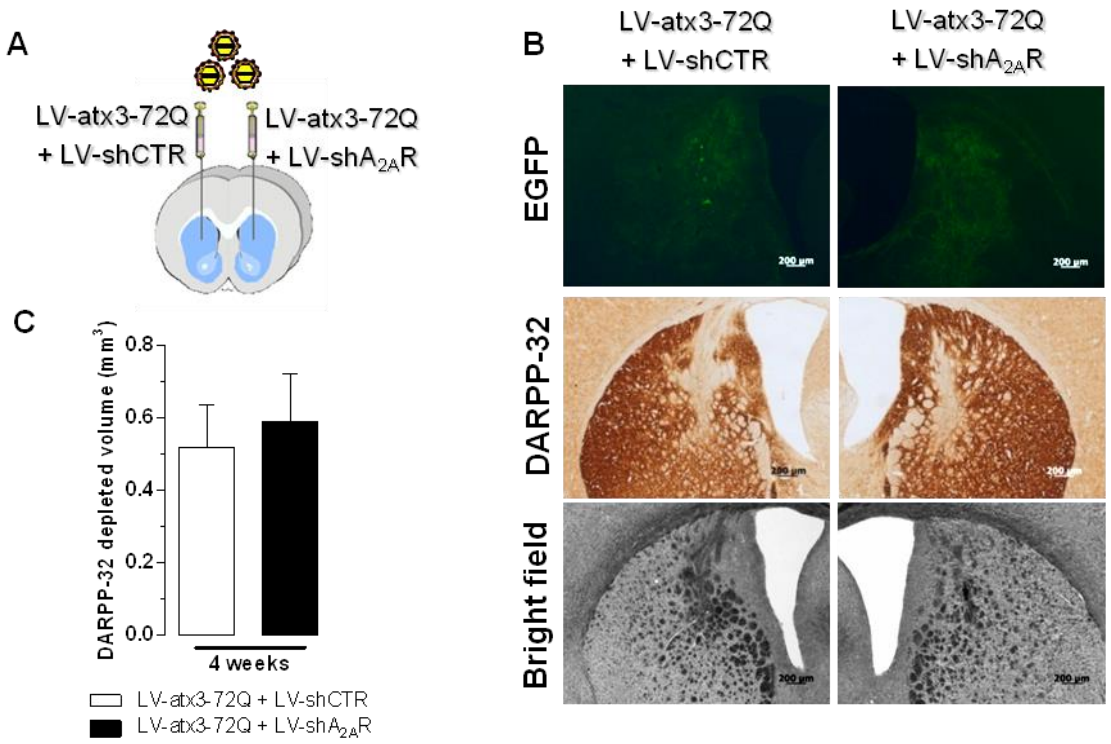


Figure 4.6: Effect of $A_{2A}R$ knockdown over striatal MJD-induced pathology. (A) Schematic representation of striatal lentivirus delivery. (B) Transduced (EGFP reporter-positive) striatum 8 weeks after lentivirus injections (green, *top*). Co-transduced striatum with LV-atx3-72Q and LV-shA_{2A}R displayed no significant differences neither on DARPP-32-depleted volume 4 weeks upon lentiviral delivery, nor on bright field photomicrographs 12 weeks post-transduction, compared to striatal delivery of LV-atx3-72Q and LV-shCTR.

Table 4.2: DARPP-32 intensity index.

100% IR	100.0 ± 4.3
LV-shCTR	42.0 ± 3.4
LV-shA_{2A}R	42.1 ± 1.6

Density analysis of DARPP-32 immunoreactivity 12 weeks after mutant ataxin-3 co-transduction with either lentivirus encoding short hairpin for $A_{2A}R$ (LV-shA_{2A}R) or control (LV-shCTR). $A_{2A}R$ knockdown displayed no modifications on DARPP-32 immunoreactivity loss relative to control.

In keeping with the fact that our lentiviral vectors are pseudotyped with the vesiculo-stomatitis G protein (VSV-G) which confers neurotropism (de Almeida et al., 2001) and thus upon striatal injection transduce mostly GABAergic medium spiny neurons (MSNs), these data suggest that $A_{2A}R$ located post-synaptically are not the main striatal neuronal compartment of $A_{2A}R$ contributing to the success of the previously shown pharmacological and genetic $A_{2A}R$ inactivation.

4.4.5 Lentiviral-mediated $A_{2A}R$ over-expression exerted an early beneficial effect on MJD-striatal pathology

We previously demonstrated that $A_{2A}R$ blockade or genetic inactivation mitigated MJD-striatal pathology (Gonçalves et al., 2013). Nevertheless, long-term silencing of striatal $A_{2A}R$ mRNA levels did not alleviate neuropathology suggesting that post-synaptic $A_{2A}R$ might not be involved in the neuroprotective effects. To test whether $A_{2A}R$ located in MSNs could have a different role, even potentially beneficial, to the striatal MJD neuropathology, we applied lentiviral vectors to up-regulate $A_{2A}Rs$ therein. We essentially followed the same experimental design as described above for the $A_{2A}R$ knockdown, and transduced the striatum of the lentiviral MJD-based model either with LV-$A_{2A}R$ or LV-EGFP (as internal control) (Fig 4.7A).

Immunohistochemical analysis of DARPP-32 as early as 2 weeks post-transduction displayed a large depleted staining volume of 0.45 ± 0.10 mm^3 (n=4) on the control hemisphere (LV-atx3-72Q and LV-EGFP), whereas the contralateral hemisphere (co-transduction of the LV-Atx3-72Q and LV-$A_{2A}R$) exhibited a one third reduction of DARPP-32 volume loss (0.30 ± 0.06 mm^3, n=4, p = 0.27; Fig 4.7B, *top*; and C). DARPP-32 loss in the control hemisphere remained unchanged until 8 weeks post-transduction, but it progressively increased until a 2 fold larger volume loss upon $A_{2A}R$ over-expression (data not shown). Nevertheless, at the later time-point of 12 weeks post-injection, mutant ataxin-3 expression resulted in clear tissue shrinkage presumably due to neuronal loss while no evident tissue shrinkage was observed upon A2AR over-expression (Fig 4.7B, *bottom*).

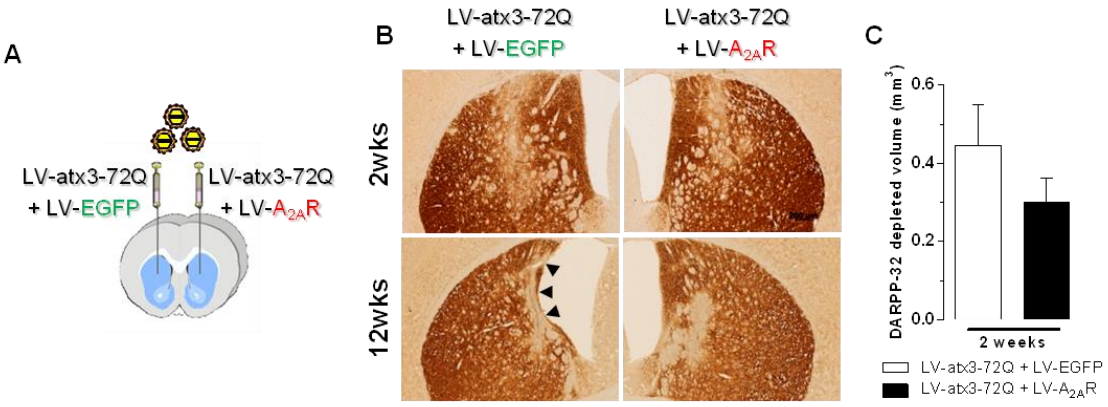


Figure 4.7: Effect of $A_{2A}R$ over-expression over striatal MJD-induced pathology. (A) Schematic representation of striatal lentiviral vectors delivery. (B and C) A reduction of DARPP-32 depleted volume in striatum was observed as early as 2 weeks upon lentiviral co-transduction with LV-atx3-72Q and LV-$A_{2A}R$ when compared to control LV-atx3-72Q and LV-EGFP co-injection (as internal control) (B, *top*), as quantified in C. 12 weeks LV-atx3-72Q and LV-EGFP co-

transduction mediated a large striatal tissue contraction which was not observed in LV-atx3-72Q and LV-A_{2A}R co-transduced striatum (B, *bottom*).

These data suggest that A_{2A}R over-expression in MSNs might even have higher beneficial than detrimental effect on MJD striatal dysfunctional phenotype, starting as early as 2 weeks after insult to a lasting amplified effect on neuronal damage.

4.5 Discussion

In the present study, we investigated if manipulation of A_{2A}Rs would modify the progression of neuropathology in a genetic viral-induced mouse model of MJD upon i) administration of the pharmacological A_{2A}R selective antagonist KW6002 ; and ii) upon localized striatal manipulation of A_{2A}R using lentiviral vectors as gene delivery tools to silence or over-express A_{2A}Rs.

We have demonstrated: i) that A_{2A}R are indeed the adenosine receptors whose antagonism mediated MJD neuroprotection, based on selective pharmacological blockade of the A_{2A}R; ii) lentiviral vectors allow efficient manipulation of A_{2A}R both *in vitro* and *in vivo* helping defining the particular A_{2A}R role in well defined compartments ; and iii) silencing of postsynaptic A_{2A}Rs in the striatum does not afford neuroprotection and instead that its up-regulation may even be beneficial, suggesting that these are not the targets of neuroprotection through antagonism.

We have previously reported the ability of caffeine, a non-selective antagonist of adenosine receptors, to yield a neuroprotective effect against neuronal dysfunction and cell damage in genetic models of MJD (Chapters 2 and 3; (Goncalves et al., 2013). Challenging A_{2A}R gene-deficient mice with mutant ataxin-3 allowed clarification that caffeine mediated MJD neuroprotection through A_{2A}R antagonism, which is in agreement with the ability of A_{2A}R, mainly targeted by chronic caffeine consumption, to afford neuroprotection against different neurodegenerative disorders, such as Alzheimer's, Parkinson's and Huntington's disease (Cunha and Agostinho, 2010; Popoli et al., 2007; Schwarzschild et al., 2006).

In the present study, we directly confirmed whether A_{2A}R blockade indeed mediated neuroprotection in MJD by testing the ability of KW6002, a selective antagonist of A_{2A}R, to prevent MJD progression. Notably, chronic treatment with KW6002 prevented neuronal dysfunction, at levels comparable to A_{2A}R genetic inactivation (Gonçalves et al., 2013), and preserved striatum from mutant ataxin-3-induced damage and consequent cell loss and tissue shrinkage. This key experiment definitely establishes A_{2A}R as a promising target to manage MJD evolution.

Therefore, we next used RNAi technology to mediate long-term efficient and stable A_{2A}R knockdown and probed for its success as a therapeutic approach to handle MJD neuropathology. The emergence of RNA interference (RNAi), a highly specific mechanism of post-translational gene silencing, has opened a huge biological application ranging from reversion of genetic disorders (Alves et al., 2008a; Nobrega et

al., 2013) to therapeutic or modifier schemes, which enabled the study of selected genes within particular compartments differently involved in physiology (Lazarus et al., 2011) and in pathophysiology (Ferres-Coy et al., 2013). Additionally, we took advantage of lentiviral vectors (LVs) to mediate $A_{2A}R$ suppression, which also allowed dissecting cell-type specific compartments where $A_{2A}R$ critically modulated MJD. Actually, LVs are in a privileged position to model CNS neurodegeneration either by recapitulating genetic neurodegenerative disorders by over-expressing disease-causing proteins (Alves et al., 2008b; de Almeida et al., 2002), or by over-expressing disease-related or -modifier genes (Blomer et al., 1998; de Almeida et al., 2001; Nascimento-Ferreira et al., 2011; Simoes et al., 2012), which makes them the ideal tools to deliver therapeutic genes to over-express or suppress relevant targets in specific cell types. LVs also exhibit several advantages, such as: i) the ability to accommodate medium size transgenes (8Kb); ii) high transduction efficiency; iii) stable transgene expression; iv) low immunogenicity (for review, see (de Lima et al., 2005)); and v) different cell-type-specific tropism (reviewed in (Waehler et al., 2007)).

Thus, we designed, cloned into lentiviral backbones and validated $A_{2A}R$ silencing constructs, and applied these into the striatum of a genetic MJD model to dissect the striatal neuronal elements where $A_{2A}R$ contributed most to MJD degeneration and that were targeted by caffeine as well as the selective inhibitor. The observation that $A_{2A}R$ knockdown in MSNs did not control MJD-associated striatal neuropathology was not completely surprising. Striatal $A_{2A}R$ arise from two distinct sources, located post-synaptically in the dendritic spines of MSNs of the indirect pathway, and pre-synaptically in glutamatergic terminals contacting the MSNs of the direct pathway (Ciruela et al., 2006). Lentiviral vectors encoding for sh$A_{2A}R$ transduced merely MSNs therein promoting only post-synaptic $A_{2A}R$ silencing, leaving out both pre-synaptic neuronal $A_{2A}Rs$ as well as $A_{2A}Rs$ in glia, whose transduction by lentiviral vectors is rather limited.

It is also worth noting that the impact of mutant ataxin-3 in $A_{2A}R$ mRNA expression and protein density still has to be fully elucidated. Consistent with previous reports heralding an $A_{2A}R$ up-regulation upon noxious stimulation (Cunha, 2005; Fredholm et al., 2005), also a transgenic mouse model of MJD with a permanent expression of a truncated form of the human ataxin-3 with 69 glutamine repeats in the mouse cerebella (Torashima et al., 2008) displayed increased expression of $A_{2A}R$ within the cerebellum (unpublished data). Hence, it remains to be confirmed whether striatal challenge with lentiviral vectors encoding mutant ataxin-3 induce an increase in

the $A_{2A}R$ mRNA levels and consequently *de novo* production of $A_{2A}R$ protein, which might partly explain the lack of effect displayed by striatal $A_{2A}R$ silencing.

Importantly, the present study revealed that MJD control through $A_{2A}R$ blockade did not result from post-synaptic $A_{2A}R$ antagonism, but rather from other striatal compartments than MSNs, strengthening even more our previous hypothesis heralding pre-synaptic and glial $A_{2A}R$ as the main elements mediating MJD progression and hence neuroprotection through antagonism, which is consistent with the $A_{2A}R$ localization in glutamatergic synapses (Rebola et al., 2005) where they control the release of glutamate (Ciruela et al., 2006; Rodrigues et al., 2005) and the activation of NMDA receptors (Azdad et al., 2009; Rebola et al., 2008; Tebano et al., 2005), as well as in astrocytes operating glutamate uptake (Matos et al., 2012; Nishizaki et al., 2002), and in agreement with a recent report showing increased aggregation of ataxin-3 upon glutamate overstimulation in induced pluripotent stem cells-derived neurons (Koch et al., 2011), which consequently leads to neurodegeneration (Simoes et al., 2012).

Notably, the present study also showed that striatal $A_{2A}R$ over-expression decreased neuronal dysfunction as early as the pathology establishes, which was later accompanied by a better neuronal survival. These results actually pointed out an early beneficial effect of post-synaptic $A_{2A}R$ in MJD progression. Such interpretation is in accordance to previous reports in another polyglutamine disorder, namely Huntington's disease (HD), where Glass and colleagues (2000) have shown that the earliest neurochemical feature was indeed a reduction of striatal $A_{2A}R$ binding sites, which might result on decreased neurotrophic factors release (Gomes et al., 2013; Gomes et al., 2006; Minghetti et al., 2007) evolving to an earlier development of cell dysfunction and damage. Moreover, Blum and colleagues (2003) showed that $A_{2A}R$ antagonists exhibited undesirable biphasic neuroprotective-neurotoxic effects in HD, displaying a dual and opposite role for pre- and post-synaptic $A_{2A}R$ upon striatal injury (Blum et al., 2003). In keeping with this scenario, other reports have showed cell type-specific $A_{2A}R$-mediated distinct effects upon cell injury (Tebano et al., 2004; Yu et al., 2008), and even diverse pharmacological selective $A_{2A}R$ antagonists were shown to display distinct pre- and postsynaptic profiles based on their affinity to $A_{2A}R$, A_1R-$A_{2A}R$ heteromers or $A_{2A}R$-D_2R heteromers (Orru et al., 2011).

In conclusion, our data bring a new insight to the $A_{2A}R$ role in MJD, particularly demonstrating that the presence of striatal post-synaptic $A_{2A}R$ in the initial stages of the disease may even have a neuroprotective effect; it also indirectly supports the mechanisms through which $A_{2A}R$ blockade mediated control of MJD, namely pre-

synaptic and glial $A_{2A}R$. Nevertheless, further studies should be performed to directly explore and fully confirm these hypotheses. Our study also extols a new methodology allowing both *in vitro* and *in vivo* $A_{2A}R$ manipulations, which might be effectively applied to other neurological disorders either to improve mechanistic comprehension or to attempt gene therapy approaches.

CHAPTER 5

Final conclusions and future prospects

5. Final conclusions and future prospects

This thesis sought to investigate whether the manipulation of a neuromodulation system operated by $A_{2A}R$ was effective in controlling the inexorable cascade of events triggered by pathogenic ataxin-3 protein involved in the aetiology of Machado-Joseph disease, the most frequent among dominantly-inherited spinocerebellar ataxias. Our findings demonstrated for the first time that $A_{2A}R$ might be a novel therapeutic target to interfere with MJD evolution. Additionally, our observations open novel areas of research to foster a better understanding of the role of ataxin-3 in the control of synaptic function and damage, and of non-neuronal brain cells contribution to the progression of neurodegenerative diseases.

We first characterized the time course of morphological and behavioral modifications triggered by mutant ataxin-3 over-expression in different genetic models of MJD with distinct brain regions affected, namely the striatum (lentiviral-based model) and cerebellum (MJD transgenic mouse model), and then explored the impact of pharmacological selective and non-selective antagonism of the adenosine A_{2A} receptors, as well as of $A_{2A}R$ genetic and molecular inactivation.

The present thesis provides direct evidence that synaptotoxicity and reactive gliosis are amongst the most precocious modifications, so far under-appreciated, in MJD. This is in line and strengths even more the crucial role of synaptic impairment and astrocytic-related metabolic imbalance in the initiation of neurodegenerative and neuropsychiatric disorders (Coleman et al., 2004; Cunha and Agostinho, 2010; Wishart et al., 2006) closely followed by a neuroinflammation process, previously implicated in MJD (Evert et al., 2001) mediating the spreading and amplification of damage until overt neuronal dysfunction and damage (Coleman et al., 2004; Gomes et al., 2011; Lobsiger and Cleveland, 2007). Notably, this study shows that the blockade of $A_{2A}R$, mainly targeted by chronic caffeine consumption, abrogated synaptotoxicity and prevented astrogliosis and microglia activation triggered by mutant ataxin-3 expression in the mouse striatum. This is in agreement with $A_{2A}R$ synaptic and non-neuronal localization (Gebicke-Haerter et al., 1996; Nishizaki et al., 2002; Rebola et al., 2005a) and the ability of $A_{2A}R$ blockade to yield robust neuroprotection in different animal models of brain degeneration (Cunha and Agostinho, 2010) through efficient normalization of synaptic function, thus preventing synaptotoxicity (Canas et al., 2009), and controlling astrogliosis (Yu et al., 2008) and microgliosis (Rebola et al., 2011).

Additionally, this thesis also shows a plethora of behavioral modifications induced by the expression of mutant ataxin-3 in cerebella, such as: i) gross motor disturbances, namely akinesia and bradikinesia (in a vertical pole and MWM); ii) fine tune body and limb movement disabilities (rotarod and beam walking); iii) muscle tone, balance and postural instability (grip strength and balance beam); and iv) cognitive impairments typified by motor learning difficulties (beam walking acquisition) and decreased object recognition; all of which resembling the human situation and directly or indirectly correlated with cerebella degeneration (Braga-Neto et al., 2012a; Braga-Neto et al., 2012b; D'Abreu et al., 2010; Kawai et al., 2004). These behavioral alterations also led us to propose that this animal model of MJD presented a broad dopaminergic dysfunction, which is in accordance with the presence of an intracerebellar dopaminergic innervation (Giompres and Delis, 2005), and in agreement with neuroimaging studies heralding a generalized impairment of the dopaminergic system in MJD patient's brains (Taniwaki et al., 1997; Wullner et al., 2005).

We demonstrate that chronic caffeine consumption by MJD transgenic animals afforded a remarkable normalization of such gross motor and cognitive impairments, which is consistent with the ability of caffeine to restore motor function (Ferre, 2008; Schiffmann et al., 2007) and memory deficits (Cunha and Agostinho, 2010; Takahashi et al., 2008a). Herein, we posed that those effects may result from 2 distinct synergistic mechanisms: i) normalization of the dopaminergic system (cerebral and cerebellar); and ii) cerebella rescue of progressive degeneration. The first hypothesis is in accordance with the ability of caffeine to restore a normal density and function of the dopamine transporter (DAT) as well as of dopamine uptake in the frontal cortex and in the striatum (Pandolfo et al., 2013) compatible to the antagonism of $A_{2A}R$, mainly targeted by caffeine (Fredholm et al., 2005). It is worth noting that the present thesis also provides the first *in vivo* demonstration of caffeine-mediated cerebella neuroprotection, which is in line with an episodic report showing the ability of caffeine and $A_{2A}R$ antagonists to confer neuronal protection against induced-toxicity in primary cultures of cerebellar granule neurons (Dall'Igna et al., 2003). This strongly supports a promising therapeutic approach to other spinocerebellar ataxias showing considerable cerebella loss, based on the consumption of caffeine.

It is worth noting that retrospective studies showed that caffeine consumption decreased incidence of neurodegenerative diseases in humans, such as Alzheimer's and Parkinson's diseases (Maia and de Mendonca, 2002; Ross et al., 2000). In turn, a recent retrospective study assessing caffeine consumption in HD patients showed greater caffeine consumption associated with an earlier age of onset (Simonin et al.,

2013). Those controversial actions of caffeine in different neurodegeneration patterns in humans together with the present findings are suggestive that it might be of greater interest to run retrospective and prospective studies also in MJD.

This thesis also shows a new and validated methodology allowing both *in vitro* and *in vivo* $A_{2A}R$ manipulations based on molecular tools designed to suppress and up-regulate the $A_{2A}R$. These tools might be effectively applied to several neurological disorders beyond Machado-Joseph disease, either to improve mechanistic comprehension or to attempt gene therapy approaches. For our study, as it remained to be elucidated whether neuroprotection mediated by caffeine was operated directly through pre- or post-synaptic sites or even glial $A_{2A}R$ antagonism, we took advantage of lentiviral vectors to *in vivo* site-specific manipulate $A_{2A}R$ and dissect cell-type specific and subcellular compartments where $A_{2A}R$ critically modulated MJD and mediated neuroprotection through its antagonism. We first targeted the post-synaptic site by introducing LVs expressing $A_{2A}R$ silencing constructs and $A_{2A}R$ mouse gene into striatum, and we have demonstrated that striatal post-synaptic $A_{2A}R$ are not relevant to caffeine-mediated MJD neuroprotection although, in turn, $A_{2A}R$ up-regulation was able to exert a beneficial effect in the early progression of the disease, which proved relevant reducing the late neuronal damage and degeneration triggered by mutant ataxin-3. These observations demonstrated that post-synaptic $A_{2A}R$ may actually have a neuroprotective role in the initial stages of the disease prompting to consider that the striatal outcome of mutant ataxin-3 expression depends on the balance between the deleterious activity of pre-synaptic and glial $A_{2A}R$ and the protective activity of post-synaptic $A_{2A}R$. This effect was previously reported in another polyglutamine disorder, Huntington's disease (HD) (Blum et al., 2003) adding special concerns when considering the blockade of $A_{2A}R$ to treat striatal neurodegeneration due to its putative neuroprotective-neurotoxic effects depending on its ability to modulate pre-synaptic over postsynaptic receptor activity.

Nevertheless, additional studies are needed to directly dissect the role of pre-synaptic and glial $A_{2A}R$ relative contributions for MJD progression and hence caffeine-mediated neuroprotection. One possibility to address the pre-synaptic $A_{2A}R$ control of MJD is the intra-striatal delivery of mutant ataxin-3 gene concomitantly with $A_{2A}R$ silencing constructs mediated again by viral vectors, now allowing axonal retrograde transport and thus elimination of $A_{2A}R$ from glutamatergic afferents. This can be achieved by using: i) lentivirus either pseudotyped with ravies virus glycoprotein (RV-G) (Mazarakis et al., 2001), or RV-G/ VSV-G chimeric envelope (Carpentier et al., 2012) or a fusion envelope glycoprotein (FuG-B) (Kato et al., 2011); and ii) adeno-associated

viral vectors of specific serotypes such as type 1 or 5 (AAV) (Lazarus et al., 2011). Another possibility is taking advantage of forebrain $A_{2A}R$ KO mice with $A_{2A}R$ deletion in the neurons of striatum as well as cerebral cortex and hippocampus (Shen et al., 2008) and perform either: i) intra-striatal delivery of lentivirus encoding mutant ataxin-3; or ii) crossbreeding with MJD transgenic mice. On the other hand, striatal transduction of GFAP gene promoter-driven $A_{2A}R$ conditional knockout mice (GFAP-$A_{2A}R$-KO) (Xu et al., in preparation) with lentivirus encoding mutant ataxin-3 would be of particular interest to dissect the putative role of $A_{2A}R$ in the glial compartment. In conclusion, the outcome of all those manipulations in morphological and behavioral modifications triggered by mutant ataxin-3 will certainly clarify the relative contribution of distinctly located $A_{2A}R$ providing sufficient detailed information to allow designing effective gene therapy interventions to handle with MJD, based on $A_{2A}R$ suppression or up-regulation.

Finally, albeit the molecular mechanism of $A_{2A}R$-mediated control of MJD remains to be determined, the present thesis provides new clues for particular compartments where such mechanisms should be explored. It also provides the first suggestion that $A_{2A}R$ might be a novel therapeutic target to interfere with MJD progression and that neuroprotective strategies based on $A_{2A}R$ antagonism are ideal candidates to manage MJD.

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