Sara Carina Duarte da Silva **Insights into pathology and neurodegeneration features in a transgenic mouse model of Machado-Joseph disease**

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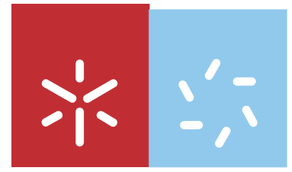

Universidade do Minho

Escola de Ciências

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Março de 2011

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TESE DE MESTRADO EM: **Genética Molecular**

E AUTORIZADA A REPRODUÇAO INTEGRAL DESTA TESE APEN AS PARA EFEITOS DE INVESTIGAÇAO, MEDIANTE DECLARAÇAO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE;

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Assinatura:

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Abstract

Machado-Joseph disease (MJD), also known as Spinocerebellar Ataxia 3 (SCA3), is the most common autosomal dominant ataxia worldwide, and is caused by a CAG repeat expansion within the coding region of the ATXN3 gene. The clinical variability of the disease phenotype as well as the age of onset depend on the length of the expanded repeat. The anticipation phenomenon is most frequently associated with repeat expansions in paternal transmission. MJD patients with a repeat expansion above 44 CAGs in the *ATXN3* gene present cytoplasmic and/or intranuclear ataxin-3 aggregates and neuronal cell loss in specific areas of the brain. However, some questions remains unanswered in this disease: why only some subpopulations of neurons are affected, although ataxin-3 is everywhere; what underlies this selective neuronal vulnerability; are these neurons dysfunctional or dying?

In an attempt to address these issues, we took advantage of studying a cDNA transgenic mouse model (CMVMJD) expressing the mutant human ataxin-3 under the regulation of the CMV promoter (pCMV), previously generated in our lab. This transgenic mouse model shows an important overlap with genetic and clinical features of MJD, namely genetic instability of the expanded CAG repeat and a motor impairment phenotype.

In this work, we performed an extensive pathological analysis of MJD mouse brains, that revealed a significant atrophy in the thalamus and in the dentate neurons. Increased GFAP immunostaining with reactive astrocytes was observed in the vestibular nuclei and substantia nigra of transgenic mice. Regarding cell death, we have searched for evidence of different cell death types (apoptosis and necrosis) by TUNEL assay, caspase-3 analysis and Fluoro-Jade B staining. We did not find any differences between wild-type and MJD transgenic mice, suggesting that probably the affected neurons are not dying, at least by apoptosis or necrosis, instead, they might just be dysfunctional. We also analysed of the somatic mosaicism in neuronal and non-neuronal tissues through aging revealed a significant increase in the mosaicism index of specific brain regions such as the pons, substantia nigra, cerebellar cortex, hipocampus, striatum, deep cerebellar nuclei and hypothalamus with age. However, there was no correlation between the extent of the mosaicism and the pathological involvement of a given region.

The results allow us to conclude that the pCMVMJD94 mouse is a good model to study the pathogenic mechanisms of MJD, mimicking an early stage of the disease.

Resumo

A doença de Machado-Joseph, também conhecida por Ataxia Espinocerebelosa tipo 3 (SCA3), é a ataxia autossómica dominante mais comum em todo o mundo, causada por uma repetição de CAGs na região codificante do gene ATXN3. A variabilidade no fenótipo da doença assim como a idade de surgimento dos sintomas depende no tamanho da repetição expandida. O fenómeno de antecipação está mais frequentemente associado a transmissões paternas. Pacientes com DMJ com expansões acima de 44 CAGs revelam agregados citoplasmáticos e /ou nucleares e perda neuronal em áreas do cérebro específicas. Algumas questões chave permanecem por esclarecer. Por exemplo, nesta doença apenas algumas subpopulações de neurónios são afectadas, embora a ataxina-3 seja expressa em todas as áreas. O que está por detrás desta vulnerabilidade neuronal selectiva? Estarão estes neurónios a morrer ou estarão disfuncionais? Numa tentativa de responder a estas perguntas, utilizámos um modelo em ratinho que expressa o cDNA da ataxina-3 humana mutada, sob a regulação do promotor CMV (pCMV), previamente gerado no nosso laboratório. Neste trabalho, fizemos uma extensa análise patológica de cérebros de ratinho DMJ que revelaram uma atrofia relevante no tálamo e núcleos denteados. Também se observou um aumento da proteína GFAP, revelando um aumento da reactividade dos astrócitos, nos núcleos vestibular e substantia nigra dos ratinhos transgénicos. Também fizemos um rastreio de diferentes tipos de morte celular (apoptose e necrose) por TUNEL, análise da activação da caspase-3 (imunohistoquímica e western-blot) e coloração com Fluoro-Jade B. Não encontrámos diferenças significativas entre ratinhos do tipo selvagem e transgénicos, sugerindo que provavelmente estes neurónios não estarão a morrer, pelo menos por apoptose ou necrose, estando possivelmente disfuncionais. Para além disso, a repetição de CAG expandida, variou em mais de 50% das transmissões nos ratinhos transgénicos, com expansões típica transmitidas em meioses paternas e contracções em maternas. A análise do mosaicismo somático em tecidos neuronais e periféricos, durante o envelhecimento, revelou um aumento no índex de mosaicismo em regiões específicas do cérebro e mostrou ser dependente da idade. Este ratinho transgénico apresenta características genéticas e clínicas importantes que se sobrepõem às da DMJ, nomeadamente instabilidade intergeracional da expansão de CAGs, características patológicas da doença (astrogliose e neurónios atrofiados, nas regiões relevantes) e um fenótipo de descoordenação motora.

Estes resultados permitiram-nos concluir que o ratinho pCMVMJD94 é um bom modelo para estudar os mecanismos patogénicos da DMJ, mimetizando estadios precoces da doença.

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List of Abbreviations

μL – Microliter

μm – Micrometer

AD – Alzheimer's Disease

AH – Anterior Horn

ALS – Amyotrophic Lateral Sclerosis

AR – Androgen receptor

AT3 – Ataxin-3 protein

AT3Q(n) – Ataxin-3 protein containing n glutamine residues

ATXN3 – Ataxin-3 gene

ATP – Adenosine triphosphate

AIF – Apoptosis Inducing Factor

Apaf-1 – Apoptotic peptidase activating factor

AMPA – Agonist of AMPA receptor

a.a. –Aminoacid

bp – Base pairs

C. elegans – Caenorhabditis elegans

C/P – Caudate/Putamen

CAG – Trinucleotide codon for glutamine

CBP – CREB binding protein

cDNA – complementary DNA

cys – Cysteine

cyt c – Cytochrome c

CREB – Cyclic AMP-response Element Binding protein

CNS – Central Nervous System

CMVp – Cytomegalovirus promoter

DN – Dentate Nucleus

DNA – Deoxyribonucleic acid

DRPLA – Dentatorubral-Pallidoluysian Atrophy

DAB – 3, 34-biiaminobenzidine

DM – Myotonic Dystrophy

ECL- Enhanced GChemiLuminescenc

ER – Endoplasmic reticulum

E.coli – Escherichia coli

FADD – Cytosolic adaptors proteins

GABA – Gamma-Amino Butyric Acid

GFAP – Glial Fribillary Acidic Protein

HD – Huntington's Disease

HDAC – Histone Deacetylase

Hdj-1 – 40-kDa heat-shock protein

Hdj2 – Constitutive form of 40-kDa heat-shock protein

H&E – Hematoxylin&Eosin

Hprt – Hypoxanthine Phosphoribosyl Transferase

Hsp – Heat shock protein

HAT – Histone Acetylase

Htt – Huntingtin Protein

H3 – Histone 3

H4 – Histone 4

IHC – Immunohistochemistry

ICE – Interleukin-1b processing enzyme

kDa – KiloDalton(s)

kb – kilobase

Lat – Dentate nucleus

LV – Lentivirus

LANP – Cerebellar leucine rich acidic nuclear protein

MJD – Machado-Joseph Disease

mL – Milliliter

mM – Milimolar

MMR – Mismatch Repair

MSH – Mismatch repair gene

MutSb – MSH2/MSH3 comlex

mRNA – Messenger RNA

mtDNA – mitochondrial DNA

MT – Mitochondria

MI – Mosaicism Index

MOMP – Mitochondrial Outer Membrane Permeabilization

n – Number of samples in the study

NEDD8 – Developmental down-regulated gene 8

NMDA – N-methyl-D-aspartic acid

NAIP – Neuronal Apoptosis Inhibitory Protein

ng – Nanogram

NI – Neuronal Inclusion

NLS – Nuclear Localization Signal

NES – Nuclear Export Signal

Nm – Namometer

OH8dG – 8 hydroxydeoxyguanosine

pCAF – Protein-associated factor

PCR – Polymerase Chain Reaction

PFA – Paraformaldehyde

PD – Parkinson's Disease

Pn – Pontine Nucleus

PCD – Programmed Cell Death

polyQ – Polyglutamine

Q(n) – stretch of n glutamine residues

Q – Glutamine

RNA – Ribonucleic acid

RNAPolII – RNA polymerase II

ROS – Reactive Oxygen Species

SCA – Spinocerebellar Ataxia

SMBA – Spinal and Bulbar Muscular Atrophy

SMA – Spinal Muscular Atrophy

SAHA – Suberoylanilide Hydroxamic Acid

SH3L3 – SH3 – containing Grb2-like protein

SOD – Superoxide dismutase

SN – Substantia Nigra

SP1 – Specificity Protein 1

SRC1 – Steroid Receptor Coactivator1

TAFII130 – TBP-associated factor

TBP – TATA box binding protein

t-test – Student's t test

TH – Tyrosine Hydroxilase

Th – Thalamus

TUNEL – Terminal deoxynucleotidyl transferase dUTP nick end labeling

TC-NER – Nucleotide excision repair

UIM – Ubiquitin-interacting motifs

UPS – Ubiquitin-proteasome system

Ve – Vestibular nuclei

VMAT2 – VVesicular monoamine transporter 2

VCP – Valosin-containing protein

Wt – Wild-type

YAC – Yeast Artificial Chromosome

1 General introduction

Chapter 1. General Introduction

1.1 Polyglutamine disorders: an overview

Expansions of repeating units of DNA, especially CAG triplet repeat expansions, are known to underlie several neurodegenerative disorders [6], including Machado-Joseph disease (MJD), or spinocerebellar ataxia 3 (SCA3) [4, 7], Huntington disease (HD) [8], spinal and bulbar muscular atrophy (SBMA)[9], dentatorubropallidoluysian atrophy (DRPLA) [10-11], and other spinocerebellar ataxias, such as SCA 1, 2, 6, 7, and 17. The clinical manifestations of each disease result from brain pathology involving a specific subset of neurons that is, for the most part, particular to each disease. A common feature among all these disorders is a progressive neuronal dysfunction/death beginning at mid-life [4].

The progressive neurodegeneration in adulthood and some common symptoms in these apparently unrelated disorders have suggested a shared mechanism for their pathogenesis. The major pathogenic mechanism of these diseases is believed to arise from a genetic gain of function related to abnormal conformation of the elongated polyQ tracts. The CAG expansion leads to an abnormally long polyglutamine (polyQ) tract within all the proteins involved in the mentioned diseases. Besides this polyQ tract, the proteins share no homology and no functional similarity, which suggests that the polyQ stretch itself confers toxic properties to these proteins through a "toxic gain of function" [5]. In agreement with this hypothesis the expression of a simple polyQ tract, in the absence of any additional protein context has been shown to be toxic, as shown in cell culture, mouse, *Drosophila* and *C. elegans* [6-10]. This toxic effect of the polyQ tract was further reinforced by a study describing the effect of the insertion of a CAG tract in the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene, encoding a metabolic enzyme in mice. These mice developed a progressive late-onset neurological phenotype, including ataxia, seizures and premature death [12].

Interestingly, and despite the ubiquitous expression pattern of polyQ mutated proteins, only some subsets of neurons are affected in each polyQ disorder (Table1), indicating that there are other factors influencing the pathogenesis [4, 21]. Apart from the polyQ tract, the disease associated proteins are unrelated to each other, sharing no homology [3, 22-23]. All these proteins are widely

expressed in the Central Nervous System (CNS) as well as in peripheral tissues, however each polyQ protein originates a different neurodegeneration profile [4].

Another interesting fact is the overlap of phenotypes of these disorders when the expansions are large: for example, juvenile-onset Huntington patients develop dystonia and seizures in addition to the classical phenotype of chorea, dementia, lack of coordination and unsteady gait seen in adult patients.

The CAG repeat tracts are normally highly polymorphic and above a certain threshold length the symptoms of the diseases are manifested (Table 1) [1, 4-5]. The disease threshold length (and the dominant toxic property) is around 35 to 40 glutamine repetitions. Above the pathogenic threshold the CAG tracts are unstable across generations, and it may expand or contract depending on the gender of the transmitter. While maternal meioses tend to lead to contractions, paternal transmissions tend to lead to expansions. This intergenerational instability leads to a phenomenon called "anticipation", where the disease symptoms appear earlier and are more severe [24-25]. At a molecular level, all polyQ diseases display proteinaceous aggregates in neurons, usually termed neuronal inclusions (NIs), mainly located in the nucleus [11]. These inclusions are a common hallmark of all these diseases and it is still controversial if they are pathogenic or not**.**

Table 1. Molecular and pathogenic features of polyglutamine diseases. Adapted from [1] [3] [4]

Disease	Gene	Locus	Protein function	Protein localization	Normal CAG	Expanded CAG	Affected brain regions
HD	huntingtin	4p16.3	unknown	Cytoplasmic	6 to 34	36 to 121	Striatum, cerebral cortex
DRPLA	atrophin-1	12q	transcription regulator	Cytoplasmic	6 to 36	49 to 84	Cerebellum, cerebral cortex,basal ganglia, Luys body
SBMA	Androgen recepetor	Xq11-12	androgen receptor	Nuclear and cytoplamastic	9 to 36	38 to 62	Anterior horn and bulbar neurons; dorsal route ganglia
SCA1	ATX1	6p22-23	synaptic plasticity	Nuclear in neurons	6 to 44	39 to 82	Cerebellar Purkinje cells,dentate nucleus; brainstem
SCA2	ATX2	12q23-24	unknown	Cytoplasmatic	15 to 31	36 to 63	Cerebellar Purkinje cells, brainstem; fronto-temporal lobes
SCA3/MJD	ATXN3	14q24.3-31	ubiquitin hidrolase	Cytoplasmatic and nuclear	12 to 41	54-86	Cerebellar dentate neurons, basal ganglia, brainstem, spinal cord
SCA6	CACNA1A	19p13	a1A- voltage-dependent-calcium channel subunit	Cell membrane	4 to 18	21 to 33	Cerebellar Purkinje cells, dentate nucleus, inferior olive
SCA7	SCA7	3p12-p21.1	unknown	Nuclear	4 to 35	37 to 306	Cerebellum, brain stem, macula, visual cortex
SCA17	TBP	6q27	transcription factor	Nuclear	29 to 42	47 to 63	Purkinje cell layer; striatum

1.2. Instability of the CAG repeat

Expanded repeat mutations do not behave strictly according to rules of Mendelian inheritance. They are unstable mutations that change in size along successive generations. In contrast, normal-sized repeats are usually transmitted stably [26][12]. This instability of the mutant alleles might explain some characteristics of these diseases such as variable phenotype [27], anticipation and parental-origin effects. The mechanisms that underlie this instability are not very well known, but several factors are known to influence it: (i) type of repeated sequence, (ii) gender of the transmitting parent [28], (iii) the original length of the repetition tract [29], and (iv) the presence/absence of interruptions in the repetitive sequence [24, 30-31]. In this type of disorders, severe juvenile-onset cases are usually paternally transmitted, due to the greater repeat instability with paternal transmission [32, 33]. For example, analysis of the repeat length in sperm from HD patients suggests that this tendency for transmitting expanded repeats is due to repeat instability during spermatogenesis [34]. Also, in MJD families the sex of the transmitting parent has a significant effect on intergenerational instability, male meioses being associated with larger variations, both contractions and expansions, of the repeat size [24, 30-31, 35].

However, repeat instability is not confined to the germline, occurring also in characteristic patterns in many somatic tissues of affected individuals [36]. The ongoing repeat instability in critical somatic tissues likely accelerates disease progression. Somatic mosaicism of the repeat sizes in triplet repeat disorders shows that different cells of the same individual carry different repeat sizes. This phenomenon has never been described for the normal allele, but it has been associated to the expanded allele for several polyQ disorders such as MJD [31] and SCA1 [37] as well as HD [38], DRPLA [39] and SMBA [40].

Tissue-specific CAG instability and severity of neuropathological features were proposed to be directly correlated in HD [38] but this was not confirmed for other diseases with CAG repeat expansions [31, 37, 39-40].

The existence of a similar threshold size for all trinucleotide repeat diseases may suggest a common mechanism for expansion. Slipped-strand mispairing during replication [41-42] has been proposed as a mechanism of mutation that could account for several features of the trinucleotide expansion. During replication of a threshold length repeat (~35 pure repeats), slippage

can occur resulting in moderate expansion. Replication of larger repeats leads to hyperexpansion. DNA mismatch repair mechanisms likely play a role in correcting errors caused by slippage, since defects in mismatch repair lead to an increased of simple sequence repeat instability in both yeast and humans (for review, see[43]).

There is no straightforward correlation between the degree of CAG instability and cell division rates (DNA replication), DNA damage (DNA repair and recombination), or disease-gene transcript levels (transcription) [44].

The age-dependent repeat instability in somatic tissues, especially in terminally differentiated neurons, strongly suggests a relevant role for pathways that are independent of DNA replication. Transcription-induced repeat instability (Figure 1) can be modulated by several DNA repair proteins, including those involved in mismatch repair (MMR) and transcription coupled nucleotide excision repair (TC-NER). Transcription was also suggested as a possible cause for repeat instability, based on observations in four lines of transgenic mice that carried a portion of the huntingtin gene containing 55 CAG repeats [45]. In three of those lines, the transgene was expressed and was unstable, whereas in the fourth the transgene was silent and the repeat was stable.

Very recent investigations have shown that genetic recombination is a powerful mechanism for generating massive expansions of trinucleotide repeat sequences. Several human genetic studies on patient materials reported haplotype analyses, especially related to myotonic dystrophy (DM) and the fragile X syndrome, which implicated gene conversion and/or unequal crossing-over (types of recombination) in genetic instabilities [46]. Studies in *E.coli* showed that the expansion of triplet repeats in vivo can occur by homologous recombination as shown by biochemical and genetic studies [47]. Gene conversion is an event in DNA genetic recombination, which occurs at high frequencies during meiotic division but which also in somatic cells. It is a process by which DNA sequence information is transferred from one DNA helix (which remains unchanged) to another DNA helix, whose sequence is altered. This conversion was suggested as the mechanism responsible for trinucleotide repeat instability during repair in yeast [48]. Recombination mechanisms were also described in mouse models and clinical cases [46, 49].

CAG repeat instability is behind some features of polyQ disorders and may also contribute to their pathogenic mechanism.

1.3 Polyglutamine pathogenic mechanisms

Several mechanisms have been implicated in polyglutamine toxicity, most of which are not mutually exclusive [50]. Here we will focus on three hypotheses: (i) disruption of proteostasis; (ii) transcriptional deregulation and (iii) mitochondrial impairment/oxidative stress.

1.3.1 Misfolded proteins and aggregation: disruption of proteostasis?

The presence of the expanded polyQ-stretch seems to confer novel properties to the mutant proteins, namely a tendency towards increased aggregation. Although protein aggregation is thought to be a central aspect of the biology of many neurodegenerative diseases, the role of two different types of aggregates in neurodegeneration is continuing to be elucidated. In polyQ disorders it is also generally accepted that aggregates or inclusion bodies are an important hallmark of polyglutamine diseases, and these aggregates are indeed found in patients' brains, but the question remains: are the visible aggregates toxic or protective?

Intranuclear inclusions were initially discovered in the first animal model of HD [16]. Interestingly, a correlation was observed between the length of the CAG repeat and the number of inclusions observed in the disease brains [51-53]. Other evidences suggested that aggregates could be protective to the cells, by stacking the toxic mutant proteins [54]. Indeed, protein aggregates are also naturally occurring species. An example are Marinesco bodies, which are eosinophilic ubiquitinated intranuclear inclusions found in pigmented neurons of the human substantia nigra and locus coeruleus as a result of the metabolism of normal aging neurons [55].

One way in which protein aggregates may harm neurons is by affecting their proteostasis. The hypothesis of cellular homeostasis disturbance suggests that the sequestration of chaperones and proteasome subunits into polyQ aggregates could result in an increased in general protein misfolding and reduced clearance of other crucial cellular proteins, leading to a proteostasis decline [56-58].

Several proteins are known to become trapped into these nuclear and/or cytoplasmic aggregates, such as ubiquitin, ubiquitin-like proteins, proteasome subunits, molecular chaperones and other polyQ-containing proteins [56, 58-60]. This suggests the involvement of the ubiquitin-proteasome degradation pathway in these pathologies, and also the activation of the heat

shock response machinery in order to either refold or degrade the mutant polyQ proteins. The mislocalization of chaperones and proteasome subunits may contribute to the progress of the disease. Molecular chaperones were shown to associate with the aggregates only transiently and to move freely [61]. However, the proteasome appears to be permanently recruited into polyQ aggregates and to be functionally impaired [62], which can interfere with the degradation of other proteins [63]. Proteasome inhibition might also increase the intracellular load of misfolded, oxidized, or otherwise damaged proteins, thereby causing neuronal toxicity. The consistent presence of normal ataxin-3 in NIs could reflect a biological feature of wild-type ataxin-3, which is translocated into the nucleus under pathological conditions and participates in the formation of aggregates [64].

1.3.2 Transcriptional deregulation

Transcriptional alteration is another unifying feature of polyQ disorders [36, 65-69]; however, the relationship between polyQ-induced gene expression deregulation and the ongoing degenerative processes remains unclear.

More than 20 nuclear proteins relevant to transcription are known to interact with polyglutamine disease associated-proteins [65]. Mutant polyQ proteins have been shown to interact abnormally with proteins involved in the transcription machinery, namely the CREB-binding protein (CBP), p300/CREBBP associated factor (PCAF), TATA-binding protein (TBP), TAFII130, and SP1 [65, 69]. Overexpression of some of these transcription regulators was shown to overcome polyQ toxicity, both in vitro in cellular models for MJD, SBMA, and HD [66, 70] as well as in vivo in a polyQ model in Drosophila [67]. This suggests an important role for transcription deregulation in polyQ pathogenesis.

Proteins that interact with polyglutamine disease associated-proteins are distributed around the core transcription machinery, which is now known to exert DNA methylation, histone acetylation and RNA modification simultaneously (see review [71]). CBP has attracted attention because CBP is a representative coactivator that possesses HAT (histone acetyltransferase) activity and interacts with numerous transcription factors, and its abnormal binding to disease proteins should affect the expression of a wide range of genes rather than that of a specific gene. Several polyQ-protein interactors have acetyltransferase activity. Acetylation of histones relaxes the DNA

structure promoting transcription, whereas hypoacetylation represses gene activity [72]. The equilibrium of histone acetylation/deacetylation is controlled by histone acetyltransferases and deacetyltransferases (HDACs). Thus, polyQ proteins may be toxic by their direct inhibition of the acetyltransferase activity of transcription regulators, leading to diminished gene expression. In fact, treatment of *Drosophila* and mouse models of HD with HDAC inhibitors has been shown to ameliorate the disease phenotype and to decrease cell degeneration, with the increase of histone acetylation and consequent transcription activation [73-75].

1.3.3 Mitochondrial impairment/oxidative stress

The mitochondria has numerous important functions in the cell, however the mitochondrial respiratory chain is also one of the major sources of damaging free radicals in human organism, and these free radicals destroy cellular macromolecules, including DNA, lipids and proteins [79]. Mitochondrial dysfunction causes a decrease in ATP production, oxidative damage and induction of apoptosis, all of which are involved in the pathogenesis of several disorders [76-77]. They all share the common features of disturbances in the buffering capacity of mitochondrial Ca^{2+}, ATP or reactive oxygen species (ROS) metabolism [78].

It has been proposed that mitochondrial impairment may be a major triggering factor of neurodegenerative diseases [80-81]. It is not clear what is the primary initiating event in the pathogenesis of each of these neurodegenerative disorders, but it seems that oxidative damage could constitute a critical factor in the propagation of injuries of the different cellular systems affected in most of them [82].

A perturbation in the mitochondrial function leads to an ionic imbalance, calcium overload and ultimately, ATP depletion (Figure 1.1). If the energy supply of the cell drops dramatically, necrotic cell death will ensue. A mild or gradual energy disturbance may also lead to the release of proapoptotic factors, particularly cytochrome c from the mitochondria, and an apoptotic cascade is initiated as well. Excessive Ca^{2+} accumulation has deleterious effects, leading to the oxidative damage of different molecules with the resulting triggering of an apoptotic cascade. In addition, it should not be forgotten that non-excitable cells, such as astrocytes and microglia, are also strongly dependent on the intracellular Ca^{2+} concentration and Ca^{2+} signaling to maintain their normal function.

The cellular processes occurring during normal ageing are to some extent similar to those involved in the pathomechanism of neurodegenerative disorders, also leading to age-dependent impairment of mitochondrial function, for instance the appearance of mutations in mitochondrial DNA. A main difference may be that the metabolic dysfunction in the "normal" ageing brain is distributed in a random fashion, while in neurodegenerative disorders, specific causes and cellular disturbances are superimposed upon the age-dependent decrease of the homeostatic reserve, and these together attack specific structures of the CNS [83].

1.4 Neuropathology and neuron-specific toxicity in polyglutamine disorders

The cause of neuron-specific dysfunction is a major unanswered question in the PolyQ field and, as such, is an active area of research.

Several studies demonstrated that this neuronal-specificity is not due to the abundance of polyQ proteins since they are ubiquitously expressed [3-4] (Figure 1.1) and must not be related to expression levels of those proteins before or after disease onset [84]. Similar expression levels of wild-type and mutant proteins were also shown in animal models and patients [85]. Another hypothesis put forward to explain this selective neuron dysfunction was the heterogeneity of polyQ length due to somatic mosaicism, resulting in loss of neurons with higher CAG repeats and survival of those neurons with less CAG-repeat length. This phenomenon was described for some of these diseases, but upon a more detailed analysis, no correlation with selective brain pathology was found [31, 37, 85]. Previous work from our lab also demonstrated, by screening the brain pathology and determining the CAG-repeat length in several brain areas (affected and spared) that there was no correlation between somatic mosaicism pattern and pathology in MJD. However, this finding could be a result of the technique used, since a gross dissection of the brain areas was performed. Also, in MJD patients, this kind of studies might be impaired by the use of post-mortem tissue in which surviving cells are those analysed. A third possibility is that in susceptible neurons, misfolding and aggregation could be prompted by cell-specific proteolytic events that release a polyQ-containing fragment [86] or by aberrant targeting of polyQ proteins to the nucleus only in certain cells [87]. Finally, specific interacting proteins are likely to contribute to selective vulnerability. For instance, certain interacting proteins may bind to disease associated-proteins in a way that promotes misfolding and aggregation, as was demonstrated for the huntingtin interacting proteins, SH3-

containing Grb2-like protein (SH3GL3) [88] and Rhes protein [89]. Other specific interacting proteins are likely to influence events downstream of misfolding, through mechanisms that are linked to the specific normal functions of the disease proteins. Thus, in each disease, a subset of specific interacting proteins may bind less or more avidly to the mutant protein, thereby altering physiological or biochemical properties of one or both proteins. The susceptibility of a neuron to the downstream effects of the mutant protein would depend, in part, on the particular interacting proteins it expresses. The ataxin-1 interacting protein LANP (cerebellar leucine-rich acidic nuclear protein) is just one of a number of identified interacting proteins that may contribute in this manner to selective vulnerability [90].

Extensive efforts are ongoing to understand how polyQ tracts may mediate neuron-specific degeneration. If the toxic gain-of-function conferred by extremely large polyQ expansions results in non-specific neurodegeneration, pathogenesis caused by more moderate polyQ expansions may be susceptible to modification. Protein context is one factor that modulates polyQ pathogenesis and by doing so, it may contribute to the specific pathology observed in each CAG-repeat disease.

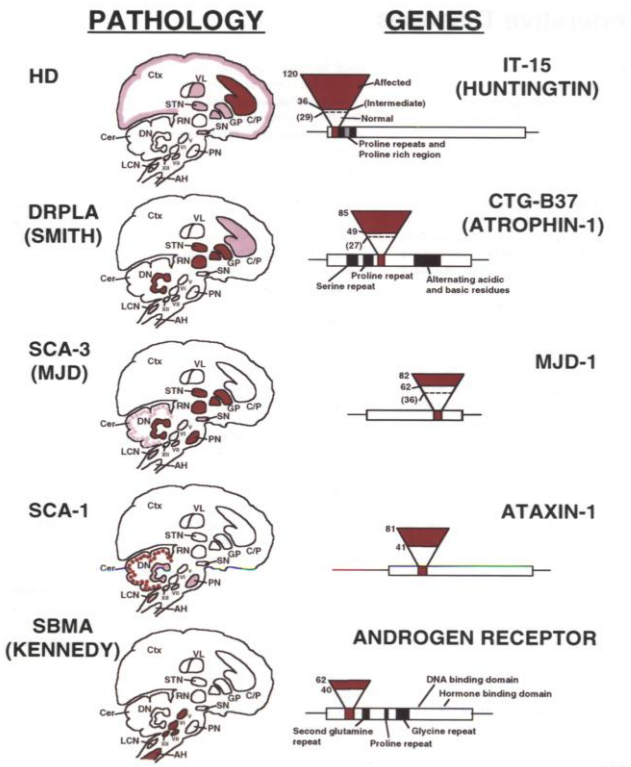

Figure 1.1. CAG-repeat neurodegenerative diseases display unique neuropathology despite a common toxic motif. This schematic diagram identifies the major sites of neuronal loss in each disease. Dark red indicates severe or selective neuronal loss; half-tone red indicates moderate or variable cell loss. The circles in the cerebellar cortex represent Purkinje cells. AH, anterior horn; Cer, cerebellar cortex; C/P, caudate/putamen; Ctx, cerebral cortex; DN, dentate nucleus; GP, globus pallidus; LCN, lateral cuneate nucleus; PN, pontine nucleus; RN, red nucleus; SN, substantia nigra; STN, subthalamic nucleus;VL, ventrolateral thalamic nucleus; V, VI, VII, and XII, cranial motor nuclei. Figure adapted from [3].

1.5 Cell death mechanisms

The relevance of cell death in neurodegenerative disorders is still a controversial topic, mainly because synaptic loss and electrophysiological abnormalities typically precede cell loss in these diseases. In addition, in chronic neurodegenerative diseases, cell death occurs over decades whereas the single cell suicide program is known to be executed within a few hours and the dead cells rapidly disappear [91-92].

Much of the attention in the cell death field has been drawn in recent years to a major cell death pathway apoptosis, which is often used as a synonymous of programmed cell death. The elucidation of additional programmed cell death pathways is gradually changing this notion. According to a recent classification, eight different types of cell death were delineated [93] and some researchers describe as many as 11 pathways of cell death in mammals, 10 of which appear to be programmed [94]. These pathways can be broadly divided into two main groups: apoptotic and non-apoptotic (see review [13]).

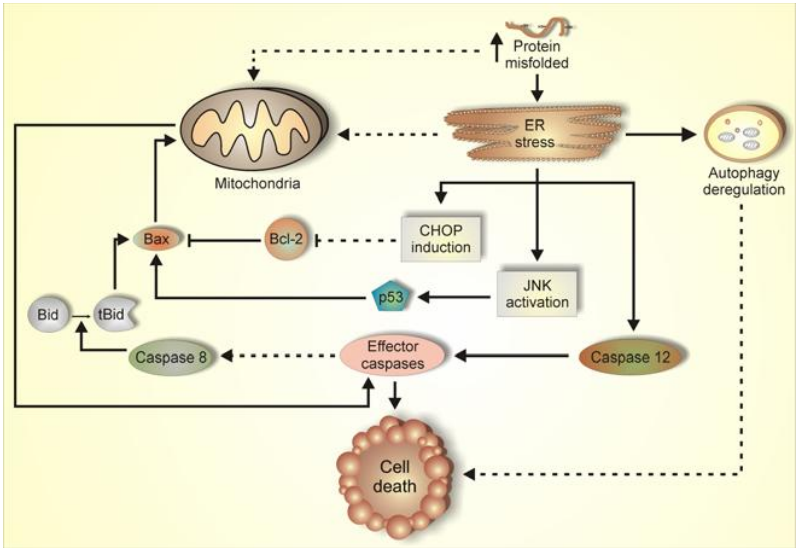

Figure 1.3. Molecular pathways of programmed cell death (PCD) operating in misfolding/aggregation diseases.

Apoptosis vs Necrosis

Apoptosis, also referred to as type I programmed cell death, is the most well defined type of cell death pathways, both morphologically and biochemically, occurring by a caspase cascade. It is characterized by membrane blebbing, cytoplasmic shrinkage and reduction of cellular volume (pyknosis), as well as condensation of the chromatin, and fragmentation of the nucleus (karyorrhexis), all of which ultimately lead to the formation of apoptotic bodies, a prominent morphological feature of apoptotic cell death [93]. As apoptosis typically does not induce inflammation or tissue scarring, it is well suited for a role in normal cell turnover during embryogenesis and in adult tissues [95]. Caspases are a family of proteins that are one of the main effectors of apoptosis. Their activation is a hallmark of apoptosis. Based on their function, the caspases can be classified into three groups: inflammatory (caspases 1, 5, 11, 12, 13, 14, involved in inflammation), initiator caspases (2 and 8 which contain a death effector receptor and 2 and 9 that possesses recruitment and activation domain) and finally effector caspases (3, 6, and 7, which are the executers) [96-97]. Recent estimates suggest that apoptosis deregulation contributes to about half of all the major medical illnesses for which adequate therapy or prevention is currently lacking [98].

The type of cell death in neurodegenerative disorders remains a matter of controversy, and it is possible that both apoptotic and nonapoptotic cell death coexist in the brains of affected patients [91]. Knock-out mice models have elucidated the researchers about the cell death pathways occurring in mammals and have revealed a prominent neuronal apoptosis defect in mice defective for caspase-3, caspase-9 and Apaf-1, suggesting that this pathway of caspase activation is important in regulating neuronal cell death in the developing brain [99].

Alzheimer's, Huntington's, Parkinson's and Prion diseases, as well as ALS, all have features of misfolded proteins. These disturbances can cause accumulation of unfolded proteins in the ER, triggering an evolutionarily conserved response termed the unfolded protein response (UPR) [100-101]. It has been proposed that these protein aggregates exhaust cellular proteasome activity in a failed attempt to degrade them, resulting in a secondary accumulation of misfolded proteins in the ER, thereby triggering ER stress (see review [102]. The UPR controls the levels of molecular chaperones and enzymes involved in protein folding in the ER. ATF6 located at the ER membrane is one of the candidates for the UPR-specific transcription factor [14]. Grp78/Bip (Bip) protein is the chaperone that increases protein folding in the ER lumen [15]. Upon induction of ER stress, ATF6 is

processed and the processed cytoplasmic region translocates into the nucleus and activates transcription of the endogenous Bip gene [14]. When these stress signals are unable to rescue cells, the apoptotic pathway is activated. Caspase-12, which is located at the outer layer of the ER, is one of the apoptotic pathways of ER stress-mediated cell death [16-17].

Necrosis has traditionally been considered an unregulated, energy- independent form of cell death, and has been well-characterized in a wide range of pathologic states. Extensive failure of normal physiological pathways that are essential for maintaining cellular homeostasis, such as regulation of ion transport, energy production and pH balance can lead to necrosis. Necrosis is characterized morphologically by vacuolation of the cytoplasm, breakdown of the plasma membrane and induction of inflammation around the dying cell due to the release of cellular contents activating the resident phagocytes and attraction of leucocytes into the necrosis area [103][18]. A classic example of necrotic conditions is ischemia that leads to a drastic depletion of oxygen, glucose, and other trophic factors and evokes massive necrotic death of endothelial cells and nonproliferating cells of surrounding tissues. Necrosis can be induced by microbial infections, neuronal excitotoxins, or ROS [104].

Many human tumors carry mutations that inactivate apoptotic pathways. Inactivation of apoptosis allows tumor cells to proliferate beyond normal homeostatic control. Necrosis represents an alternative pathway for these cells to be eliminated. The inflammatory component of necrotic death has the potential advantage of stimulating an immune response that could increase the efficiency of tumor cell death [105]. The balance between apoptotic and necrotic cell death may be modulated to potentiate a patient's immune response to a tumor.

Adult neuronal death also occurs by necrosis, an unregulated cell death that is the direct result of external insults such as physical injury, energy depletion, toxic insults, hypoxia and/or ischemia. Necrosis of adult neurons is independent of caspases, and although the precise molecular mechanism underlying this type of cell death is unknown, it might be mediated by increases in intracellular calcium, which activates calpains and cathepsins (cytosolic calcium-activated cysteine proteases), leading to degradation of cytoplasmic proteins [106].

Another way to die: autophagic programmed cell death

Autophagy is an evolutionary conserved and genetically controlled turnover of cellular constituents that occurs in all eukaryotic cells [107]. Autophagy is defined morphologically by the appearance of numerous cytosolic autophagosomes, which are formed by the assembly and expansion of double layered, membrane bound structures of unknown origin around whole organelles and isolated proteins. The origins of autophagosomes are difficult to determine because they contain a mixture of markers from the ER, endosomes and lysosomes. The autophagosomes encapsulate cytosolic materials and subsequently dock and fuse with lysosomes or other vacuoles, resulting in the degradation of their contents [108].

Autophagic cell death is thought to represent an alternative pathway to cell death when apoptosis is impeded, but there are indications that autophagic cell death and apoptosis are not mutually exclusive death pathways, and can cross talk with each other [108]. Evidences for this purpose are (i) the fact that apoptosis regulating molecules such as Bcl 2, Bcl xL, Bax and Bak were implicated in the regulation of autophagy, both independently and through interaction with Beclin 1 [109-110] and (ii) that some of the endonucleases that take a part in apoptosis associated DNA fragmentation may originate in lysosomes [111].

Autophagy impairment has been reported in neurodegenerative diseases including Parkinson, Huntington, and Alzheimer diseases [112-114]. In these diseases, the pathological accumulation of autophagosomes/ autophagosome-like structures and abnormalities in the endosomal-lysosomal pathway were documented by electron microscopy (EM) in human postmortem brain tissue [115-119].

While the role of autophagy in neurodegenerative diseases is far from being understood, the available data indicate it plays an integral role in the cellular response to intracellular protein aggregation common to these diseases. The effect of autophagy in neurons during disease can be broadly divided into two classes: autophagosomal degradation is either impaired or excessively activated, leading to an apparent disruption of the intracellular organelle organization and accumulation of autophagosomes in neurons over long periods of time.

Autophagy plays a crucial role in maintaining neuronal homeostasis through clearance of defective organelles and unfolded/aggregating proteins.

1.6 Machado-Joseph Disease

Machado-Joseph disease (MJD), also known as Spinocerebellar Ataxia 3 (SCA3), is the most common autosomal dominant ataxia worldwide [24]. The disease was first described in families from the Azores Islands, being reported some years later in other countries and in families with no Portuguese ancestrality. This late-onset disorder was first described as an autosomal dominant ataxia in William Machado's family, and thus named Machado disease [120]. In the same year, another case was reported (in the Thomas family) with similar clinical symptoms, and this disease was entitled as "Nigro-spino-dentatal degeneration with nuclar ophthalmoplegia" [121]. Later on, in 1976 was described a "particular type of autosomal dominant hereditary ataxia" in the family of Antone Joseph, which was designated as Joseph disease [122].

Two years later, after an intensive study in Azorean families and regarding the common features of the three families described above, a new "autosomal dominant system degeneration in Portuguese families of the Azores Islands" was introduced [123]. In the 80's, this disease was named Machado-Joseph disease (MJD) and some clinical criteria for diagnosis were introduced [124]. The prevalence of this disease was from the beginning thought to be the highest among people of Portuguese/Azorean descent. For immigrants of Portuguese Azorean ancestry in New England, the prevalence was described to be around 1:4000. In Portugal the prevalence of Machado-Joseph disease is 3,1:100,000 (*P.Coutinho, Personal Communication*), with clusters in several mainland regions, reaching a value of 1:140, on the small Azorean island of Flores [125].

Later, however, researchers, based in DNA studies, have identified MJD cases in many ethnic backgrounds. Its relative frequency among the spinocerebellar ataxias is higher in Portugal (49%), China (49%), Brazil (44%), the Netherlands (44%), Japan (43%), and Germany (42%); its relative frequency is lower in France (33%), the United States (21%), and Australia (12%); and it is rare in the United Kingdom (5%), India (3%), and Italy (1%) [126-137].

1.6.1 Clinical definition

MJD patients suffer from a progressive neurodegenerative disorder appearing more frequently between the ages of 20 and 50 years, in which the intellect is preserved [123]. The

preservation of cognitive function is a key feature of MJD in its differential diagnosis among the vast group of spinocerebellar ataxias. MJD is characterized by motor uncoordination and weakness in the arms and legs, spasticity, gait ataxia, difficulty with speech and swallowing, altered eye movements, double vision, and frequent urination. Some patients have dystonia (sustained muscle contractions that cause twisting of the body and limbs, repetitive movements, abnormal postures, and/or rigidity) or symptoms similar to those of Parkinson's disease. Others have twitching of the face or tongue, or peculiar bulging eyes due to lid retraction. The severity of the disease is related to the age of onset, an earlier onset being associated with a more severe and rapidly progressive form of the disease. Symptoms can begin any time between early adolescence and old age up to 70 years of age [125]. MJD is also a progressive disease, meaning that symptoms get worse with time. Life expectancy ranges from the mid-thirties, for patients with severe forms of MJD, to a normal life expectancy for those with mild forms. For patients who die early from the disease, the cause of death is often aspiration pneumonia due to immobility and poor coordination of swallowing and breathing. The clinical spectrum of SCA3 is highly pleomorphic and led to the definition of four clinical sub-phenotypes: type I, characterised by the dominance of pyramidal and extrapyramidal anomalies, in addition to ataxia and other signs, with an early age-at-onset and fast progression; type II, with typical cerebellar ataxia, progressive external ophtalmoplegia and pyramidal signs appearing at an intermediate age; type III, with late onset and slow progression of peripheral signs, such as loss of proprioception and muscle atrophies; and type IV, the rarest, characterised by the presence of Parkinsonic signs, associated to the core clinical features [123-124, 127].

1.6.2 Pathology

Pathological examination of post-mortem MJD patients' brains showed a depigmentation of the substantia nigra, a considerable atrophy of the cerebellum, pons, and medulla oblongata, as well as in motor cranial nuclei [3, 129]. More recently, it was shown that all the precerebellar nuclei and the thalamus were also affected in MJD patients [126, 130]. In addition, in the majority of these patients the post-mortem brain weight is lower than that of individuals without medical histories of neurological or psychiatric diseases [131].

Intracytoplasmic inclusions although, observed to a lesser extent than intranuclear ones, are found in MJD patients' brains [128], constituting a pathological feature of MJD. *In vivo* and *in vitro* studies suggests that disease protein ataxin-3 accumulates in ubiquitinated intranuclear inclusions in

neurons of affected brain regions [6, 19-20]. Several MJD mouse models were generated in the last years and nuclear and /or cytoplasmic aggregates were observed in the mice brains [21-25].

1.6.3 Genetics

The fact that MJD is a hereditary autosomal dominant disease means that the presence of the mutation in one single allele is sufficient to cause the disease. This implies that an affected individual has a 50% chance of passing the disease on to their offspring. Like in other CAG repeat diseases the phenomenon of anticipation is quite common. The children of affected parents tend to develop symptoms of the disease earlier in life, have a faster progression of the disease and experience more severe symptoms. A longer expansion is associated with an earlier age-of-onset and a more severe form of the disease. However, it is impossible to predict precisely the course of the disease for an individual based solely on the repeat length. Many years after the first description of the disease, its causative gene was described and mapped in the chromosome 14.q32.1 [132]. The MJD1 gene (later named *ATXN3*) was cloned one year later, and the authors observed that the mutation (expansion) of the CAG tract was only present in patients [7]. This knowledge allowed the establishment of the molecular diagnosis of MJD, based on the determination of the CAG repeat length, and the consequent confirmation of the disease in families of different origins [24, 133]. The genomic structure of the *ATXN3* gene and the subsequent knowledge of the number and size of exons and introns was only established in 2001. The gene has around 48 Kbp and was described to contain 11 exons, the CAG tract being in exon 10, and to encode at least four different transcripts with variable sizes: 1.4, 1.8, 4.5, and 7.5 Kb, probably due to differential splicing and polyadenylation signals. Very recently 56 additional splicing variants of *ATXN3* gene, some of which observed only in MJD patient's, were reported [134-135]. The biological relevance of these variants remains to be determined. In the brain, *ATXN3* is preferentially transcribed in neurons although low levels are also present in glial cells. Most importantly, neurons susceptible to cell death in MJD/SCA3 express *ATXN3*, although not selectively. Also, *ATXN3* mRNA levels do not differ between controls and patients and are not correlated with the clinical severity or repeat length [136].

1.6.4 *ATXN3* gene product: ataxin-3 (ATXN3) protein

Ataxin-3 has an approximate molecular weight of 42 kDa in normal individuals, but is significantly larger in affected patients, confirming that the expanded CAG repeat is translated into a polyQ stretch. This polyQ tract is located in the C-terminus region that is variable in length, depending on the isoform, and whose expansion is intrinsically related with MJD. [7]

Different isoforms of ataxin-3 have been described: isoform 1 (NP_004984) has 361 aa, with a hydrophilic C-terminal and corresponds to the clones MJD1-1 and MJD5-1 [137]; isoform 2 (P54252) has a distinct C-terminal region when comparing with the first one and it has a hydrophobic nature. It is formed by 365 aa and matches the MJD2-1 clone [137]; the third variant, MJD1a (S50830) contains 349 aa (it lacks 16 aa within the C- terminus region comparatively to isoform 2, due to a premature stop codon [7]. Finally, the variant 4 (NP_1093376) contains less 55 aa than isoforms 1 due to the lack of exon 2 in the H2 clone [134]. This ATXN3 variant lacks the catalytic aminoacid (Cys14), essential for ATXN3 deubiquitylating in vitro activity against poly-ubiquitin chains [135, 138]. The human ataxin-3 protein has a conserved N-terminal josephin domain (1-198 aa) containing the putative catalytic triad aminoacids cysteine (C14), histidine (H119) and asparagine (N134). The josephin domain, which derives from the name of the disease, is followed by two or three (in isoforms 1 and 4) ubiquitin-interacting motifs (UIMs) and the polyQ stretch and also has a conserved nuclear-localization signal (NLS). Similarly, 6 putative nuclear export signal (NES) sequences were found within the ataxin-3 primary structure [135]. Considering the splicing variants recently reported [26], more ataxin-3 isoforms could exist, and given that fact, is still unclear which ones would be more relevant in the disease context.

Ataxin-3 is found in the genomes of several species, ranging from nematodes to human, and including plants [138]. It possesses a globular N-terminal domain with a sequence motif named Josephin (residues 1-198 in the human protein). The sequence of the C-terminus, which contains low complexity sequences, is less conserved among species.

The search for protein motifs suggested that ataxin-3 might be an ubiquitin-binding protein [139]. Functional assays in vitro supported this function and showed predominant binding to K48-linked tetra-ubiquitin through its ubiquitin interaction motifs (UIM) located near the polyglutamine domain [60, 140-141]. A crucial breakthrough in the understanding of ataxin-3 function was the discovery of its DUB activity in vitro [140, 142], however the biological consequence of this function hasn't been characterized extensively yet and the cellular substrates of ataxin-3 remain unknown.

Histone ubiquitylation results in heterochromatin relaxation and assembly of transcription complexes on the promoter, and ubiquitylation of transcription factors enhances their transcriptional-activation function (see review [143]. Therefore, deubiquitylating enzymes as ataxin-3 can also modify transcriptional regulation through the removal of ubiquitin from histones. A role of ataxin-3 in proteasomal protein degradation has been supported by the identification of its interaction partner, valosin-containing protein (VCP) [142]. Recently, several reports have been described interactions between ataxin-3 and numerous other proteins, implicating it in various cellular functions and pathways [144-146].

1.7 Different mouse models for the same disease

Although there is no treatment available at present to cure or delay the onset of MJD, mouse models have been generated to facilitate the understanding of the disease and the development of a therapy. Mice are the preferred animal model because they are small and easy to genetically manipulate and can be generated in relatively large numbers, kept in a controlled environment, and used for invasive procedures. Being mammals, they have important genomic, anatomical, and physiological similarities to humans [147]. The technical problems encountered in the use of patient brain tissue, such as autolysis caused by long postmortem delays, can be avoided using mouse models.

The first transgenic mouse model of MJD was generated using truncated and full-length cDNAs of the *ATXN3* gene, with the L7 promoter directing expression specifically in Purkinje cells (poorly affected in MJD). In these constructs, the cDNA encoded for Q79C, Q79, Q35C (truncated forms with or without the C-terminal) or MJD79 (full-length context). The transgenic mice expressing truncated forms (Q79C, Q79) of the protein with an expanded polyQ tract showed an ataxic phenotype, whereas the animals expressing the full-length protein (MJD79) or a polyQ length present in human control individuals (Q35C) did not reveal any characteristic symptom of the disease. The onset of the symptoms in the affected animals was around 4 weeks of age and the strong ataxic phenotype was more prominent in animals with a higher copy number of the transgene. Histological analysis of these ataxic mice brains showed an atrophic cerebellum and

massive loss of Purkinje cells. This work showed that the expanded polyglutamine tract is responsible for neuronal loss and degeneration [15].

Some years later, another mouse model was created in an attempt to properly mimic the temporal and spatial expression of the human disease gene, using yeast artificial chromosome (YAC) constructs carrying the full-length *ATXN3* gene with expanded polyglutamine tracts, containing all the enhancers and long-range regulatory elements needed for cell-specific expression at physiological levels. Two additional genes were also part of the cloned genomic region.

Homologous recombination in the yeast host was used to generate three YAC constructs with 15, 76 and 84 repeats, corresponding to the human wild-type, intermediate and early-disease-onset MJD alleles, respectively. The mice carrying expanded alleles showed abnormal gait, tremor, hypoactivity, limb clasping, an inability to correct geotaxis, reduced grip strength, abnormal toe pinch responses and progressive loss of weight. These symptoms aggravated with repeat length and with the gene dosage. Regarding the onset of the disease, homozygous mice showed an earlier onset, with faster and worse disease progression. Analysis of brain sections showed degeneration, mild gliosis of the dentate and pontine nerve nuclei (affected in MJD patients) and increased number of reactive astrocytes in the cerebellum. Ubiquitinated nuclear inclusions were also shown in symptomatic mice [148]. These mice are representative of MJD and can be a valuable resource for the detailed analysis of the roles of repeat length, tissue specificity and level of expression in the neurodegenerative processes underlying MJD pathogenesis and could also be used to test therapeutic strategies. However, for this later purpose, their phenotype may be considered too mild.

In 2004, a third mouse model of MJD was published. This transgenic mouse expresses human mutant (Q71B and Q71C) or normal (Q20) ataxin-3 MJD1a under control of the mouse prion promoter, driving expression throughout the brain and spinal cord. Homozygous but not heterozygous (Q71B and C) animals displayed a phenotype that included progressive postural instability, gait and limb ataxia, weight loss, premature death, neuronal intranuclear inclusions, and decreased TH-positive neurons in the substantia nigra. The phenotype manifested only when the mutated protein was expressed above a critical concentration, i.e., only in their Q71C line. The heterozygous animals were indistinguishable in appearance and behavior from wild-type mice. This line also had problems in breeding, since homozygous animals were infertile. Brains from affected Q71-expressing transgenic mice contained an abundant mutant ataxin-3 putative-cleavage fragment

(Fragment ~35kDa), which was scarce in normal Q20 transgenic mice. Reactivity of the Fragment with several antibodies and analysis of co-migration with truncations of mutant ataxin-3 revealed that it contained C-terminal residues spanning from amino acid 221, including the polyglutamine expansion. The Fragment was also shown to be more abundant in the two affected brain regions in MJD patient's post-mortem (dentate nuclei and substantia nigra). Thus these authors developed a new murine model for mutant ataxin-3 toxicity and identified a putative cleavage fragment of the disease protein that appeared to be cytotoxic above a critical concentration [149].

In the last few years, more mouse models were generated and other clues have arisen from these models. In 2007, a mouse model was generated using full-length ataxin-3 constructs (isoform 1, clone MJD1-1, with a third UIM at the C-terminus) containing 15, 70, or 148 CAG repeats under control of murine prion protein promoter. A nuclear signal (NLS) was also introduced as well as a nuclear export signal (NES) to transport ataxin-3 into the cytoplasm. Transgenic mice carrying 70 CAG repeats revealed a severe and rapidly progressive phenotype (tremor, wide-based hindlimbs to stabilize the body in a resting position as well as markedly reduced activity and grooming, resulting in a disheveled appearance) displaying a large number of NIIs and dying prematurely. This study demonstrated, not unexpectedly, that both the size of the expanded CAG repeat length and the level of transgene expression are of major importance for disease onset and disease progression in mice. Mice with a less strong expression of ataxin-3 with 70 CAG repeats developed a milder phenotype than lines with a stronger expression. Similar, transgenic mice but with 148 CAG repeats merely survived the first months and had major problems to produce offspring, whereas mice with stronger expression of this transgene died very early without producing any offspring. More important, the artificially induced nuclear localization of ataxin-3 with 148 polyglutamine repeats accelerated and intensified the phenotype of transgenic mice even further, whereas the addition of a nuclear export signal ameliorated the phenotype. This model thus demonstrated that nuclear localization of ataxin-3 plays an essential role in MJD [150].

In 2008, another transgenic mouse model was created using cDNA of human wild-type ataxin-3-Q22 or disease-causing ataxin-3-Q79 (ataxin-3 mjd1a isoform) under the control of mouse prion protein promoter [151]. Mice from two ataxin-3-Q79 transgenic lines displayed various symptoms of motor dysfunction with an onset age of about 5–6 months, and the severity of

neurological phenotypes progressively increased in the following months. The symptoms included forelimb clasping, impaired motor coordination and shorter latency to fall in the rotarod behavioural test, less activity, incorrect body posture, ataxic gait, reduced weight and reduced pelvic elevation. Authors also found ubiquitinated nuclear inclusions in the neurons of dentate nucleus, pontine nucleus and substantia nigra (as in human MJD patients). Some stainings demonstrated a mild reduction in the number of neurons of the cerebellar dentate nucleus, granular, molecular and Purkinje cell layers. These mice showed a motor dysfunction, although there was an absence of cell death, suggesting that instead of neuronal loss, mutant ataxin-3 causes neuronal dysfunction. In order to test the involvement of neuronal deregulation in this model, the authors performed a microarray analysis in the cerebellum of the mice where they found a variety of deregulated gene expression, down (genes involved in glutamatergic transmission, transcription factors, genes involved in Ca2+ signalling, heat shock proteins, GABA receptors) or upregulated (pro-apoptotic proteins, proteasome subunits, RNA polymerase subunits).

Finally, also in 2008, a different model was generated; these authors used rats instead of mice, and take advantage of the lentivirus' (LV) features. cDNAs encoding the human wild-type ataxin-3 (Atx3-27Q) or mutant ataxin-3 (Atx3-72Q, isoform mjd1a) were used. Lentiviral vectors encoding human wild-type (Atx3-27Q) or mutant ataxin-3 (Atx3-72Q) were stereotaxically injected into the substantia nigra, cortex or striatum of the brain of these rats. Pathological changes were observed in the substantia nigra, such as nuclear inclusion bodies in animals injected with mutant ataxin-3 cDNA. These nuclear inclusions co-localized with ubiquitin and α-synuclein. Authors also tested the involvement of ataxin-3 in neuronal dysfunction, by measuring two markers of dopaminergic neurons, the tyrosine hydroxylase (TH) and the vesicular monoamine transporter 2 (VMAT2). They found a loss of TH' and VMAT2' positive neurons. A reduction of TH immunoreactivity was also observed in the striatum of animals injected with LV encoding mutant ataxin-3, suggesting dysfunction of the TH nigro-striatal projecting fibers.

In order to investigate the involvement of striatum in MJD, pathological analysis was performed in this brain region, and a condensation of the cell nucleus, loss of neurons, condensation of the internal capsule of the striatum and fluorojade B (cell death marker) positive cells was observed [152]. These results indicate that brain delivery of mutant ataxin-3 with lentiviral vectors provides a new genetic model of MJD which may help elucidate the molecular mechanism of mutant ataxin-3 toxicity and may facilitate the evaluation of new therapeutic strategies.

In our lab, we also generated a novel transgenic mouse model, using a human cDNA (isoform 1, MJD1-1 clone)) carrying the repeat tract coding for 83 polyglutamines, under control of the pCMV (cytomegalovirus) promoter, a strong and general promoter. From two different microinjections two female founders (background FVB/N) were obtained, A and B. These two founders were able to transmit the transgene through the generations leading to the establishment of two transgenic mice lineages that were backcrossed with C57Bl/6 mice 9 and 7 times for the creation of experimental animal groups for lineage A and B, respectively (Silva-Fernandes, submitted for publication).

This novel transgenic mouse model for MJD has revealed some important features that recapitulate the disease. Namely, hemizygous transgenic animals developed a motor phenotype in agreement with the dominant feature of the disease; there was a correlation between CAG repeat length and disease manifestation; MJD transgenic mice displayed intergenerational instability and somatic mosaicism in different neuronal and non-neuronal tissues; MJD mouse brains showed neuronal atrophy in specific regions such as the thalamus, dentate nuclei, substantia nigra and the vestibular nuclei. Although the possibility existed that overexpression of ataxin-3 would give rise to some form of pathological consequences, no pathology was observed in homozygous transgenic mice from lineage B, which express similar levels of mutant AT3 to those of hemizygous animals of lineage A. Since the founder B carried a shorter CAG tract than founder A (83 vs 94 CAGs), this data allowed to us to conclude that at this level of expression of mutant AT3 the minimum CAG tract length to induce phenotype in transgenic mice was located between 83 and 94 CAG repeats. Behavioural studies revealed a number of findings in the mice from lineage A that differed from non-transgenic and transgenic from lineage B. We found that hemi and homozygous animals of lineage A displayed an uncoordination phenotype. In lineage A, which manifests MJD-like symptoms it was possible to establish a phenotype-genotype correlation: animals with higher CAG repeat tract spent less time on the rod. Pathological examination of the brains of MJD transgenic mice with expanded (CAG)n alleles originated from founder A also showed atrophy and/or apparent cell loss in several areas, including the substantia nigra, thalamus, dentate and vestibular nuclei as well as astrogliosis in specific areas such as the vestibular nuclei and substantia nigra. This is a promising model for SCA3 since it mimics in many aspects the disease-like features and could also be useful for dissecting the initial cellular and molecular events in the pathogenesis of MJD.

2 Objectives

Chapter 2. Objectives

Regarding the impact of neurodegenerative diseases in the society and bearing in mind that the "why and how" of these diseases are still unclear, we aimed to contribute for the characterization of a novel mouse model of MJD. Our specific goals were:

1. To study the neuropathology of the CMVMJD1-1 transgenic mice;

2. To map and screen the cell death mechanisms in the brain of this mouse model;

3. To determine the CAG repeat instability pattern of the MJD transgenic mouse model, across generations, both in maternal and paternal meioses, in two genetic backgrounds (C57Bl/6 and FVB/N);

4. To analyse the degree of somatic mosaicism in distinct areas of the Central Nervous System and in peripheral tissues of MJD transgenic mice and correlate it with their observed pathological involvement.

3 Pathological analysis

Chapter 3. Brain pathology in MJD transgenic animals

3.1 Background

In MJD only restricted populations of neurons are affected even though ataxin-3 is ubiquitously expressed in the central nervous system [59]. Since the first description of the neuroanatomical alterations observed in MJD patients [27-28] several studies has been published to better understand the pattern of central nervous dysfunction and/or degeneration [153][29-32].

The initial studies suggested that central nervous neurodegeneration in SCA3 is restricted to the cerebellar dentate nucleus, the pallidum, the substantia nigra, subthalamic, red, and pontine nuclei, select cranial nerve nuclei and the anterior horn and Clarke's column of the spinal cord [33-34]. More recent studies allowed the identification of a more widespread degeneration in the CNS of MJD patients, including the cerebellar cortex, the thalamic nucleus, the vestibular and the oculomotor systems [130, 146].

Pathoanatomical analysis of SCA3 brains helps to understand the structural basis of this neurodegenerative disease and offers explanations for a variety of disease symptoms. This better understanding of the neuropathology of the condition has implications for the treatment of SCA3 patients and represents a basis for further biochemical and molecular biological studies aimed at deciphering the pathomechanisms of this progressive ataxic disorder.

The cerebellum has been suggested to be a major target of degenerative processes in MJD [4, 156]. It receives somatosensory, somatomotor and oculomotor afferents from the neocortex, the brainstem and the spinal cord, and subsequent to cerebellar processing for the most part projects these data back to the neocortex and the vestibular nuclei via its cortical Purkinje cells, deep nuclei (fastigial, globose, emboliform, and dentate nuclei) and the thalamic ventrolateral nucleus [157-160]. Owing to these anatomical connections the cerebellum is integrated into neuronal motor feedback loops which, via long descending corticonuclear and corticospinal projections, ultimately control the activity of premotor and motor neurons in the lower brainstem and spinal cord and thus it participates in the fine coordination of movements of the limbs, trunk, head and eyes. All these connections suggests that probably there are other brain areas and different subregions involved in this disease such as precerebellar nuclei (red, pontine, arcuate, prepositus hypoglossal, superior vestibular, lateral vestibular, medial vestibular, interstitial vestibular, spinal vestibular, vermiform,

lateral reticular, external cuneate, subventricular, paramedian reticular, intercalate, interfascicular hypoglossal, and conterminal nuclei, pontobulbar body, reticulotegmental nucleus of the pons, inferior olive, and nucleus of Roller) which has been recently demonstrated [161].

In order to validate our transgenic MJD mouse model we studied the pathological features present in the transgenic mouse brains. We observed neuronal atrophy of some brain regions analysed, with the presence of dark neurons in the thalamus, dentate and pontine nuclei, astrogliosis in the vestibular nuclei and substantia nigra, and ataxin-3 positive-perinuclear aggregates, suggesting that this mouse model mimics some of the pathological findings observed in MJD patients.

3.2 Materials and Methods

DNA extraction and mouse genotyping

For mouse genotyping, DNA was isolated from tail tissue using Puregene DNA isolation kit (Gentra systems, inc). For PCR genotyping, the primers TR1 (5'-GAA GAC ACC GGG ACC GAT CCA G-3') and TR2 (5'-CCA GAA GGC TGC TGT AAA AAC GTG C-5') were used to amplify the transgene (454 bp) and as an internal control of the PCR the mouse homologous Atxn3 gene was amplified (800 bp) using the primers mmMJD8 (5'-CAA AGT AGG CTT CTC GTC TCC T-3') and mmMJD24 (5'-AGT GCT GAG AAC ACT CCA AG- 3'). The fragments were amplified in a reaction mixture containing 2□M dNTPs, 10□M of primers mmMJD8 and mmMJD24, 5□M of primers TR1 and TR2, 1.5□M MgCl2 , buffer (10mM Tris-HCl, pH 8.3, 50 mM KCl) and 0.75 U of DNA Taq polymerase (Fermentas). PCR cycling conditions are as follows: 95°C for 5 minutes followed by 35 cycles of denaturing at 95°C for 1 minute, annealing at 60°C for 1 minute, extension at 72°C for 1 minute and final extension at 72°C for 10 minutes. The PCR products were loaded on 1.5% agarose gels and electrophoresed at 120 V for 1 hour. Gels were visualized with AlphaImager 2200 (AlphaInnotech) and analyzed densitometrically with the corresponding AlphaEase software.

To discriminate between hemyzygous and homozygous transgenic mice a semi-quantitative PCR was performed, in which transgene amplification was performed using the TR1 and TR2 primers described above. As a reference gene, an intronic fragment (546 bp) of the mouse homologous Atxn3 gene was amplified using the primers mmMJD89 5' GCT AGC TAG AGC TAC TTA

TTG 3' and mmMJD54 5' GAC TCC AGA GAG CAC CTG 3'. Briefly, to determine the number of cycles in which the amplification was at the middle of the exponential phase, a sequential series of PCRs using both pairs of primers were performed for each lineage of transgenic mice as described above, differing only in the number of cycling times.

Animals

The C57Bl/6 and transgenic animals used in this study were all sacrificed by exsanguination and posterior perfusion using 100 mL of fixative solution (4% paraformaldehyde (PFA) in 0,1M phosphate buffer) per animal, under anaesthesia (1mL Imalgène, 200 L Domitor and 5.9 mL saline). The tail and the brain were collected and the brain was immersed in PFA 4% for 2 days for posterior processing.

Immunohistochemistry

After two days in PFA 4% the brains were embedded in paraffin and sections were cut with a thickness of 4 m.

Slides with paraffin sections 4 μm thick were steamed for antigen retrieval and then incubated with GFAP antibody (1:500) or polyclonal antibody for ataxin-3 (1:40). A secondary antibody (anti-rabbit) was applied, followed by ABC coupled to horseradish peroxidase (Dako) and DAB substrate (Vector Laboratories). The slides were counterstained with hematoxylin according to standard procedures and analyzed under a microscope (Olympus) using visible light. For morphological brain analysis we performed hematoxylin&eosin and cresil violet staining.

3.3 Results and discussion

As shown in figure 3.1/3.2, pathological examination of the brains of middle age MJD transgenic mice with expanded CAG from lineage A showed neuronal atrophy in several areas, including the substantia nigra, thalamus, dentate and vestibular nuclei as well as astrogliosis in specific areas such as the vestibular nuclei, substantia nigra. These findings are in agreement with the pathological findings reported in MJD patients, where the degeneration involves neuronal loss

and gliosis and the commonly affected regions include the dentate and pontine nuclei, substantia nigra and vestibular nuclei [153, 165-167].

Astrocytes are CNS cells that respond to injuries in the brain. The presence of mutated ataxin-3 may represent an insult and astrocytes are able to react. Astrogliosis is also characterized by rapid synthesis of GFAP intermediate filaments [154]. Increased astrogliosis in transgenic mice and absent in wild-type animal, may indicate this insult of ataxin-3, showing that mutated ataxin-3 is deregulating the tissue homeostasis.

We observed shrunken cells with pyknotic or small nuclei in the transgenic animals but not in the wild-type, this morphology could be associated with dark neurons which are considered a manifestation of neuronal injury and although they are associated with various grades of damage their mode of formation is not yet clear. It has been suggested that the dark neurons may reflect early damage in the cytoskeleton such as microtubules or microfilaments [155].

The NIs could be important both in pathogenesis and in the selective vulnerability of each disease. However several reports show that this link between NIs and vulnerability may not be so straightforward. In HD, for instance, mouse models suggest dissociation between NIs and degeneration: transgenic mice expressing a fragment of mutant huntingtin have massive numbers of NIs, with relatively little neurodegeneration [162] whereas mice expressing full-length mutant huntingtin undergo significant striatal degeneration without many NIs [163]. Most importantly, recent results in the study of SCA1 transgenic mice strongly suggest that visible nuclear aggregates are not required for the initiation of pathogenesis [164].

We performed anti-ataxin-3 immunohistochemistry to analyse the subcellular localisation of ataxin-3. It is possible to observe (Figure 3.3) that ataxin-3 expression was increased in adult transgenic mouse brains, both in intensity and number of stained cells; in wild-type animals we observed less stained cells with diminished intensity. We could not find marked nuclear protein aggregation or inclusions, the sub localisation of ataxin-3 being predominantly perinuclear, but in this location it was possible to see aggregates (Figure 3.3). This sub-cellular localisation might correspond to an earlier phase of aggregation while later phases may be associated with nuclear aggregates. Formation of perinuclear aggregates in the presence of mutated ataxin-3 was very recently demonstrated in vitro: AT3Q72 transfected cells showed aggregates in the perinuclear zone at an earlier stage whereas after a longer post-transfection time the researchers could observe some nuclear aggregates [168].

Neuropathology of transgenic mouse model unveils the presence of atrophic neurons...

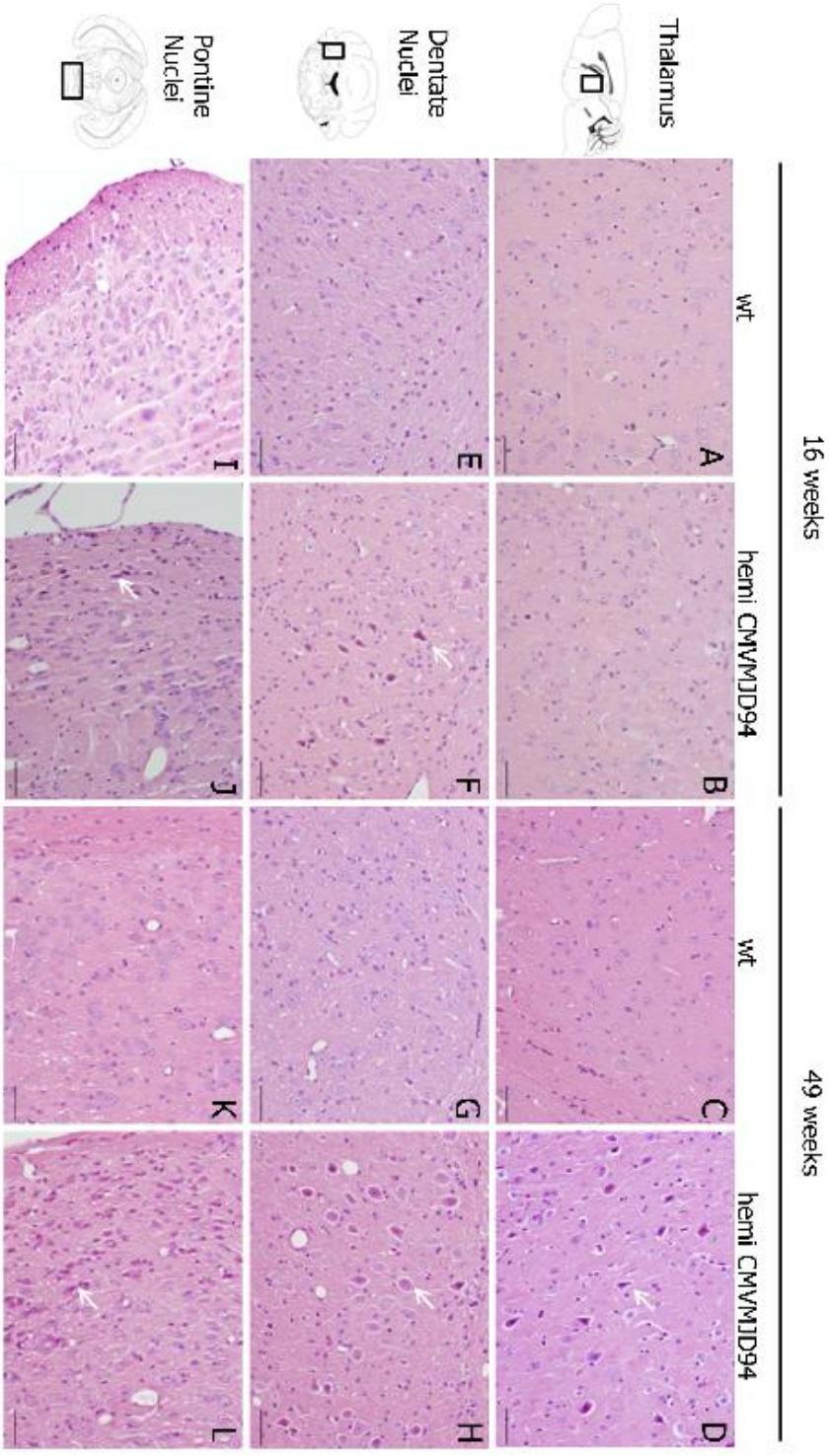

Figure 3.1. Neuropathology of CMVMJD94 mice. Comparative sections stained with H&E of wild-type and hemi CMVMJD94 mice thalami (A-D), dentate nuclei (E-H) and pontine nuclei (I-L) at 16 and 49 weeks of age. Transgenic CMVMJD94 mouse neurons in the thalamus and dentate nuclei were observed by H&E staining as scattered dark, shrunken cells with pyknotic nuclei and a basophilic cytoplasm (arrows) in comparison with normal cells observed in wild-type animals. wt, wild-type; hemi, hemizygous. Scale bar 50 μm.

... and astrogliosis in distinct areas of the CNS

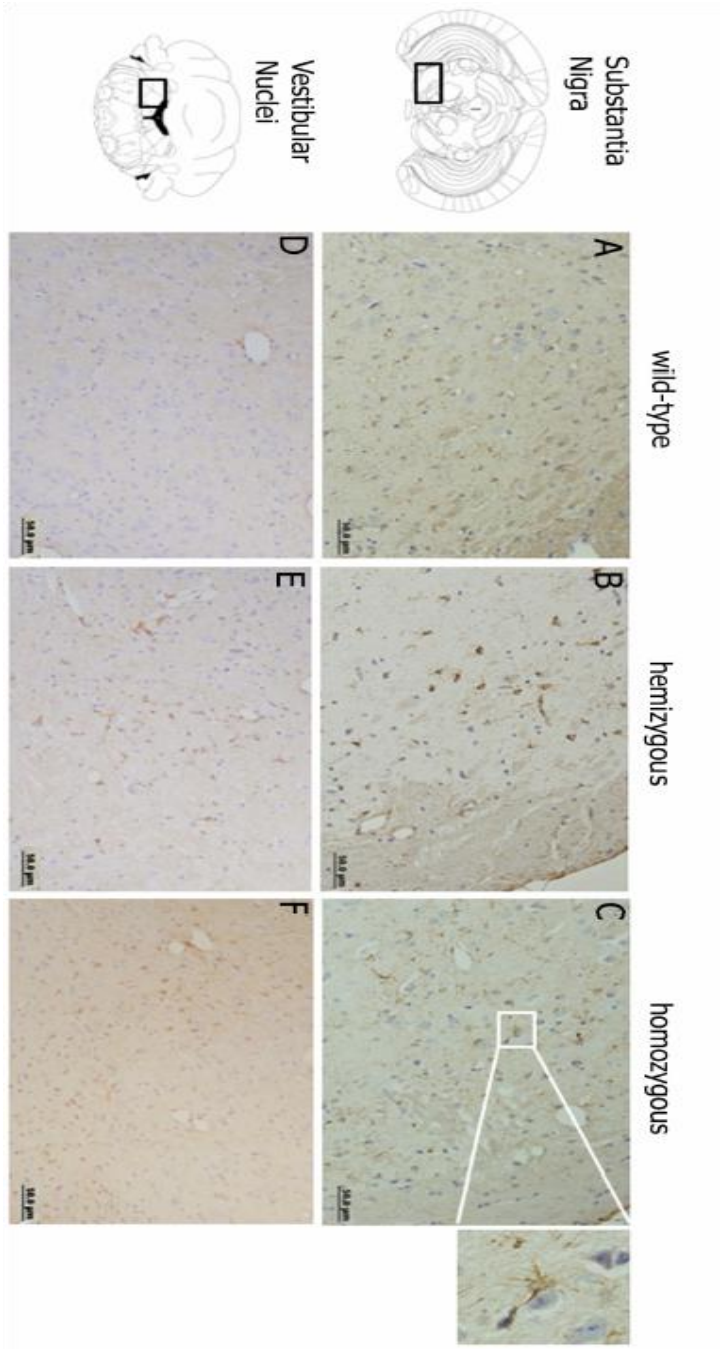

Figure 3.2. Astrogliosis in MJD transgenic mice brains. GFAP immunostaining of the substantia nigra (A-C) and the vestibular nuclei (D-F) at 12 months of age. Hemizygous and homozygous transgenic pCMVMJD94 mice (B, C, E, F) revealed an increase in immunostaining and the presence of reactive astrocytes in comparison with wild-type animals (A, D). Original magnification: × 200. The insets represent a high magnification (original magnification × 600) demonstrating the pycnotic neurons and the reactive astrocytes.

Aggregated proteins with perinuclear localization in affected areas of MJD

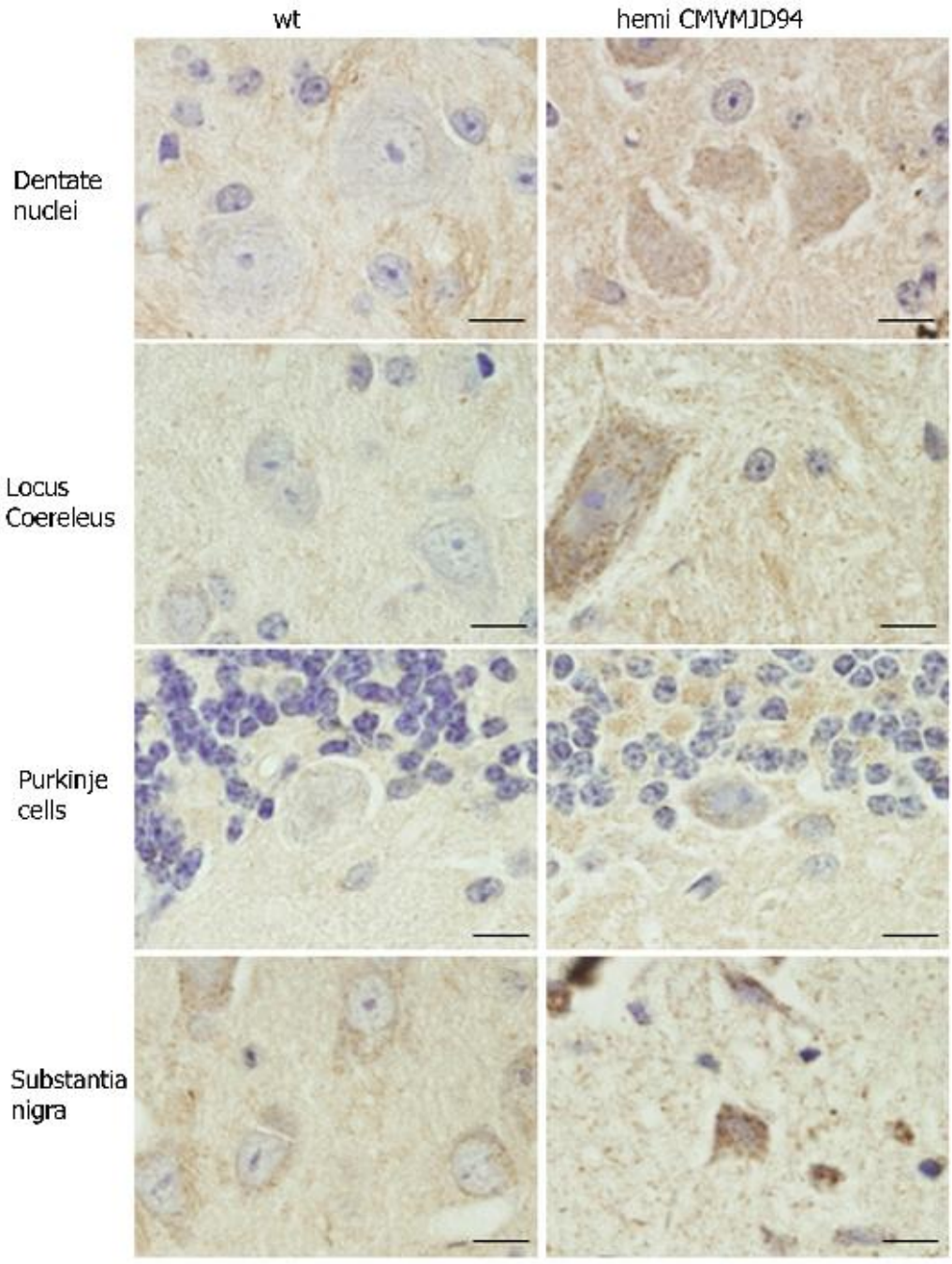

Figure 3.3. Absence of large intranuclear inclusions in transgenic mice with motor impairment. Anti-ataxin-3 immunohistochemistry of wild-type and transgenic CMVMJD94 mice at late stages of the disease (49 weeks). Perinuclear localization of ataxin-3 was observed in CMVMJD94 transgenic mice. wt, wild-type; hemi, hemizygous. Scale bar 10 μm.

3.4 Conclusion

In this chapter we have described some brain pathological features found in our MJD transgenic mouse model.

The presence of dark, shrunken cells with pyknotic or small, staining nuclei and eosinophillic cytoplasm observed after H&E staining, showed us that these cells may be dying or may be atrophic. In the next chapter we will focus on these hypotheses. We also showed increased reactive astrocytes by the immunostaining with GFAP.

Another interesting observation is the presence of perinuclear aggregates and increased intensity of staining in transgenic mice cells when anti-ataxin-3 IHC is performed. This result shows that cytoplasmic aggregates may be important in the pathogenesis of MJD.

All together, these results demonstrate that this MJD transgenic mice model has important features regarding pathology, and we think that this model is a great tool to study the early stages of MJD pathogenesis.

4 Cell death mechanisms

Chapter 4. Neurodegeneration in MJD mice: mapping and screening the cell death mechanisms

4.1 Background

Cell death is essential for embryonic development and maintenance of tissue homeostasis in multicellular organisms.

It has long been evident that cell death is a carefully programmed process. Apoptosis is biochemically defined as a form of programmed cell death executed by a family of zymogenic proteases known as caspases that dismantle the cell in an orderly fashion by cleaving an array of intracellular substrates [169]. Activation of the caspase cascade in a chain reaction like fashion and subsequent cleavage of its downstream targets are therefore considered the biochemical hallmark of apoptotic cell death.

Apoptosis occurs in a well choreographed sequence of morphological events [170]. The dying cell undergoes nuclear and cytoplasmic condensation with blebbing of the plasma membrane, and eventually breaks up into membrane-enclosed particles, termed apoptotic bodies, containing intact organelles, as well as portions of the nucleus leading to nuclear DNA breakdown into multiples of ~200 bp oligonucleosomal size fragments. These apoptotic bodies are then rapidly recognized, ingested and degraded by professional phagocytes or neighbouring cells. Moreover, in some pathological conditions a combination of cell death by necrosis and apoptosis may occur. Besides these two types of cell death, other cell death mechanisms may be involved in polyQ diseases, such as autophagy (see review [173]). Apoptosis has been proposed as a possible mechanism for neuronal death in neurodegenerative diseases. However, there is no direct and convincing evidence of apoptosis in human brains of patients with these disorders, and the mechanisms of neuronal death in neurodegenerative diseases are still unknown. Apoptosis has been implicated in Alzheimer disease (AD). Neuropathologic studies of AD human brains show that the rate of apoptosis is increased 30- to 50-fold over that of age matched controls. Since apoptotic cells appeared to be cleared rapidly by the body, rapid clearance of apoptotic neurons in the AD-affected brain might lead to an underestimation of the total amount of apoptosis occurring in the disease. However, the significance of the increased apoptosis is unclear because the apoptosis could be one of many harmful processes occurring in the AD-affected brain. Some of the stimuli that induce apoptosis have also been implicated in the pathogenesis of Huntington's disease (HD), and typical apoptotic

DNA fragmentation was shown to be increased in brains from post mortem HD patients [174]. Some reports demonstrate that apoptosis is a type of cell death involved in HD [175] and it correlates with disease progression [176]. The ability of the mutant Huntingtin protein to induce apoptosis suggests that apoptosis could play an important role in the pathology of Huntington disease. In other polyQ diseases, including MJD no clear evidence of cell death has been reported, therefore it is very important, in the context of the pathogenesis, to clarify this hypothesis.

Are the atrophic neurons found in our MJD transgenic mouse model dying?

In this study we used late-stage MJD transgenic mice with 86-100 weeks of age in order to screen and map the entire brain for neurodegeneration processes. We performed TUNEL assay to detect DNA fragmentation, which is the latest event in apoptosis; we used Fluoro Jade B, a fluorescent staining that can be used to detect necrotic-like cell death and we performed IHC anti-active-caspase-3 to screen specifically for apoptotic cell death. We also performed western-blot to quantify the levels of active caspase-3 in mouse brains.

4.2. Materials and methods

DNA extraction and mouse genotyping

As described in section 3.2.

Animals

The C57Bl/6 animals used in this study were all sacrificed, under anaesthesia (1mL Imalgène, 200 µL Domitor and 5.9 mL saline) by exsanguination and posterior perfusion using 100 mL of saline solution per animal,. The tail and the brain were collected and the brain was emerged in a viscous solution (OCT) and frozen at -80°C for posterior processing in the cryostat.

Brain processing

The slides were prepared in a freezing cryostat at a 10µm thickness. The slides were kept at -20°C until use.

TUNEL assay

Frozen tissue sections (10µm) of mice aged 86 to 100 weeks were fixed in 1% PFA for 30 minutes and post-fixed in precooled Ethanol:Acetic acid (2:1) at -20°C for 5 minutes. The sections

were washed in saline twice. Endogenous peroxidase was inactivated by immersing the tissue sections in 3% H2O2 in saline solution for 30 min and rinsing again with water and saline several times. In situ DNA end-labelling was accomplished using the Apoptag Peroxidase In situ Apoptosis Detection Kit (Chemicon) according to the manufacturer's instructions.

In brief, sections were treated with 1× equilibration buffer, followed by application of terminal deoxynucleotidyl transferase enzyme solution and incubation in a humidified chamber at 37°C for 1 h. The slides were transferred to a stop buffer, rinsed in saline solution and covered with anti-digoxigenin–peroxidase for 30 min at room temperature. After several washes in saline the color reaction was developed using freshly made substrate solution (0.05% 3,34-diaminobenzidine, 0.03% H2O2 in saline solution) and stopped in double distilled H2O. Nuclear counterstaining was performed with hematoxylin.

The slides were analyzed under a microscope (Olympus) using visible light.

Immunohistochemistry anti-active-caspase-3

For caspase-3 detection, frozen sections (10 μm) of brain mice with 86 to 100 weeks were fixed in 4% PFA and incubate with rabbit anti-active caspase-3 (R&D systems AF835, 1:250, diluted in saline solution) overnight at 4°C. Detection of active-caspase-3 was assessed using LabVision kit according to the manufacturer's instructions.

The slides were analyzed under a microscope (Olympus - BX61) using visible light.

Western-Blotting

Brain tissue (brainstem and cerebellum) of old animals (80 weeks) was homogenized in cold 0.1 M Tris-HCl, pH 7.5, 0.1 M EDTA, and a mixture of protease inhibitors (Complete; Roche). Protein concentration was determined using the Bradford assay (Biorad). Samples were sonicated for 10 sec, heated for 3 min at 100°C, and microfuged for 10 sec before loading. Seventy five micrograms of total protein was loaded into SDS-Page gels and then transferred to nitrocellulose membranes. After incubation with the primary antibodies: rabbit anti-active caspase-3, (1:200, R&D systems AF835) rabbit anti-calbindin (1:1000, Millipore) and mouse anti-alpha-tubulin (1:100, DSHB) the secondary antibodies were incubated at the following dilutions: anti-rabbit (1:500, Pierce) and anti-mouse (1:500, Pierce). Antibody affinity was detected by chemiluminescence (ECL kit, Santa Cruz). Band quantification was performed using ImageJ according to the software manufacturer's instructions using alpha-tubulin as the loading control.

Fluoro Jade B

Frozen tissue sections (10μm) of mice with 86 to 100 weeks first immersed in a solution containing 1% sodium hydroxide in 80% alcohol (20 mL of 5% NaOH added to 80 mL absolute alcohol) for 5 minutes. This was followed by 2 minutes in 70% alcohol and 2 minutes in distilled water. This was followed by 2 minutes in 70% ethanol an 2 minutes in distilled water. The slides were then transferred to a solution of 0.06% of potassium permanganate for 10 minutes in a shaker table, followed by two washes in distilled water. The slides were emerged in staining solution, 0,001% of Fluoro-Jade B (Chemicon) solution for 20 minutes and then rinsed three times in distilled water one minute each.

The slides were analyzed under a microscope (Olympus) using FITC filter.

4.3 Results and discussion

Transgenic and wild-type mice show the same pattern of TUNEL staining

The TUNEL assay is an in situ method to detect cells with DNA strand breaks. The Terminal Deoxynucleotide Transferase dUTP Nick End Label (TUNEL) allows detection of free 3'-OH termini present in the DNA of apoptotic cell by enzymatic labeling. DNA fragmentation is a late event in apoptosis and there are some data suggesting that DNA fragmentation occurs not only by apoptosis but also in necrotic cells [177-178] and there is evidence that this may be the case for neurons [179-180].

In this work we investigate the apoptotic cell death using the TUNEL assay. MJD transgenic mice with 86-100 weeks of age were used in this study. We have screened the entire brain in order to obtain the maximum of information. Generally, the TUNEL assay did not reveal differences in its pattern between wild-type and transgenic animals (Figure 4.1), and the staining observed was probably related to the age of the animals and not with pathology itself. Interestingly, in the areas that we have found pathological features - dentate and pontine nuclei, substantia nigra, vestibular nuclei and thalamus – (see chapter 3) we have also found no TUNEL positive cells, suggesting that apoptosis may not be the type of cell death occurring in these cells.

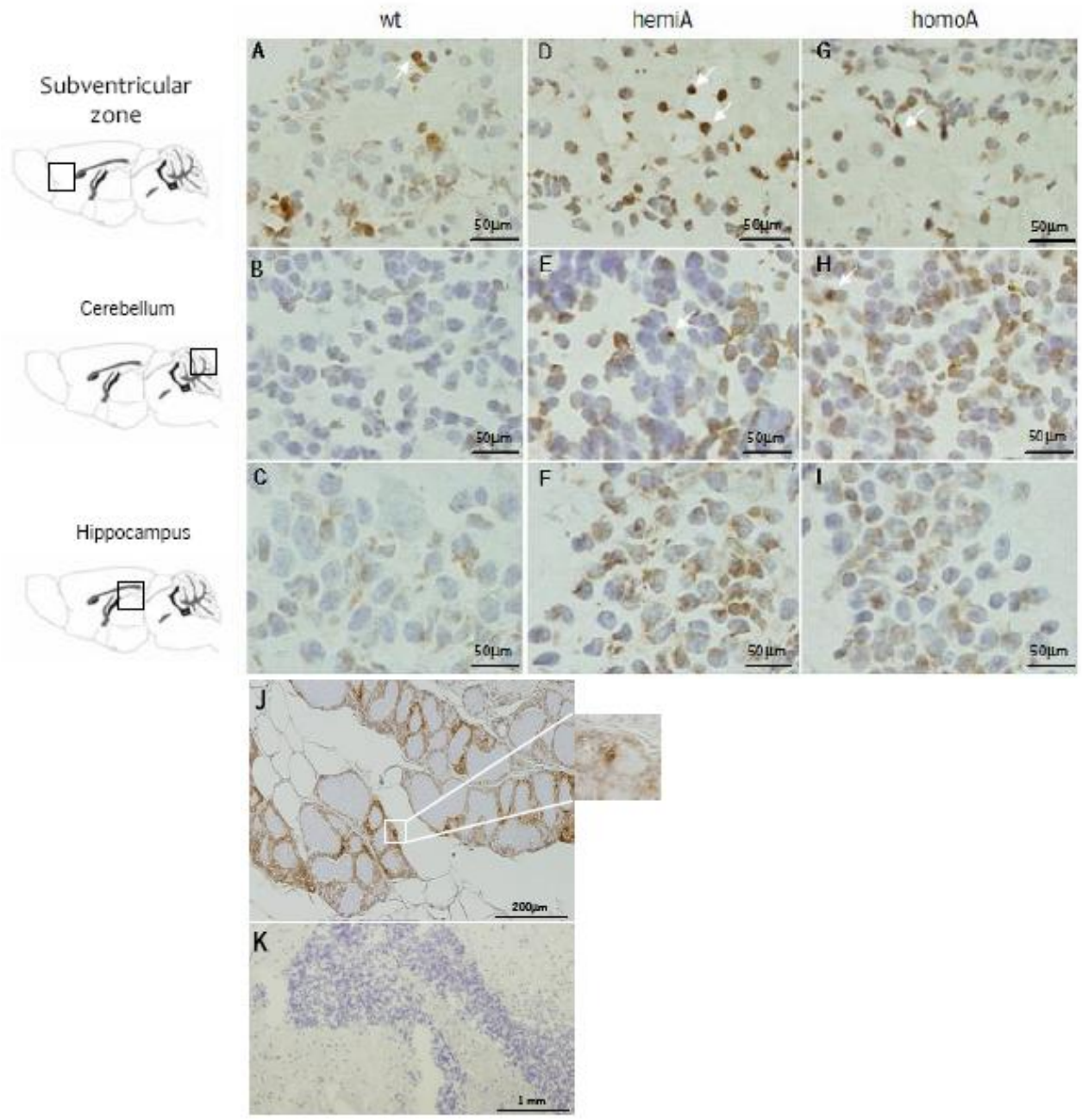

Figure 4.1. TUNEL assay of non-transgenic, hemi and homozygous MJD transgenic mice at 86-100 weeks of age in subventricular zone, cerebellum and hippocampus. Microscopic sections were cut at 10 µM and were subjected to TUNEL assay. (A-I) Wild-type (wt), hemizygous (hemiA) and homozygous (homoA) mice from lineage A show few TUNEL positive cells (arrows) in the brain areas shown in this panel but the same is true for the entire brain. N=4 (J) mammary glands of female rats after weaning as a TUNEL positive control. (K) TUNEL assay negative control.

Caspase-3 activation is similar between normal animals and MJD animals

The apoptotic cell death pathway is executed by caspases, a family of aspartyl-specific cysteine proteases [181-182]. Caspase-3 and 9 are the main executioner caspases in the brain and their activation has been described in HD mouse models and in HD human brains [183-184].

In order to further exclude apoptosis as a cell death type occurring in our MJD transgenic mice, we performed immunohistochemistry anti-active caspase-3 in the same animals and in serial sections of the entire brain. Our results, in accordance with those of the TUNEL assay, showed that caspase-3 is not activated in wild type nor in transgenic mice (Figure 4.2) both in spared areas and in those where we found pathology.

We also performed quantitative anti-active caspase-3 Western-blot in the cerebellum and brainstem of 80 weeks old animals, to confirm our IHC result, and we did not see any significant differences in these two areas between wild-type and transgenic animals (figure 4.3).

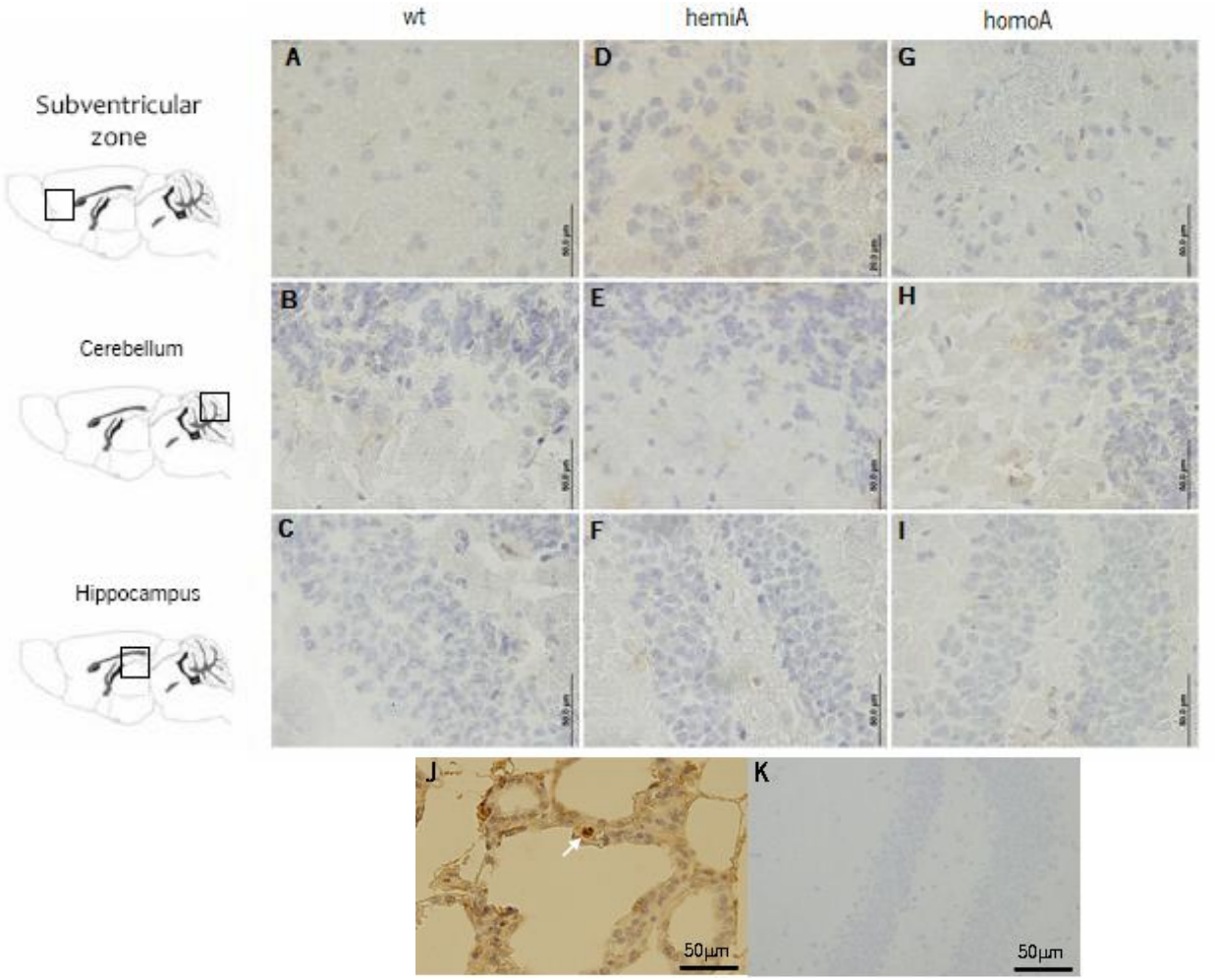

Figure 4.2. IHC anti-active caspase-3 of non-transgenic, hemi and homozygous MJD transgenic mice at 86-100 weeks of age in subventricular zone, cerebellum and hippocampus. Microscopic sections were cut at 10 µM and were subjected to IHC. (A-I) Wild-type (wt), hemizygous (hemiA) and homozygous (homoA) mice from lineage A show no active caspase-3 cells in the brain areas shown in this panel but the same is true for the entire brain. N=4 (J) mammary glands of female rats after weaning as a caspase-3 positive control. (K) IHC negative control (without primary antibody).

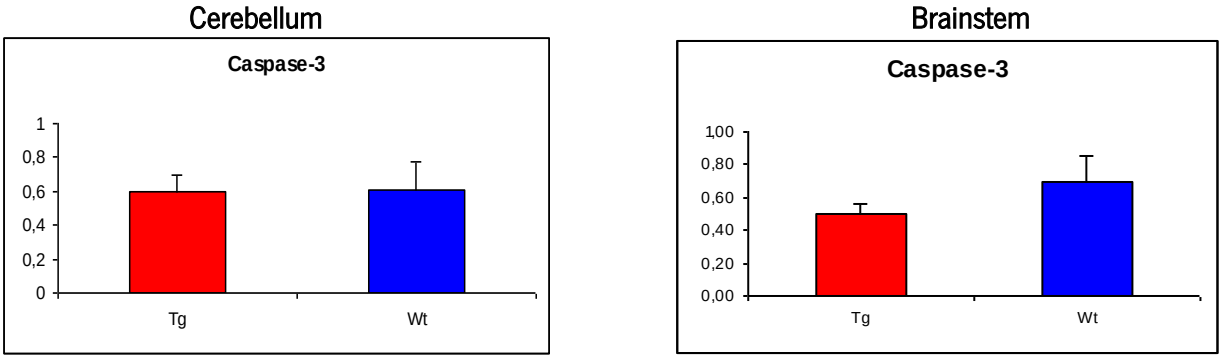

Figure 4.3. Western-blot anti-caspase-3 of non-transgenic and hemizygous MJD transgenic mice at 80 weeks of age in cerebellum and brainstem. At the protein level there is no differences between wild type and transgenic mice for caspase-3. t test analysis, $p<0.05$. N=4

Necrosis is not increased in MJD transgenic mice

Our results suggest that apoptosis is not occurring in the regions containing atrophic neurons that we observed in chapter 3. However necrosis has also been described to be implicated in HD [175] and other neurodegenerative disorders, and it could be occurring also in MJD.

Fluoro-Jade is an anionic fluorochrome capable of selectively staining degenerating neurons in brain slices. The histochemical application of Fluoro-Jade results in a simple, sensitive and reliable method for staining degenerating neurons and their processes [185].

Once again, we used 80 weeks old animals and we screened the entire brain performing Fluoro-Jade B staining. Our results show that there is no difference in this staining between wild-type and transgenic mice (Figure 4.4) and again we did not found correlation with pathological brain areas. In contrast a recent report using a model of MJD in rat, generated using lentiviral vectors, showed Fluoro-Jade positive cells in the striatum, suggesting neuropathology in this "spared" area of the disease [152]. The use of lentiviral vectors as an alternative way to obtain a disease model, lead in this case to a localized strong overexpression of the mutant protein, possibly promoting neurodegeneration in these usually spared brain regions.

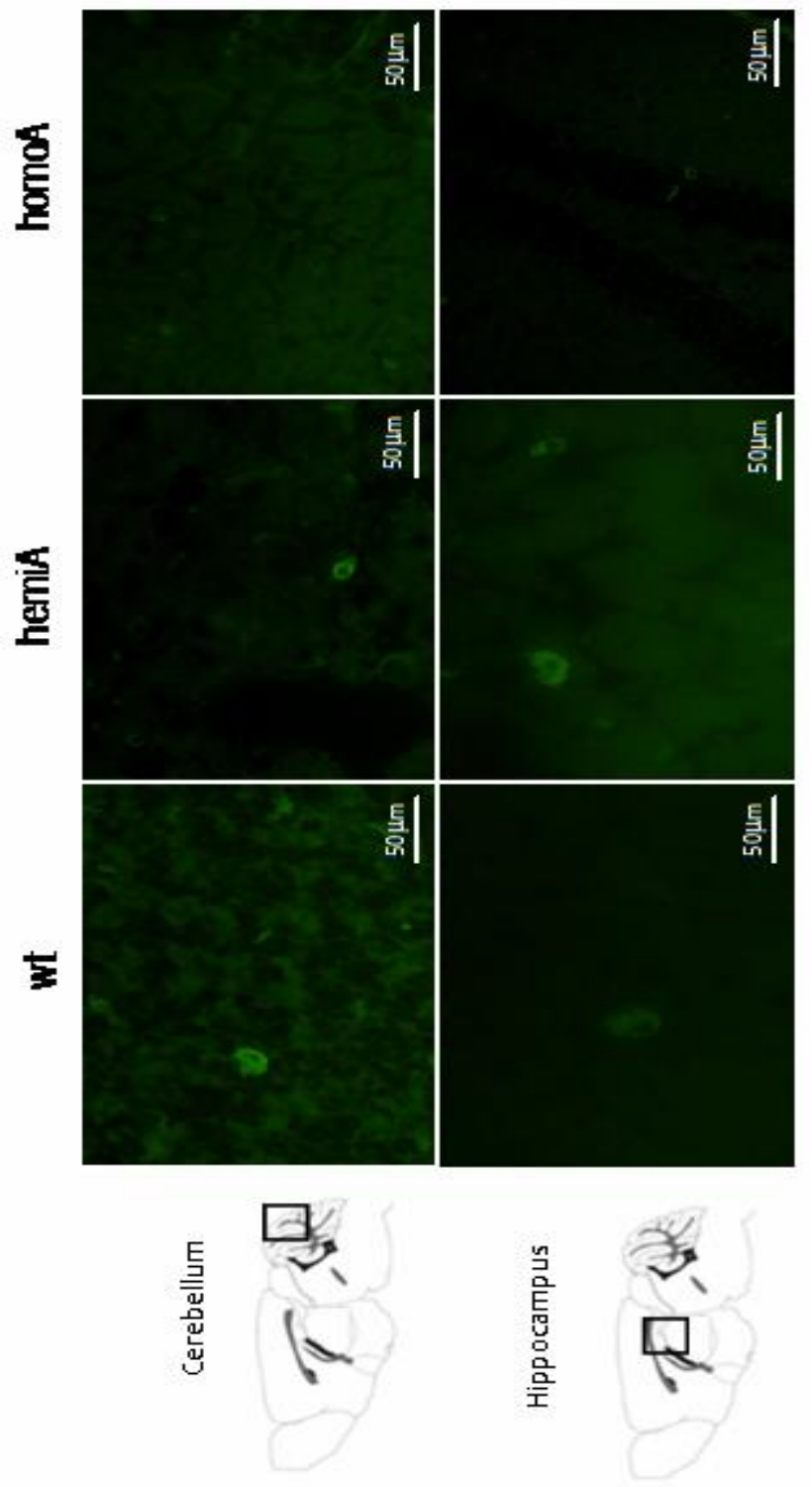

Figure 4.4. Fluoro-Jade B staining of non-transgenic, hemi and homozygous MJD transgenic mice at 86-100 weeks of age in cerebellum and hippocampus. Microscopic sections were cut at 10 µM and were subjected to this staining. Wild-type (wt), hemizygous (hemiA) and homozygous (homoA) mice from lineage A show no differences in FJB pattern. N=4

All together, our results suggest that another cell death type might be happening or instead, that these are dysfunctional, rather than dying, neurons.

Transcriptional deregulation has been proposed in the ataxin-3-Q79 transgenic mouse model of MJD [151], where the authors did not find neuronal loss in the cerebellum, as well as in other mouse models of MJD [149-150]. In a microarray study of the ataxin-3-Q79 mouse model, the expression of several genes was found to be altered, including the downregulation of genes involved in the intracellular Ca^{2+} signalling [151]. The same was described for other SCAs and for DRPLA [186-189].

Glutamate-mediated 'excitotoxicity', a term coined to describe an excessive release of glutamate, and a subsequent over-activation of excitatory amino acid (NMDA, AMPA, and kainite) receptors may occur first via a rapid influx of Ca^{2+} through voltage-gated calcium channels, caused by the depolarization of the membrane after AMPA receptor activation (and through Ca^{2+} permeable AMPA receptors themselves), and second, via a delayed release of Ca^{2+} from intracellular stores. Excessive Ca^{2+} influx can detrimentally alter dendritic spine morphology via interactions with the neuronal cytoskeleton, and thus can perturb normal synaptic function. Calcium-binding proteins, such as calbindin-D28K may help to prevent glutamate from exerting deleterious effects [190].

In order to evaluate the relevance of this pathway in MJD, we performed a western-blot detection of calbindin, a calcium-binding protein present in dendrites, soma, and axons of Purkinje cells, using 80 weeks old transgenic mice. Both in cerebellum and brainstem we could not observe any statistical difference in the intensity of staining between wild type and transgenic animals (Figure 4.5). Until now we only tested this protein, although several other molecules should be tested in order to obtain an answer, such as calcineurin B and myosin Va.

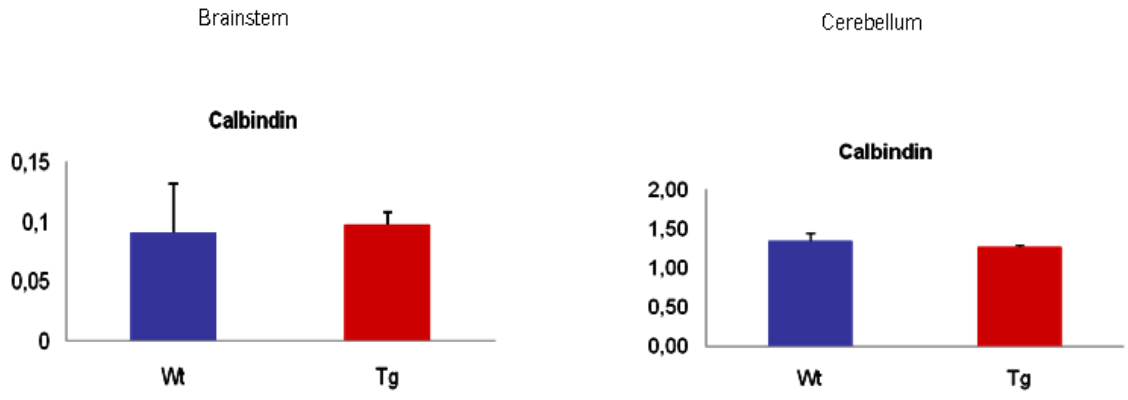

Figure 4.5. Western-blot anti-calbindin of non-transgenic and hemizygous MJD transgenic mice at 80 weeks of age in cerebellum and brainstem. At the protein level there are no difference between wild type and transgenic mice for calbindin. t test analysis, $p<0.05$. N=4

4.4 Conclusion

This chapter represents a novel study, since the analysis of different pathways of cell death using different has not been described in the literature, in MJD models or human brains.

We have shown that apoptosis and necrosis are two cell death processes that are not happening in our mouse model; it is still possible that autophagy, another way for cells to dye, may be behind this neurodegeneration process [112-114, 191]. Alternatively, processes such as synaptic dismantling may be involved.

Another possibility could be the abnormal functioning of neurons without cell death. So far we have not detected any neuronal death, the observed pathological features mentioned before, may support the hypothesis of dysfunctional neurons. However, we also need to perform a rigorous stereological analysis and to study autophagy in order to exclude the neuronal death hypothesis.

5 CAG repeat instability

Chapter 5. CAG repeat instability in a MJD transgenic mouse model

5.1 Background

A triplet repeat disease, including MJD, can be defined as a disease caused by the unstable expansion of a triplet repeat tract [192].

In the first description of the MJD gene in 1994, screening for CAG repeat expansions in 12 Japanese SCA patients with clinical signs of MJD revealed a repeat expansion from 68 to 79 CAGs in 11 of the patients whereas 72 healthy Japanese controls harboured between 13 and 36 repeats [7]. This early evidence showed that in mutant as in normal alleles, these triplet repeats are quite polymorphic.

A large number of genetic studies over the last 15 years finally defined the normal range of CAG repeats in the MJD gene fewer than 44 and the expanded repeat size of more than 45 [193], 86 being the largest expanded repeat described [156]. Studies also indicated that the expanded CAG repeat is widely unstable during paternal transmission [194], showed evidence for somatic mosaicism of the expanded allele [31], and revealed that, besides the expanded CAG repeat length, additional genetic factors exist manifesting the age at onset of MJD [194-195].

Germline mutations are involved in triplet repeat instability. Progressively larger expanded CAG repeat sizes are correlated with the earlier age at onset in successive generations, which is known as anticipation. Intergenerational instability in MJD is more prominent in paternal than in maternal transmission [35, 194]. Processes that are specific to sperm or oocyte development may be involved in these biases [44, 196]. Female cell production occurs before birth and requires a limited number of cell divisions [197] while the majority of male germline production occurs after birth with regular spermatogonial stem cells cycles [198]. However, as other highly proliferative tissues show lower levels of instability than sperm, there must be other factors, rather than the rate of cell division, contributing to repeat instability. Somatic mosaicism of the repeat sizes in CAG repeat disorders describes that different cells of the same individual carry different repeat sizes. Somatic instability has been described for MJD in different brain areas [31] with smaller expanded repeat sizes in the cerebellar cortex than in other brain regions such as the frontal cortex [199]. Repeat length heterogeneity has been observed both in proliferating and in non-proliferating tissues [37-38, 200-201], suggesting that other factors, besides proliferation status, are contributing to the extent of somatic mosaicism.

A logical question that arises then is whether this somatic mosaicism of repeat length has any impact in the specificity of cell death: does polyglutamine load contribute to cell-specific vulnerability in these disorders?

In HD, this question was addressed in humans [38] and in a mouse model; indeed this seemed to be the case, since the CAG tract was more expanded in the striatum, the most affected area in this disease [202]. This simplistic correlation was not, however, confirmed in other polyglutamine diseases.

In this chapter we tried to answer the following questions: does our transgenic mouse model display intergerational instability? And somatic mosaicism? Is mosaicism age-dependent? Is there a correlation between the degree of mosaicism and pathological involvement?

In order to answer to the first question we used DNA from tail tissue of male and female transgenic C57Bl/6 and FVB/N mice and analysed them and their progeny. We used two different genetic backgrounds in order to see if this instability was background-dependent. To investigate the second and third questions we used DNA from several brain regions of males C57Bl/6 transgenic mice with 5, 24, 60 and 72 weeks of age.

5.2 Materials and methods

5.2.1 Intergerational instability

DNA extraction and genotyping

As described in section 3.2.

Molecular analysis of the CAG repeat

The (CAG)2CAAAAGCAGCAA(CAG)$_n$ repeat tract and a 75 bp flanking region of the ATXN3 gene was amplified by PCR using the primers MJD25a (5'GGC TGG CCT TTC ACA TGG AT 3') and MJDcDNA (5'CGG AAG AGA CGA GAA GCC TAC 3').

Relative CAG variation across generations was analysed by PCR amplification of DNA extracted from tail biopsies with the primers described above, using incorporation of [203] dATP, and separation in a denaturating polyacrylamide gel as described [204]. For determination of the major allele size the films were visualized with AlphaImager 2200 (AlphaInnotech, San Leandro, CA, USA) and bands were analyzed densitometrically with the corresponding AlphaEase software to detect the highest peak. CAG variation was determined by comparison with the migration of the highest peak band from PCR products generated from parental and progenitor DNAs.

For analysis of the CAG repeat, namely the determination of the CAG repeat number and the analysis of the somatic mosaicism in transgenic mice, DNA was extracted from several brain regions (motor and cerebellar cortex, deep cerebellar nuclei, hippocampus, hypothalamus, amygdala, striatum, substantia nigra and pontine nuclei) and also from tail tissue and amplified by PCR; primer MJDcDNA was fluorescently labelled with 6-FAM and products were displayed on an ABI 310 automated DNA sequencer (Applied Biosystems).

The analysis of the degree of mosaicism in the different tissues of mutant mice was performed by calculations the mosaicism index (MI) as described by [205-206]. The number of bands with peak areas of at least 10% that of the major band were counted and their surfaces summed. MI was defined as the ration between this sum and the area of the major size band. In order to avoid inclusion of PCR artifacts, only bands larger than the major bands were taken into

account. The CAG tract length of the highest peak was determined using an equation from the calibration curve obtained with cloned alleles containing known numbers of CAG repeats.

Statistical analysis

Continuous variables with normal distribution (K-S test $p>0.05$) were analyzed with the Student t-test and ANOVA. All statistical analyses were performed using SPSS 16.0 and a critical value for significance of $p< 0.05$ was used throughout the study.

5.3 Results and discussion

Parental gender and genetic background-dependent pattern of CAG intergenerational instability

To investigate whether, as human patients, MJD transgenic mice displayed intergenerational instability, we determined the CAG repeat tract length in both maternal and paternal meioses of animals from lineage A. This analysis was performed in lineage A because it carries only two copies of the transgene, making this analysis of length variation easier. In order to identify genetic effects upon repeat instability, we used hemizygous male and female transgenic from lineage A in two different genetic backgrounds, C57Bl/6 and FVB/N.

Studies regarding MJD patients showed no significant difference in frequency of contractions, stable inheritance or expansions between male or female meioses, but an increased variability was found in the CAG repeat tract length when paternally transmitted [24]. We observed that CAG repeat length in transgenic mice varied through generations in more than 50% of the transmissions: the expanded allele had the tendency to expand when transmitted through the male progenitor and to contract when transmitted from the female progenitor (Figure 5.1 A). This gender bias has also been demonstrated in other polyQ diseases, such as HD [207]. The genetic background seemed to affect the pattern of instability and, this gender observation reached statistical significance in the C57Bl/6 background ($p<0,05$) (Figure 5.1 B). Distribution of CAG length variation in the two strains suggests that background-specific modifier loci might affect the degree of intergerational instability.

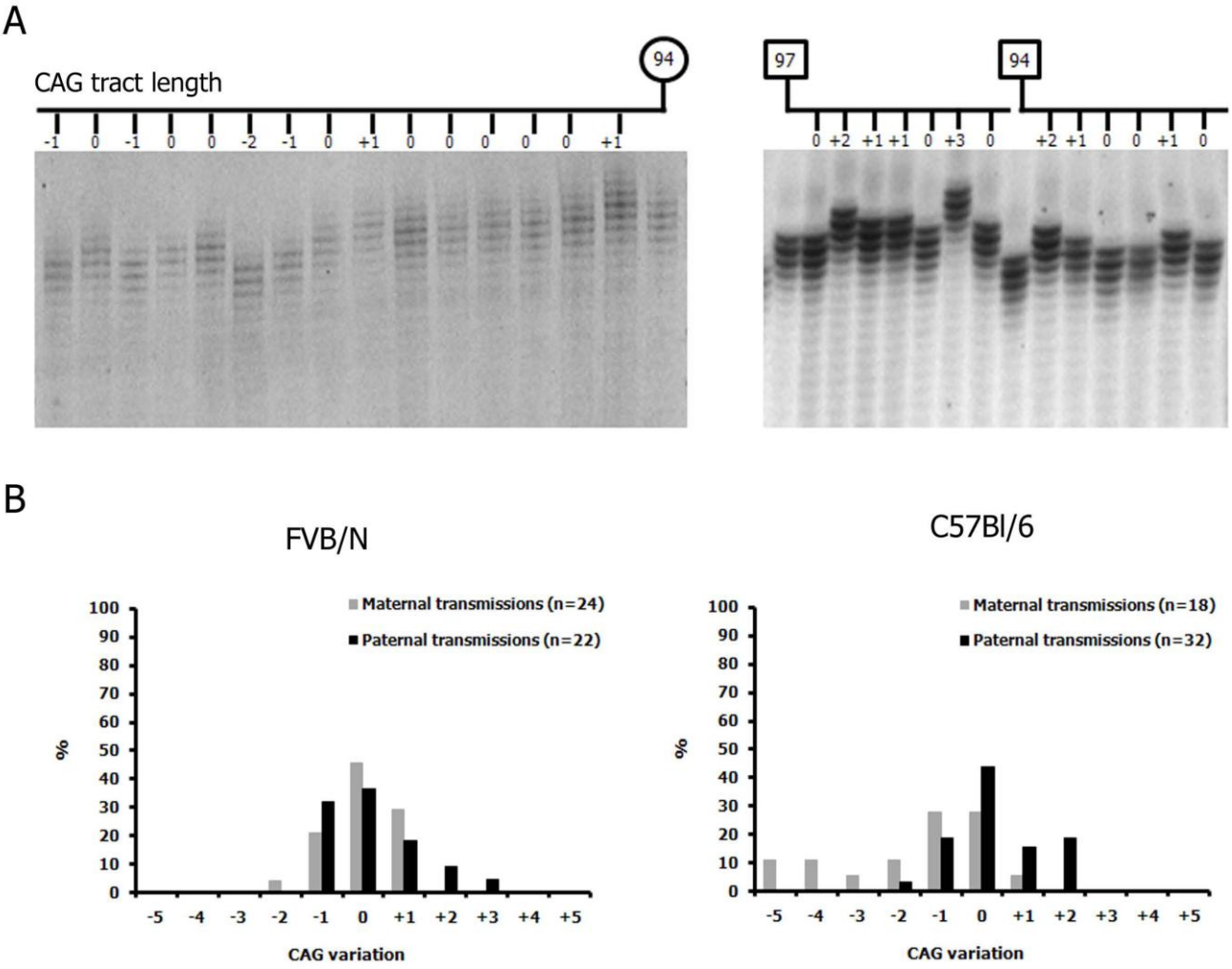

Figure 5.1. Intergenerational instability of the expanded CAG repeat in MJD transgenic mice. (A) Polyacrilamide gels showing the variation of the CAG repeat tract length of male and female transgenic mice progenitors and their respective descendants. (B) Differential pattern of CAG repeat variation in MJD transgenic mice throughout male and female meioses in the FVB/N and C57Bl/6 genetic background.

Progression of CAG repeat variability in somatic tissues of MJD mice during aging

The analysis of the CAG repeat in different tissues of transgenic mice revealed the existence of somatic mosaicism of the expanded allele: several bands, larger and smaller than the major band were observed; however, to avoid confounding by polymerase slippage and incomplete amplification effects, only the larger bands were considered in the analysis as proposed by Cancel and collegues [205].

When we planned this experiment, we decided to analyse the degree of instability in different neuronal areas and also in peripheral tissues. As show in figure 5.2, somatic mosaicism was present in all tissues analysed, except heart. In some of the areas affected in MJD, such as the pontine nuclei and substantia nigra, the MI was higher, however this was also observed in areas thought to be spared in the disease, such as the striatum and the liver. In the liver this probably happens due to the well-known capacity of this organ to regenerate and proliferate [208]. Regarding other non neural tissues in MJD patients, the muscle exhibited a lesser degree of CAG instability, while the liver, kidney and colon displayed a greater diversity of extra bands and more extended major band compared to CNS [211].

Each CNS tissue is predominantly composed of post-mitotic neuronal cells and glial cells that can continue to divide in the postnatal period. The proportion of these two main cell types differs among the tissue analyzed. This fact may explain the different pattern of MI observed the neuronal tissues that we have analysed. Given that in HD, DRPLA, SCA1 and MJD patients the cerebellar cortex has the smallest repeat size and least mosaicism of all CNS regions studied [37-38, 209], and because the ratio of neuronal to glial cells in cerebellar cortex is higher than elsewhere, it was suggested that the main source of expanded repeat instability in the CNS would be the glia that continue dividing, whereas neurons are postmitotic [37]. This hypothesis is supported by a study in DRPLA post mortem brain specimens [210], where a wider range of repeat sizes was observed in older individuals. It was postulated that the continuous mitotic division of the glial cells throughout life leads to this increased somatic mosaicism. Besides proliferative capacity, other mechanism may be behind this instability, such as the involvement of the mismatch repair system described very recently in HD [207].

Our results suggests that the somatic mosaicism is a relevant feature of our MJD transgenic model, mimicking what is happening in MJD patients, and indeed this instability is an age-dependent process (Figure 5.2). Generally, in all tissues analysed, age seems to be an important factor, since we demonstrate that at 5 weeks the mosaicism is lower and increases with age. This

was also observed in other triplet diseases such as Myotonic dystrophy and SCA1 [212-213]. However, somatic mosaicism seems to be the result of differences in the pattern of development and/or cell composition of each structure in CNS, and not to be directly correlated with the specific pattern of cell death. Thus, these results do not completely exclude the possibility that glial cells might be the main contributors to the somatic mosaicism of expanded repeat size: more detailed studies, using microdissection and single-cell PCR, would be needed in order to improve our understanding of the origin and biological relevance of somatic mosaicism in this disease.

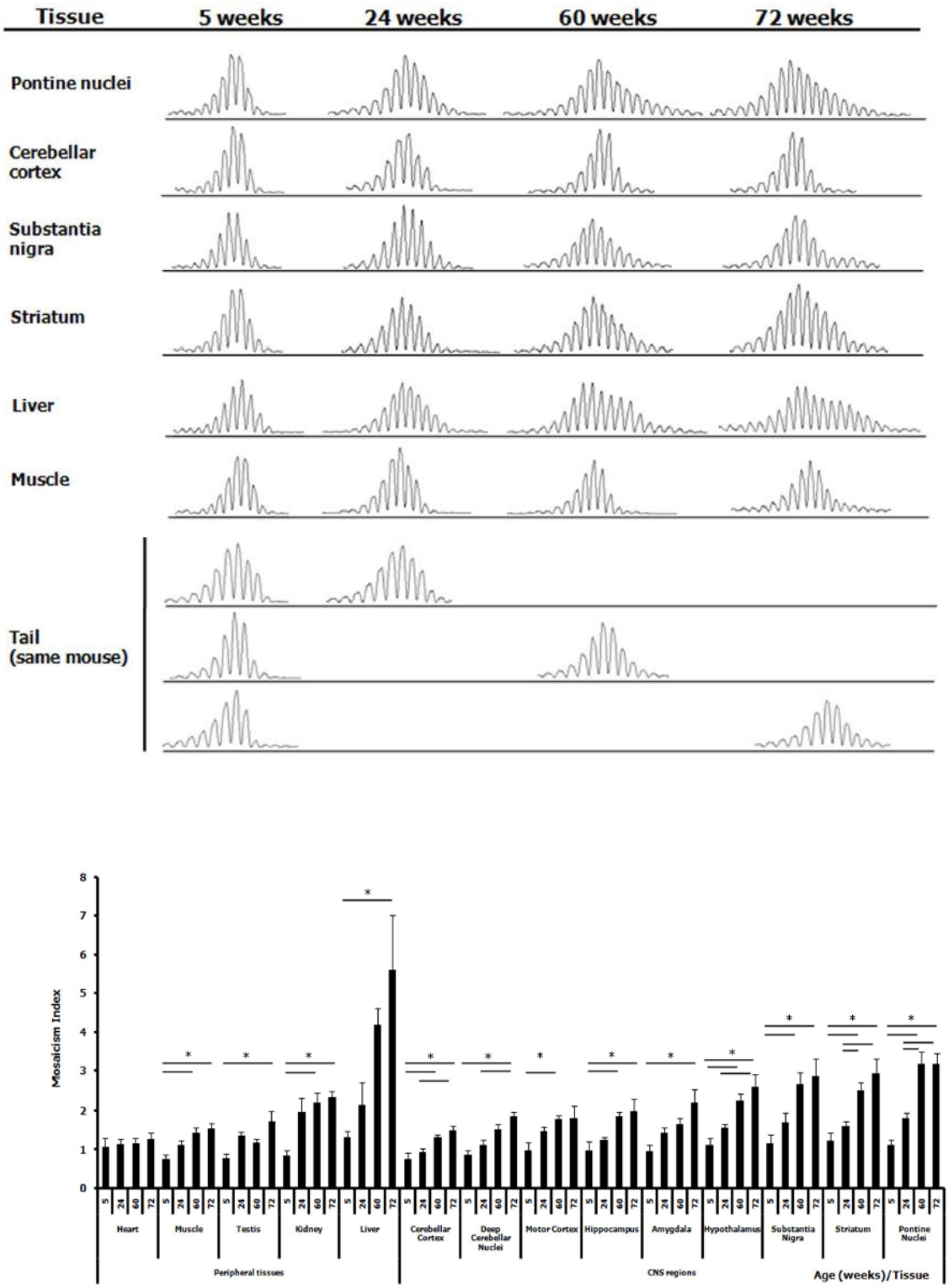

Figure 5.2. Age-dependent somatic mosaicism of the expanded CAG repeat in neuronal and non-neuronal tissues MJD transgenic mice. (A) Representative genescan tract diagrams of the CAG repeat pattern of neuronal and non-neuronal tissues from MJD transgenic mice at different ages. (B) Differential pattern of mosaicism index increase through aging for pCMVMJD94 hemizygous mice. Mean (at least three independent samples) ± SE, * $p<0,05$

5.4 Conclusion

In this chapter we demonstrate that the human CAG mutation in our MJD transgenic mouse model is not stable, but a dynamic mutation. Our model displays intergerational instability which is genetic background specific and is dependent on gender. In order to study the intergerational instability in our mouse model, we assess the CAG repeat tract length both in paternal and maternal meioses. The amplification of the transgene by PCR, showed a pattern of bands similar to those found in heterozygous human patients [31].

We also found differences in the CAG instability behaviour between male and female transmissions, and the mild instability found in our model was in agreement with that described in MJD patients [24].

We observed that different tissues exhibited different patterns of MI during aging. The cells from the liver, pontine nuclei, substantia nigra and striatum displayed the highest MI increases and the cells from the cerebellar cortex and muscle the lowest MI increases trough age.

We have found pathological involvement in the pontine nuclei and the substantia nigra but we did not observe anomalies in the striatum which led us to conclude that the specific increase of somatic mosaicism in these areas was not clearly correlated with specific cell vulnerability in this mouse model. Age has been described as a CAG repeat instability modifier [214-216], as so our results are in accordance with those obtained in other animal models of different polyQ diseases.

Although a correlation between somatic mosaicism and pathology was not clear, our results concerning the CAG variation pattern in transgenic mice from lineage A, revealed that this mouse model can be useful for the study of the molecular basis of the CAG repeat instability, as well as of MJD pathogenesis.

6 General discussion

Chapter 6. General Discussion

In the last years, a lot has been done in the attempt to understand the pathogenic mechanism of polyQ diseases, including MJD. The common features between different polyQ diseases, make researchers believe that probably there a common mechanism underlying all these disorders since each of the expanded CAG repeats encodes polyglutamine and the pathogenic threshold for disease is roughly the same, at around 40 copies of the repeat in most of the different subtypes.

It is assumed that the common toxic gain-of- function mechanisms for the polyglutamine-containing protein depend on aggregation and deposition of misfolded proteins leading to neuronal dysfunction and eventually cell death [217].

Enigmatically, despite the fact that the majority of the proteins associated with spinocerebellar neurodegenerative disease are expressed systemically, the resulting cytotoxicity appears restricted to a few neuronal subtypes of the CNS [19]. Selective cellular conditions and specific protein–protein interactions might confer local insolubility conditions, leading to oligomerization and fibrillization in vulnerable neurons.

Despite well-described clinical and pathological phenotypes, the molecular and cellular events that underlie neurodegeneration in these disorders are still poorly understood. Compelling evidence points to major aetiological roles for interference with transcriptional regulation, protein aggregation and clearance, the ubiquitin-proteasome system and alterations of calcium homeostasis in the neuronal loss observed during the neurodegenerative process. But novel molecular routes that might be disrupted during disease progression are also being identified. These pathways could act independently or, more likely, interact and enhance each other, triggering the accumulation of cellular damage that eventually leads to dysfunction and, ultimately, the demise of neurons through a series of multiple events [2]. This suggests that simultaneous targeting of several pathways might be therapeutically necessary to prevent neurodegeneration and preserve neuronal function. Understanding how dysregulation of these pathways mediates disease progression is leading to the first attempts of obtaining effective therapeutic strategies in vivo, which may prove beneficial in the treatment of polyQ diseases.

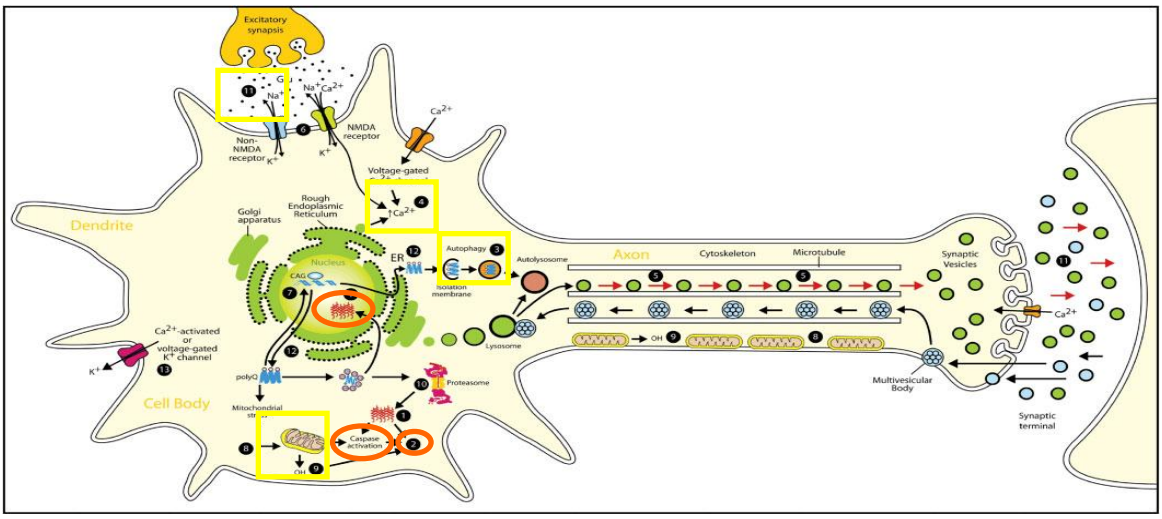

Figure 6.1. Molecular mechanisms of neurodegeneration in spinocerebellar ataxias. 1, aggregation; 2, apoptosis; 3, autophagy; 4, Ca^{2+} homeostasis alterations; 5, disruption of axonal transport and vesicle trafficking; 6, excitotoxicity; 7, interference with gene transcription; 8, mitochondrial impairment; 9, oxidative stress; 10, alterations of proteasome degradation; 11, synaptic dysfunction; 12, unfolded protein response (UPR); 13, potassium channel dysfunction; Ca^{2+}, calcium ions; ER, endoplasmic reticulum; Glu, glutamate; K^+, potassium ions; Na^+, sodium ions; Q, glutamine; Ub, ubiquitin. Adapted from [2]

As shown in figure 6.1, several pathways may be involved in polyQ pathogenesis and may act together rather than individually. In our work we explored some of these pathways (orange circles) however we still need to check other pathways that can lead to MJD pathogenesis (yellow squares). We could observe atrophic neurons and perinuclear inclusions in MJD transgenic mouse brains, as described in chapter 3, and we screened for different types of cell death in the entire brain of these mice (chapter 4). We could conclude that the types of cell death analysed are not happening in our model; we will next address autophagy, and eventually other pathways must be studied in other to have more clues about this crucial topic. Evidences of autophagic cell death have been proposed in several neurodegenerative diseases [112-114, 191]. Rapamycin, an inducer of autophagy, reduces the aggregation of expanded polyQ in transfected cells [218], protects against neurodegeneration in a fly model of Huntington disease, and improves performance on behavioural tests and decreases aggregate formation in a mouse model of Huntington disease [219]. Autophagy could be the next step in this study and may be a good target for therapeutically strategies [35].

Another common feature of polyQ diseases is the dynamics of the trinucleotide repeats. Tissue-specific instability occurs during human [38, 220-221] or animal [45, 202] development in

trinucleotide repeats disorders. Somatic variation in repeat tract length is observed in the brains of individuals affected by HD [38], Machado-Joseph disease, SCA1 [209] and spinobulbar muscular atrophy (SBMA) [222]. Intergerational instability is present is this type of disorders and is responsible for the onset of the disease and when transmitted by paternal meiosis tend to aggravate the symptoms on the offspring [35, 194]. Another aspect of the dynamics of the mutation is seen in somatic tissues and this fact could be related to the vulnerability of the areas affected in the polyQ diseases. To address this question in our model we performed an extensive study, using several neuronal and non-neuronal tissues from transgenic mice during age, at 5, 24, 60 and 72 weeks of age. Although in some brain areas such as the pontine nuclei and the substantia nigra there was a tendency to an increased somatic instability, we could not find a clear correlation between vulnerable areas in the disease and a higher mosaicism index.

Finally, although this work is focused on Machado-Joseph disease, we believe that some of our results may be transposed to other polyglutamine disorders.

7 Conclusions

Chapter 7. Main conclusions of the work

- Pathological analysis of CMVMJD94Q transgenic mouse brains showed atrophic neurons and increased astrogliosis in regions such as substantia nigra and vestibular nuclei.
- The presence of perinuclear aggregates, but not nuclear inclusions, was detected in CMVMJD94Q transgenic mouse brains by reactivity with ataxin-3;
- No differences were observed in TUNEL positive cells, caspase-3 positive cells, Fluoro-Jade B staining and calbindin protein levels between wild-type and CMVMJD94Q transgenic mice;
- This model shows a generally homogeneous pattern of somatic mosaicism in several CNS and non-neuronal tissues, showing a tendency towards higher instability in some disease affected areas of such as the pontine nuclei and the substantia nigra but also in disease spared areas such as the striatum, and non-affected organs such as the liver.

8 Future perspectives

Chapter 8. Future perspectives

The CMVMJD94Q transgenic mouse model shows an important overlap with genetic and clinical features of MJD, namely intergenerational instability of the expanded CAG repeat, pathological hallmarks of the disease (atrophic neurons and astrogliosis) and motor coordination impairment.

This work opened some questions that we want to address soon and gave us the opportunity to begin other studies such as the development of therapeutic strategies using this mouse as a model, and pathology findings as biomarkers for assessment of effectiveness of therapies.

We want to analyse the neuronal dysfunction further, studying markers of some pathways that may be involved on this process, such as markers of calcium signalling, GABAergic and glutamatergic transmission (VGLUT2, IP3-RI), and autophagy (autophagosomes, Bax, Bcl-2), among others.

We will also focus our attention and energy into studying the effect of a selection of drugs that target different pathways: (1) transcription deregulation; (2) oxidative stress; (3) autophagy.

We will test the effects of compounds targeting the potential pathogenic pathways: transcription deregulation (phenylbutyrate, SAHA and resveratrol), oxidative stress (creatine) and autophagy (lithium and rapamicyn) in this mouse model for MJD. The compounds that improve significantly the motor phenotype and show to be non toxic, could be interesting candidates for future clinical trials in MJD patients and can also contribute for the knowledge of the pathogenic mechanism(s) of this disease.

9 References

Chapter 9. References

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