Escola Superior de Tecnologia da Saúde do Porto
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Rastreio de compostos terapêuticos num modelo da doença de Machado-Joseph em *C. elegans*: tendo como alvo a mitocôndria.

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Key words: Machado-Joseph disease; ataxin-3; protein aggregation; neuronal dysfunction; mitochondrial dysfunction; creatine; small molecules.

Abstract

Since the early 90's a new class of inherited neurodegenerative diseases has been characterized, the polyglutamine (polyQ) expansion diseases. This group is composed by nine progressive and finally fatal disorders. The mutation underlying each one of these disorders is an expansion of a CAG trinucleotide repeat that encodes a polyQ tract in the respective disease proteins. This polyQ expansion causes the appearance of misfolded protein species, which ultimately lead to the formation of aggregates and neuronal loss. Although polyQ diseases present different clinical features and neuronal degeneration pattern, all these diseases have in common the fact that the associated gene products are widely expressed but affect only specific subsets of neurons. This specificity suggests that protein misfolding and its toxic outcomes may be determined by the polyQ-flanking sequences of the specific disease-associated proteins. Machado-Joseph disease (MJD) or spinocerebellar ataxia type 3 (SCA3) is the most frequent autosomal dominant ataxia worldwide. Ataxin-3 (ATXN3) is a polyQ protein and expansion of its repetitive glutamine tract causes MJD. The economic and social impact of these neurodegenerative diseases has led several researchers worldwide to investigate the pathogenesis mechanism and therapeutic strategies for polyQ diseases. Animal models, like *Caenorhabditis elegans* (*C. elegans*), have proved to be an essential tool in this field due to their importance in the development of therapeutic trials.

C. elegans offers unique advantages for examining the aggregation dynamics of aggregation-prone proteins and its toxic effects on individual neurons, since the transparency of all 959 cells allows easy detection of fluorescent proteins in live animals. Despite having relatively few neurons, *C. elegans* display a wide array of complex behaviors and a clear link exists between the behavior and the function of neuronal subsets.

In this work, we used a *C. elegans* model of MJD for a screening of therapeutic compounds. First, we have tested the effect of a candidate compound targeting mitochondrial toxicity: creatine (Cr). We showed that, in our *C. elegans* model of MJD pathogenesis, Cr food supplementation had limited effect in mutant ATXN3-mediated neuronal dysfunction and aggregation. Further experiments will be required to determine

the treatment effectiveness. Next, in a hypothesis-free approach, we have tested 20 compounds from an FDA-approved out-of-patent library, and the small molecules Prestw-38 and Prestw-227 significantly reduced mutant ATXN3-mediated motor impairment.

In summary, with this work we have identified two compounds that reduced the percentage of mutant ATXN3 animals that present locomotion defects, and one of which showed a significant reduction in the number of aggregates per area and in the area of aggregates per area. It also made available a valuable *C. elegans* model/tool for drug discovery and target identification that can be very useful in future therapy development in MJD.

Resumo

Desde o início dos anos noventa, foi caracterizada uma nova classe de doenças neurodegenerativas hereditárias, as doenças de expansão de poliglutaminas (poliQ). Este grupo é constituído por nove doenças progressivas e fatais. A mutação causadora de cada doença é a expansão de uma repetição de um trinucleótido de CAG que codifica um segmento de poliQ na proteína causadora da doença. Esta expansão de poliQ está associada a uma alteração de conformação das proteínas, com consequente formação de agregados e perda de células neuronais. Embora as doenças de poliQ apresentem diferenças clínicas e no padrão de neurodegenerescência, todas estas doenças têm em comum o facto de as suas proteínas causadoras terem uma expressão ubíqua, mas somente afectarem populações específicas de neurónios características de cada uma das doenças. Esta especificidade sugere que a agregação proteica e os seus efeitos tóxicos podem ser determinados pela sequência aminoacídica de cada proteína. A doença de Machado-Joseph (DMJ) ou ataxia espinocerebelosa do tipo 3 (SCA3) é a ataxia autossómica dominante mais frequente em todo o mundo. A ataxina-3 (ATXN3) contém um segmento de poliQ cuja expansão está na origem da DMJ. O impacto sócio-económico destas doenças neurodegenerativas tem levado vários grupos de investigação pelo mundo inteiro a investigar o mecanismo patogénico assim como estratégias terapêuticas para as doenças de poliQ. Os modelos animais para as doenças de poliQ, por exemplo os *Caenorhabditis elegans* (*C. elegans*), são uma ferramenta essencial no estudo destas doenças devido à sua enorme importância para o desenvolvimento de ensaios terapêuticos.

O nemátode *C. elegans* proporciona grandes vantagens no estudo dos efeitos tóxicos de proteínas poliQ em neurónios, uma vez que a transparência das suas 959 células facilita a detecção de proteínas fluorescentes *in vivo*. Apesar de apresentarem um número reduzido de neurónios, os *C. elegans* apresentam inúmeros comportamentos complexos, existindo uma relação clara entre a função de determinados subtipos neuronais e os comportamentos regulados por esses grupos de neurónios.

Neste trabalho, usamos um modelo transgénico da DMJ em *C. elegans* para um rastreio de compostos terapêuticos. Em primeiro lugar, testamos o efeito da creatina (Cr), um composto-candidato que tem como alvo a toxicidade mitocondrial. Demonstramos que, no modelo da patogénese da DMJ em *C. elegans*, o tratamento com Cr teve um efeito reduzido na disfunção neuronal e agregação mediados pela ATXN3-mutante. Serão necessários novos ensaios para determinar a eficácia deste tratamento. Por fim, testámos 20

compostos de uma biblioteca comercial de compostos terapêuticos. Os compostos Prestw-38 e Prestw-227 demonstraram reduzir significativamente o fenótipo motor do modelo da patogénese da DMJ em *C. elegans.*

Em resumo, este trabalho permitiu identificar dois compostos que reduziram o defeito de locomoção presente no modelo da patogénese da DMJ em *C. elegans*, um dos quais demonstrou também uma redução significativa no número de agregados por área do animal e na área de agregados por área do animal. Estes resultados permitiram validar este novo modelo animal da DMJ para o rastreio de compostos terapêuticos e identificação de alvos que poderão ser úteis para o desenvolvimento de terapias para a DMJ.

Table of contents

List of abbreviations

AD: Alzheimer's Disease
ADP: Adenosine Diphosphate,
AGAT: Glycine amidinotransferase
ALS: Amyotrophic Lateral Sclerosis
ATP: Adenosine Triphosphate,
ATXN3: Ataxin-3 protein
***ATXN3*:** Ataxin-3 gene
BBB: Blood–Brain Barrier
***C. elegans*:** *Caenorhabditis elegans*
CAG: Trinucleotide codon for glutamine
CB: Cerebellar cortex
CK: Creatine Kinase
CKMi: Mitochondrial CK
CNS: Central Nervous System
CoQ_{10}: Coenzyme Q_{10}
Cr: Creatine
Crn: Creatinine
CrT: Cr transport system
DMSO: Dimethyl Sulfoxide
DN: Dentate Nucleus
DNA: Deoxyribonucleic acid
DRPLA: Dentatorubral-pallidoluysian Atrophy
ER: Endoplasmic Reticulum
FRAP: Fluorescence Recovery After Photobleaching
GAMT: Guanidinoacetate methyltransferase
GP: *Globus Pallidus*
HD: Huntington's Disease
HSF1: Heat Shock Factor 1
HSP: Heat Shock Protein
Htt: Huntingtin Protein
IIS: 1-like signaling
JD: Josephin domain
kDa: kiloDalton
LCN: Lateral Cuneate Nucleus
min: Minute
MJD: Machado-Joseph Disease
mm: millimeters
mM: Milimolar
moPrP: Mouse Prion Protein Promoter
MPP+: 1-methyl-4-phenyl pyridinium
MPT: Mitochondrial Permeability Transition
MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRC: Mitochondrial Respiratory Chain
mtDNA: Mitochondrial DNA
n: Number of samples in the study
N2: Bristol Laboratory *C. elegans* strain (wild-type strain)
NAGE: Aative Agarose-gel Electrophoresis

NGM: Nematode Growth Medium
NIs: Nuclear inclusions
NLS: Nuclear-localization Signal
OD: Optical Density
pCMV: Cytomegalovirus Promoter
PCR: Polymerase Chain Reaction
PCr: Phosphocreatine
PD: Parkinson's Disease
PN: Pontine Nucleus
polyQ: Polyglutamine
Q: Glutamine
qRT-PCR: quantitative real time PCR
RN: Red Nucleus
RNA: Ribonucleic acid
ROS: Reactive Oxygen Species
SB: Sodium Butyrate
SBMA: Spinobulbar Muscular Atrophy
SCAs: Spinocerebellar Ataxias
SD: Standard Deviation
SDD-AGE: Semi-denaturating Detergent Agarose Gel Electrophoresis
SN: *Substantia Nigra*
SNpc: SN pars compacta
STN: Subtalamic Nucleus
TSEs: Transmissible Spongiform Encephalopathies
***t-test*:** Student's *t* test
ub: Ubiquitin
UIM: Ubiquitin-interacting motifs
UPR: Unfolded Protein Response
UPS: Ubiquitin-proteasome system
WT: Wild-type
YAC: Yeast Artificial Chromosome
YFP: Yellow Fluorescent Protein
µL: Microliter
6-OHDA: 6-hydroxydopamine

Chapter I

General Introduction

1.1. Neurodegenerative diseases

Neurodegenerative diseases are devastating disorders, affecting thinking, skilled movements, feelings, moods, cognition and memory. This group of diseases includes common disorders such as Alzheimer's disease (AD), Parkinson's disease (PD) and rarer diseases such as Huntington's disease (HD), spinocerebellar ataxias (SCAs), transmissible spongiform encephalopathies (TSEs) and amyotrophic lateral sclerosis (ALS) (Soto, 2003). Selective neuronal loss, synaptic abnormalities, neuronal inflammation and the presence of cerebral deposits of misfolded protein aggregates are some common characteristics of these diseases (Martin, 1999; Soto, 2003). These aggregates are a typical disease mark and although the main protein component is different in each disease, they have some similar morphological, structural and staining features (Soto, 2003). In addition, the area of the brain that is most affected differs among diseases and determines the distinct clinical symptoms of each. Though it was initially thought to be the most important problem in neurodegeneration, different diseases suggest that extensive neuronal death may not be the initial cause of the diseases (Haass, 2007). Certainly, clinical symptoms have been described before significant neuronal loss and a better temporal and topographic correlation is found with synaptic dysfunction (Haass, 2007).

1.2. Protein folding and misfolding

The presence of misfolded proteins within the cellular environment may presents two types of critical obstacles to cellular function. Firstly, the function of the misfolded proteins is practically always inevitably lost and in the second place, misfolded proteins often have a tendency to self-associate and form aggregates. To assist the folding and to supervise the maintenance of the folded structure, cells have evolved a highly efficient protein folding quality control system, which regulates protein homeostasis and prevents accumulation of misfolded protein in the cell (Horwich & Weissman, 1997; Morimoto, 2002; Nollen & Morimoto, 2002). Some of the foremost identified quality control system include: the Heat Shock Protein (HSP) expression, the Unfolded Protein Response (UPR), the Ubiquitin-Proteosome System (UPS) and autophagy. The HSPs can assist in the correct folding by binding to exposed hydrophobic regions on folding proteins therefore protecting

them from aggregation (Hartl, 1996). Within the endoplasmic reticulum, the UPR operates as a positive feedback loop increasing the activity of many quality control systems in response to an increase in misfolded protein (Kostova & Wolf, 2003). The UPS is a surveillance mechanism which primarily functions to target and degrade misfolded or aberrant proteins (Ciechanover & Brundin, 2003). Autophagy is a mechanism of degradation of intracellular components inside lysosomes (Wang & Klionsky, 2002; Cuervo, 2004). Nevertheless, under particular pathological conditions, the ability of this machinery is exceeded and aggregation and deposition in tissues are the consequence of the failure of the protein folding quality control system (Dobson, 1999; Soto, 2003). This failure may result from: mutations affecting the activity of specific chaperones (Evgrafov et al., 2004); the damage of the UPS, as occurs in ubiquitin protein catabolic disorders (Layfield et al., 2001); the tendency for a specific protein, or a set of proteins, to misfold and aggregate, producing disturbances in protein homeostasis, as occurs in a broader class of folding disorders called *proteinopathies*.

Protein aggregation is the hallmark of an ever-increasing number of diseases. These folding diseases, also referred to as *protein conformational disorders*, include AD, PD, HD, SCAs, like spinocerebellar ataxia type 3 (SCA3), type II diabetes, ALS and prion diseases such as Creutzfeld-Jakob disease (Dobson, 1999; Soto, 2003).

1.3. Polyglutamine (PolyQ) diseases

In the early 1990s, a new mutational mechanism was identified: the expansion of unstable trinucleotide repeats (Fu et al., 1991; La Spada et al., 1991; Ross & Poirier, 2004). The identification of expanded trinucleotide repeats in various genes has been found to be the last cause of a growing number of neurodegenerative diseases. The largest group of trinucleotide repeat of neurodegenerative diseases are those due to expansion of a CAG repeat located within the coding region of the affected gene. These repeats are translated into an extended polyglutamine (PolyQ) tract in the expressed protein, which results in protein aggregation within the cell. Of this group, nine diseases associated to expanded coding $(CAG)_n$ tracts have been described: HD, caused by expansion of $(CAG)_n$ tracts in the HD gene (Gusella et al., 1983), Kennedy disease or spinobulbar muscular atrophy (SBMA), caused by a mutation in the androgen receptor (La Spada et al., 1991),

dentatorubral-pallidoluysian atrophy (DRPLA) which is caused by a mutation in atrophin-1, a transcriptional regulator (Li et al., 1993; Nagafuchi et al., 1994), and several forms of SCAs: SCA1, caused by a mutation in ataxin-1 (Orr et al., 1993); SCA2, caused by a mutation in ataxin-2 (Pulst et al., 1996); SCA3, also known as Machado-Joseph disease (MJD), caused by CAG-repeat expansion in the C-terminal of ataxin-3 (ATXN3) (Kawaguchi et al., 1994); SCA6, caused by a mutation in the α_{1A} calcium channel (Zhuchenko et al., 1997), SCA7 (David et al., 1997); and SCA17, caused by a CAG expansion in TATA-binding transcription factor (Zoghbi & Orr, 2000; Nakamura et al., 2001). Chromosomal locations of the relevant genes and the names of the translated protein products are listed in Table 1.1.

Apart from the expanded polyQ tract, that confers toxic properties to the protein, these proteins are different from each other. They vary in size and function and enclose the glutamine segment at different location within the protein sequence. In spite of generally ubiquous patterns of expression, each polyQ containing protein originates a disease-specific neurodegeneration pattern, suggesting that the protein context plays a role in each disease (Burright et al., 1995; Sharp et al., 1995; Trottier et al., 1995; Zoghbi & Orr, 2000).

Table 1.1- Plolyglutamine Disorders.
Adapted from Tarlac & Storey, 2003.

Disorders	**Locus**	**Protein**	**Normal $(CAG)_n$**	**Expanded $(CAG)_n$**
Huntington's disease	4p16.3	Huntingtin	6-38	36-121
Spinocerebellar ataxia type 1	6p23	Ataxin-1	6-44	40-81
Spinocerebellar ataxia type 2	12q24	Ataxin-2	15-29	35-59
Spinocerebellar ataxia type 3	14q32	Ataxin-3	12-41	55-84
Spinocerebellar ataxia type 6	19p13	α_{1A} Ca^+ channel	4-17	20-30
Spinocerebellar ataxia type 7	3p14	Ataxin-7	4-35	37-220
Spinocerebellar ataxia type 17	6q27	TATA-binding protein	25-42	45-63
Dentatorubral-pallidoluysian atrophy	12p13	Atrophin-1	3-35	49-88
Spinobulbar muscular atrophy	12p13.31	Androgen receptor	11-38	38-62

With the exception of SBMA (which is X-linked), all polyQ diseases are autosomal dominant inherited disorders, with a late clinical onset characterized by progressive neuronal degeneration with loss of coordination, motor and, in some of these diseases, cognitive impairment (reviewed in Zoghbi & Orr, 2000). The transition in polyQ sequence

length from benign to pathological is quite sharp. In general, the pathological threshold lengths range from 35 to 50 glutamine residues. The only exception is SCA6, in which the threshold ranges from 15 to 20 glutamines (Myers et al., 1998). Within the expanded ranges, there is an inverse correlation between the number of CAG repeats and the age of disease onset (Gusella & MacDonald, 2000) (see Figure 1.1). The highly expanded CAG repeats appeared to be unstably transmitted to succeeding generations with a tendency for further elongation, typically in paternal transmission, providing the molecular basis of a phenomenon called *anticipation* (Maciel et al., 1995; Rubinsztein et al., 1996).

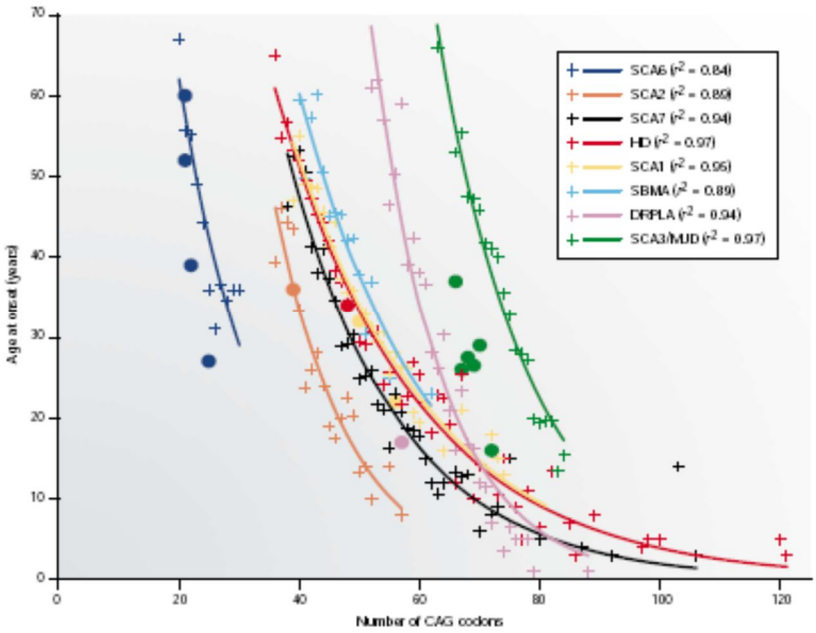


Figure 1.1- Correlation curves between age-at-onset and number of CAG-repeat length for the different polyQ diseases. '+' represent age at onset associated with various CAG repeats lengths. The age at onset for homozygotes for the various disorders is also shown (filled circles), plotted according to the longer of their two expanded CAG repeats. Adapted from Gusella & MacDonald, 2000.

1.3.1 PolyQ aggregates

Nuclear inclusions (NIs) or aggregates have been observed in mice (Ikeda et al., 1996) and in cell cultures (Chai et al., 1999; Evert et al., 1999) expressing truncated forms of MJD proteins. Consistently, Davies and co-workers (1997) reported the presence of an age-dependent appearance of NIs in HD exon-1 transgenic mice model and suggested that the inclusions were formed through self-aggregation of the polyQ repeat (Davies et al., 1997). In 1997, DiFiglia and co-workers reported that the polyQ length influenced the

extent of huntingtin (Htt) aggregation and accumulation in the HD cortex and striatum (DiFiglia et al., 1997). At the same period, Paulson and co-workers (1997) discovered ATXN3 aggregates in *post-mortem* brain tissue of MJD patients (Paulson et al., 1997). Moreover, several studies have verified that aggregation occurs in most animal models of polyQ diseases, such as *Caenorhabditis elegans* (*C. elegans*) (Faber et al., 1999; Satyal et al., 2000), flies (Perez et al., 1998; Warrick et al., 1998), mice (Davies et al., 1997; Martindale et al., 1998) as well as in cellular models (Scherzinger et al., 1997; Cooper et al., 1998). However, the role of NIs of mutant proteins in neurodegeneration remains controversial (Ross, 2002) as several other studies suggest that aggregates can function as a protective cellular mechanism for stacking the toxic mutant proteins, facilitating their degradation (Saudou et al., 1998; Fujigasaki et al., 2001).

These aggregates are usually detergent-insoluble, and in each disease, are known by different names, for example Lewy bodies in PD and plaques or tangles in AD. PolyQ aggregates typically have a fribrillar structure under electron microscopy, and aggregates are occasionally strained by amyloid binding dyes such as Congo-red or thioflavin-T, suggesting that the aggregates take an amyloid-like form (Scherzinger et al., 1997; Huang et al., 1998; Chen et al., 2002). And also in each disease aggregates have different aspects and locations.

1.4. Machado-Joseph disease (MJD)

MJD is the most common SCA subtype in most populations. The phenotype is one of the most variable among SCAs. This disease was first described in North-American families of Azorean ancestry, in the 1970s, later in the mainland and then in non-Portuguese families from all continents (Sequeiros & Coutinho, 1981; Sequeiros & Coutinho, 1993).

Nakano and co-workers reported the first cases, in the Machado family from São Miguel in Azores, in 1972; the disease presented as an autosomal dominant ataxia, and was named by the authors, “Machado disease” (Nakano et al,. 1972). In the same year, Woods and Schaumburg observed the Thomas family, also of Portuguese-Azorean ancestry; although resembling that reported by Nakano and co-workers (1972), some clinical particularities led the authors to define it as a new clinical entity, and they named “nigro-

spino-dentatal degeneration" (Wood & Schaumburg, 1972). In 1976, Rosenberg and co-workers defined a new hereditary ataxia by observation of the Joseph family, originated from Flores Island in Azores, which they named as "Joseph disease" (Rosonberg et al., 1976). A fourth Azorean family was reported to have an "Azorean disease of the nervous system" in 1977 (Romanul et al., 1977). Finally, Coutinho and Andrade (1978) studied the first 15 families from Azores and proposed that the above mentioned diseases were indeed variations of the same clinical disease (Coutinho & Andrade, 1978). Two years later, Lima and Coutinho (1980) proposed the name "Machado-Joseph disease" for this clinical entity (Lima & Coutinho, 1980). In our days, with families described all over the world, MJD is known to be the most common form of dominantly inherited ataxia (15- 45% of all forms in different countries and ethnic populations) (Margolis, 2002; Schols et al., 2004; Sequeiros et al., 2007).

1.4.1 Clinical features of MJD

MJD is a progressive neurodegenerative disease, affecting mainly the motor function. Patients usually present with ataxia, pyramidal and extrapyramidal signs, peripheral amyotrophies, progressive external ophthalmoplegia, intention fasciculation-like movements of facial and lingual muscles, rigidy, and bulging eyes (Coutinho & Andrade, 1978; Lima & Coutinho, 1980; Barbeau et al., 1984; Coutinho, 1992). Importantly, no cognitive impairment is observed in MJD patients; this may be a main criterion in the differential diagnosis with other SCAs including alterations of the intellect (Coutinho, 1992). Usually, patients will become confined to a wheelchair and will later be bedridden. The median survival time after onset is 20 years. Diseases manifestations typically start during adulthood, but the distribution of age at onset is very wide, ranging from 5 to 73 years (Sequeiros & Coutinho, 1993).

The high phenotypic variability associated with MJD, led to the organization of the disease into four sub-types, occasionally present in the same family, sorted by the age of clinical onset and major symptoms of the disorder (Lima & Coutinho, 1980). Sub-type 1 includes the MJD forms with early age of onset (10-30 years of age), a faster progression, and more intense pyramidal and extrapyramidal signs. Sub-type 2 is the most frequent, and is characterized by intermediate age-of-onset (20-50 years of age) and progression, with patients exhibiting the usual ataxia and ophthalmoplegia and the possible evolution to either sub-type. In sub-type 3, patients show the latest age at onset (40-70 years of age), slower disease progression and more peripheral signs. A fourth sub-type was added by

Rosenberg in 1983; it is the rarest and comprehends MJD patients exhibiting Parkinsonic symptoms associated with the more typical MJD symptoms (Rosenberg, 1983; Margolis, 2002).

1.4.2 Neuropathological features of MJD

In the 1980s, Coutinho and co-workers defined the pathological criteria of MJD, nevertheless, the mechanism of neurodegeneration still remains unknown (Coutinho & Sequeiros, 1981). The neuropathological features also show a large variability. Pathological examination of *post-mortem* brains from MJD patients revealed neuronal loss and degeneration of specific brain regions, such as cerebellar dentate nucleus, *pallidum*, *stratium*, *substantia nigra* (*compacta* and *reticulate*), subthalamic, red and pontine nuclei, select cranial nerve nuclei and the anterior horn and Clarke's column of the spinal cord (see Figure 1.2) (Robitaille et al., 1997; Schmidt et al., 1998; Iwabuchi et al., 1999; Gilman, 2000; Yamada et al., 2004; Koeppen, 2005). Structures such as the cerebral and cerebellar cortex and inferior olives are usually spared.

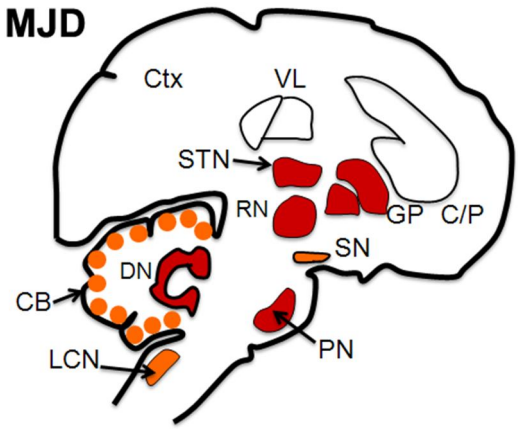


Figure 1.2- Brain regions affected in MJD (illustrated in orange). STN: subtalamic nucleus, GP: *globus pallidus*, RN: red nucleus, SN: *substantia nigra*, PN: pontine nucleus, DN: dentate nucleus, LCN: lateral cuneate nucleus, CB: cerebellar cortex. Adapted from Ross, 1995.

Besides neuronal loss, MJD as other polyQ diseases, is characterized by neuronal NIs containing expanded polyQ, molecular chaperones, ubiquitin, some components of the proteasome and both normal and pathogenic ATXN3 (Paulson et al,. 1997; Perez et al., 1998; Chai et al., 1999; Nagaoka et al., 2003). In contrast to previous neuropathological studies, recent pathoanatomical investigations demonstrated widespread damage to the cerebellum, thalamus, midbrain, pons, *medulla oblongata* and spinal cord in MJD, providing suitable explanations for a variety of less understood clinical MJD symptoms

(Rub et al., 2003a; Rub et al., 2003b; Rub et al., 2003c Rub et al., 2004; Rub et al., 2005; Rub et al., 2006a; Rub et al,. 2006b; Rub et al., 2007).

1.4.3 Genetics features of MJD

MJD is transmitted in an autosomal dominant manner, meaning that one copy of the defective *ATXN3* gene is sufficient for the disease to be transmitted to the offspring (Sequeiros & Coutinho, 1981). Intergenerational instability in the transmission of the mutated allele is common in MJD. The affected offspring commonly manifest the disease earlier in life, with faster progression and aggravated symptomatology. This phenomenon of *anticipation* is common in MJD families (Sequeiros & Coutinho, 1981; Sequeiros & Coutinho, 1993). A longer expansion is associated an earlier age of onset and a more severe form of the MJD.

1.4.4 The *MJD1/ATXN3* gene

The mutation associated with MJD is the expansion of a $(CAG)_n$ tract in the coding region of the *MJD1/ATXN3* gene, mapped to the long arm of chromosome 14 (14q24.3-32.1) in 1993 (Takiyama et al., 1993). The molecular diagnosis of MJD subsequently became possible, and the mutation was then confirmed in families of different origins (Maciel et al., 1995; Higgins et al., 1996; Lopes-Cendes et al., 1996; Gaspar et al., 2001). MJD is caused by the expansion of a CAG repeat in the 3' coding portion of the gene. The repeat is highly polymorphic, ranging from 12-41 repeats units in normal-sized alleles, and 55-86 in the expanded allele of MJD patients (Maciel et al., 2001). Even though the range of repeat lengths with reduced penetrance is not strongly established in MJD as in other SCAs, individuals with alleles carrying a CAG repeat between 45-51 have been described that may or may not manifest the disorder (Maciel et al., 2001; Gu et al., 2004; Padiath et al., 2005). The human *MJD1/ATXN3* gene spans 48 Kbp and comprises 11 exons; the CAG repeat is located in exon-10 (Ichikawa et al., 2001).

1.4.5 The ATXN3 protein(s)

The *MJD1/ATXN3* gene encodes the protein ATXN3, the smallest of the proteins known to be involved in polyQ diseases with a molecular weight around 42kDa in normal individuals. The human ATXN3 protein has a conserved N-terminal Josephin domain (JD), which is a conserved module named after MJD, containing the putative catalytic triad aminoacids cysteine (C14), histidine (H119) and asparagine (N134). Depending on the

isoform, ATXN3 is followed by two or three ubiquitin-interacting motifs (UIMs) (Young et al. 1998). Bioinformatics analyses showed that ATXN3 also contains a conserved nuclear-localization signal (NLS) directly upstream of the polyQ sequence (Tait et al., 1998). The polyQ stretch of variable in length is in the C-terminus region of ATXN3 (Kawaguchi et al., 1994).

Northern blot analysis revealed ubiquitous *ATXN3* expression and suggested the existence of four different transcripts with different sizes (1.4, 1.8, 4.5 and 7.5 Kb), thought to result from alternative splicing and polyadenylation events, occurring in exons 2, 10 or 11 (Ichikawa et al., 2001). Five *ATXN3* cDNA variants were described: MJD1a, MJD1-1, MJD5-1, MJD2-1 and H2 (Kawaguchi et al., 1994; Goto et al., 1997); the latter lacks exon 2, but preserves the open reading frame, identical to variant MJD1-1 (Ichikawa et al., 2001). A recent study reported fifty-six novel splicing variants of the *ATXN3* gene from which nineteen are likely to be translated into at least twenty different protein isoforms (Bettencourt et al., 2010). The physiological and clinical relevance of these new variants remains to be determined.

ATXN3 is expressed throughout the body and in all regions of the brain, including areas generally spared by the disease (the cerebral cortex) (Trottier et al., 1995; Paulson et al., 1997; Wang et al., 1997). ATXN3 is highly present in the cytoplasm of most cells, but can be present also in the nucleus and has been associated with the nuclear matrix, endoplasmic reticulum (ER) and mitochondria (Paulson et al., 1997; Schmidt et al., 1998; Tait et al., 1998; Trottier et al., 1998; Fujigasaki et al., 2000). As in other polyQ diseases, the abnormal and normal allele products were shown to be equally expressed in lymphoblastoid cells and in the brain of MJD patients (Paulson et al., 1997; Wang et al., 1997). Isoform 1 (3 UIMs) is the most abundant ATXN3 isoform in brain of patients and transgenic mice (Harris et al., 2010).

ATXN3 homologous genes are present in different organisms, including plants, nematodes and humans. Several proteins have been identified that display high homology to human ATXN3 in other species, namely, Rattus norvegicus 88% (Schmitt et al., 1997), Mus musculus 88% (Costa et al., 2004), Gallus gallus 82% (Linhartova et al., 1999), *C. elegans* 38% (Rodrigues et al., 2007), and other organisms. Curiously, the long polyQ tract seems to be human-specific because it is absent in other species such as mouse and *C. elegans*, which have only six and one glutamines in their *ATXN3* homologues, respectively.

1.5. Pathogenic mechanisms of polyQ diseases

PolyQ disorders are characterized clinically by the constant progression of the symptoms and by the protein accumulation inside neurons causing their dysfunction and ultimately death. Although it has been years since the identification of the genetic basis of polyQ diseases, their molecular basis is still controversial. Several cellular and molecular mechanisms have been proposed to explain polyQ pathogenesis (see Figure 1.3).

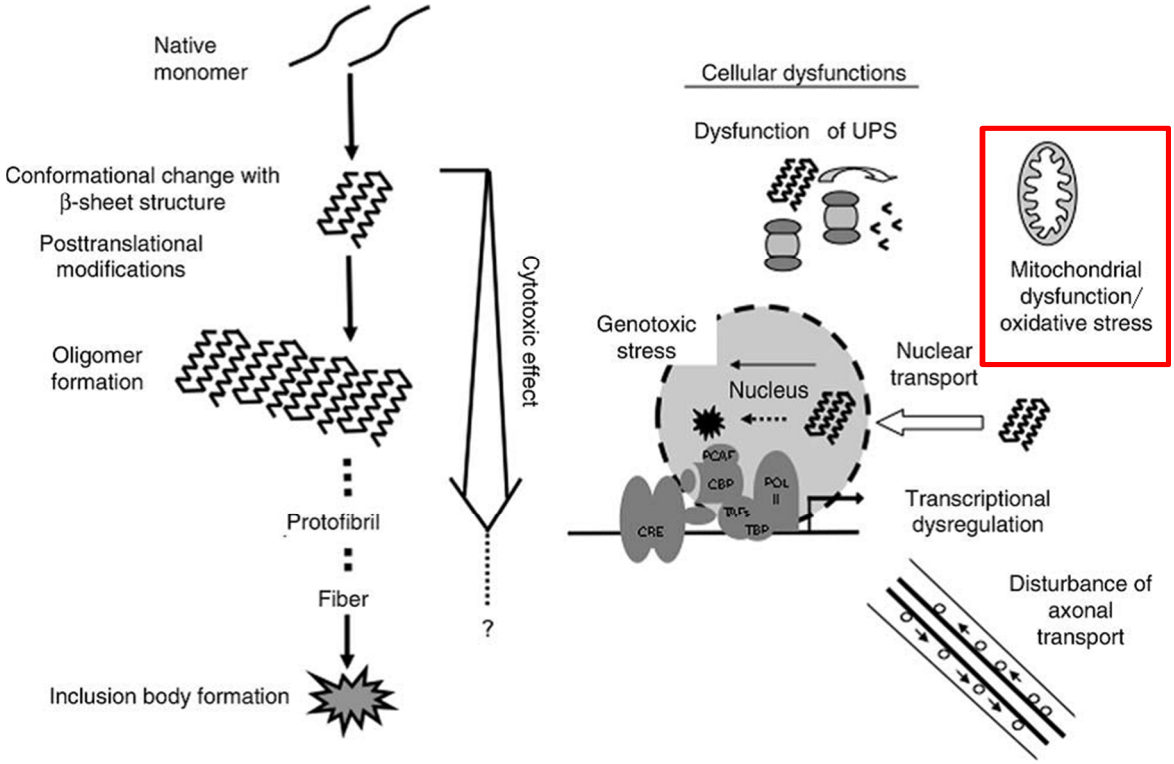


Figure 1.3- Molecular mechanisms underlying pathogenesis of polyQ diseases. Hypothetical pathway of conformational change, oligomerization and formation of inclusion bodies. The native expanded polyQ monomer undergoes conversion to a misfolded β-sheet conformation, resulting in formation of NIs through assembly of oligomeric intermediates. Expanded polyQ proteins may exert toxicity as a misfolded monomer or toxic oligomers. The major toxic effects of these structures may involve alterations in transcription, impairment of the UPS, mitochondrial dysfunction, dysregulation of intracellular Ca^{2+} homeostasis, impairment of axonal transport and genotoxic stress in polyQ diseases. Adapted from Takahashi et al., 2010.

1.5.1 Mitochondrial dysfunction in polyQ diseases

Considering that mitochondrial dysfunction may originate from mutations in more than 1000 genes (Larsson & Clayton, 1995), from the deleterious effects of many toxic compounds (Kovacic et al., 2005), and even occur spontaneously during ageing (Reeve et al., 2008), it is almost not surprising that mitochondrial disorders are much more frequent

than previously thought (Elliott et al., 2008). The mitochondrial theory of neurodegeneration and aging implies that reactive oxygen species (ROS), mitochondrial DNA (mtDNA) damage, and progressive respiratory chain defect are mutually interacting in a vicious circle (Harman, 1972; Bender et al., 2006).

Protein aggregates might induce oxidative stress by producing free radical species, resulting in protein and lipid oxidation, elevation of intracellular calcium levels and mitochondrial dysfunction (Behl et al., 1994; Hsu et al., 2000). Oxidative stress has been shown to contribute to the pathogenesis of many polyQ diseases (Bence et al., 2001; Butterfield & Kanski, 2001; Imaizumi et al., 2001; Martindale & Holbrook, 2002). A cellular condition of oxidative stress develops when the mitochondrial oxidative phosphorylation and the cell's antioxidative capacity become overloaded. In these conditions, ROS are generated in excess and damage to the cell and its DNA, RNA, lipid and protein constituents may possibly occur. In this context, misfolded protein structures may be particularly susceptible to oxidative modifications, which may promote unfolding and thus increase the susceptibility to further modifications that exaggerate the stress responses (Dukan et al., 2000). Although the exact mechanisms and order of events may be quite different in the various polyQ diseases, the result seems similar: chronic stress and ultimately death of the cell.

Oxidative stress related with deficiency of the oxidative phosphorylation and the induction of stress responses are well established as part of the gain-of-function occurring in neurodegenerative diseases. Deficient activity of components of the mitochondrial respiratory chain (MRC) has been found in brain cells of patients with AD, PD and HD (Butterfield & Kanski, 2001; Mattson et al., 2002). Biochemical analyses of the brain of a HD patient showed abnormal aspects of energy metabolism, including reduced glucose metabolism, decreased mitochondrial complex activity and increased lactate concentration (Browne et al., 1997). Reduced concentrations of creatine (Cr) and phosphocreatine (PCr) have been observed in the basal ganglia of HD patients (Sanchez-Pernaute et al., 1999). In addition, mitochondria from HD patients and HD transgenic mice exhibited lower membrane potentials and required lower calcium loads for depolarization than mitochondria from controls (Sawa et al., 1999; Panov et al., 2002). These findings reinforcing the idea that oxidative stress and mitochondrial dysfunction play an important role in polyQ diseases pathogenesis.

Yu and co-workers (2009) demonstrated that mtDNA copy numbers are decreased in mutant cells and MJD patient's leukocytes samples compared with the normal controls. Furthermore, the amount of the common mtDNA 4,977-bp deletion is higher in MJD patients compared with that in normal individuals. These results suggest that the cell damage caused by greater oxidative stress in MJD mutant cells plays an important role, at least in part, in the disease progression. (Yu et al., 2009).

1.6 Therapeutic approaches for polyQ diseases

There is no effective cure for MJD and all other polyQ diseases. The lack of therapeutic approaches that effectively prevent neurodegeneration in MJD patients prompted us to search for compounds that modulate mutant ATXN3 aggregation and the associated neurological dysfunction.

Potential therapeutic approaches for polyQ diseases could be divided into two classes: those targeting directly the mutant proteins and those targeting the toxic effects of the mutant proteins such as mitochondrial dysfunction and oxidative stress, transcriptional abnormalities, autophagy, UPS impairment, excitotoxicity, or apoptotic pathways such as preventing the cellular damage (Bauer & Nukina, 2009) (see Figure 1.4).

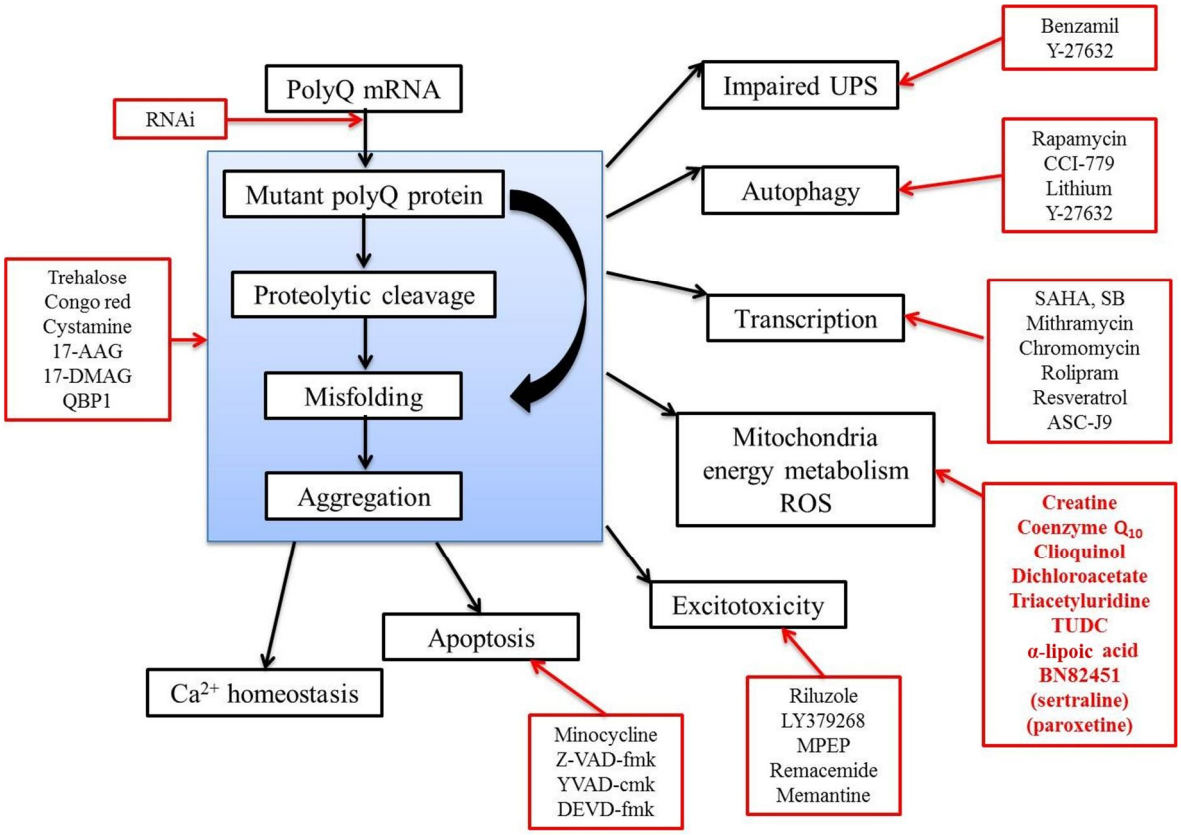


Figure 1.4- Summary of therapeutic approaches for polyQ diseases. Blue background highlights the hypothetic processing of the mutant polyQ protein. Molecules targeting different events in polyQ disease pathogenesis (black boxes) are displayed in red boxes. Written in red are molecules targeting mitochondrial dysfunction. Adapted from Bauer & Nukina, 2009.

Few research papers focused on developing therapies for MJD have been published to date. Chen and co-workers (2008) found that administration of dantrolene (a clinically relevant stabilizer of intracellular Ca^{2+} signaling) improved motor performance and prevented neuronal cell loss in the pontine nuclei and *substantia nigra* of the transgenic mouse model SCA3-YAC-84Q (Chen et al., 2008). Their results indicate that deranged Ca^{2+} signaling may play an important role in MJD pathology and that Ca^{2+} signaling stabilizers such as dantrolene may be considered as potential therapeutic drugs for treatment of MJD patients.

Menzies and co-workers (2010) showed that cell cycle inhibitor-779 (CCI-779) treatment (a water soluble rapamycin ester which demonstrates the same activity as rapamycin) significantly improved motor performance in a transgenic mouse model of MJD (a transgenic mice expressing mutant full-length ATXN3 with an expanded polyQ repeat region of 70 residues), decreased the number of ATXN3-positive aggregates seen in

the brains of transgenic mice and also decreased soluble mutant ATXN3 levels (Menzies et al., 2010). CCI-779 is designed for long-term use in patients and therefore represents a possible therapeutic strategy for the treatment of MJD.

Chou and co-workers (2011) tested the hypothesis that HDAC inhibitor sodium butyrate (SB) alleviates ataxic symptoms of MJD transgenic mice by reversing ataxin-3-Q79-induced histone hypoacetylation and transcriptional repression. SB treatment significantly reversed ATXN3-Q79-induced histone hypoacetylation and transcriptional downregulation in the cerebellum of ATXN3-Q79 transgenic mice and also delayed the onset of ataxic symptoms, improved neurological phenotypes and improved the survival rate of ATXN3-Q79 transgenic mice (Chou et al., 2011). Their results suggest that SB might be a promising therapeutic agent for MJD.

1.6.1 Therapeutic approaches to polyQ diseases: targeting mitochondria

As mentioned before, damage of mitochondrial functions is one of the key events in polyQ diseases leading to cell death by activation of apoptotic cascades. The process of mitochondrial dysfunction is accompanied by impaired respiration; stress-induced mitochondrial depolarization; increased free radical production, with oxidative damage; and globally abnormal energy metabolism in polyQ diseases (Grunewald & Beal, 1999; Panov et al., 2002; Browne & Beal, 2006).

Different compounds improving energy metabolism defects or reducing oxidative stress in polyQ diseases have been successfully tested in mouse models. Antioxidants such as α-lipoic acid, coenzyme Q_{10} (CoQ_{10}) clioquinol, tauroursodeoxycholic acid (also with antiapoptic affects), and BN82451 have been proven effective in the R6/2 mouse lines of HD (Giampa et al., 2009). As mentioned before, reduced concentrations of Cr and PCr were observed in basal ganglia of HD patients (Sanchez-Pernaute et al., 1999); accordingly, in two mouse models of HD, Cr administration stabilized the mitochondrial permeability transition (MPT), prevented ATP depletion, increased the protein synthesis (Ferrante et al., 2000; Andreassen et al., 2001) and also ameliorated motor symptoms and the brain pathology.

1.6.2 Creatine (Cr), Phosphocreatine (PCr) and Creatine Kinase (CK)

Cr [α-methyl-guanidinoacetic acid] is a natural substance in vertebrates and helps to supply energy to muscle and nerve cells. In humans, Cr is taken up from the food, especially meat and fish, (approximately 1-2 g/day) by intestinal absorption (Khan & Cowen, 1977; Dvorak, 1981). Cr is also synthesized endogenously mainly in the kidney, pancreas, and liver (approximately 1-5 g/day) by the action of arginine: glycine amidinotransferase (AGAT) and guanidinoacetate methyltransferase (GAMT).

Cr is taken up into the muscle and brain by a sodium-dependent active transmembrane creatine transport system (CrT) and becomes physiologically active when it is transformed enzymatically into PCr. The Cr/PCr system, regulated by the mitochondrial creatine kinase (CKMi), plays an essential role in maintaining the high energy levels necessary for brain development and functions, through regeneration and buffering of ATP/ADP levels (Wallimann et al., 1992, 2007; Wyss & Kaddurah-Daouk, 2000; Brosnan & Brosnan, 2007; Andres et al., 2008). Intracellular Cr and PCr are non-enzymatically converted, at an almost constant rate constant (of 1.5%/day) of body Cr, into creatinine (Crn), which passively diffuses out of the cells and is excreted by the kidneys into the urine. The daily urinary Crn excretion is directly proportional to the total Cr stores in the body (Greenhaff ,1997). Cr loading by adults at a rate of 5 g/day for 5 days increases arterial Cr levels (Poortmans et al., 1997). In muscle, PCr levels can rise an average of 20% accompanied by a 20% mean increase in peak work performance (Greenhaff, 1997) and a loss of fat, and increase of lean body mass (Vandenberghe et al., 1997).

As mentioned before, the Cr/PCr system is thought to be physiologically important in tissues with high and variable energy requirements, like brain and muscle (Wallimann & Hemmer, 1994). In these cells, high-energy PCr serves not only as an immediate temporal energy buffer, but also as an energy shuttle from subcellular sites of energy production (mitochondria and/or glycolysis) to sites of energy consumption (ion pumps and various other ATPases) where creatine kinase (CK) isoenzymes are specifically localized in a compartmented fashion (Bessman & Geiger, 1981; Wallimann et al., 1992) (see Figure 1.5). In the cytoplasm and membrane compartments of neurons, as well as glial cells in culture (Manos et al., 1991; Molloy et al., 1992) and *in vivo* (Hemmer et al, 1994; Kaldis et al., 1996), CK isozyme BB catalyzes the reversible conversion of PCr and ADP to ATP and Cr to manage different aspects of demands in the brain (Hemmer & Wallimann, 1993; Chen et al., 1995). On the other hand, CKMi catalyzes the reversible conversion of Cr and

ATP to ADP and PCr to generate energy reserves in the cytoplasm (Wyss et al., 1992). PCr reserves are depleted even more rapidly than ATP during ischemia (Passonneau & Lowry, 1971) owing to their conversion to ATP by CK.

Although the brain constitutes only 2% of the body mass, it can be responsible for up to 20% of total energy consumption under resting conditions. Accordingly, it necessitates a significant ATP turnover in order to maintain membrane potentials as well as the signaling activities of the central and peripheral nervous system. The presence of CK isoforms in the brain and spinal cord underlines the energetic role of Cr/PCr system on central nervous system (CNS) (Andres et al., 2008).

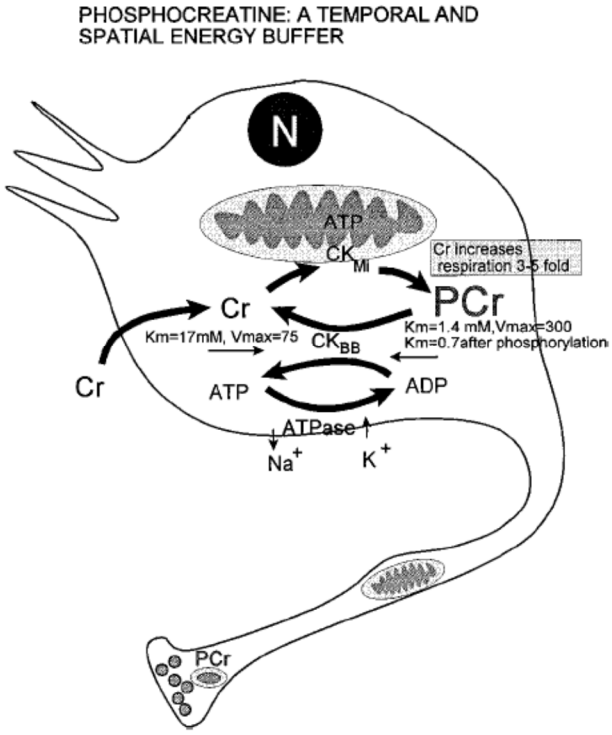


Figure 1.5- Relationship of Cr and PCr to ATP in the neuron. Exogenous Cr should be readily transported into neurons by a CrT or synthesized if precursors are available (Dringen et al., 1998). Under resting levels of ADP, ATP produced in the mitochondria is converted to PCr by CKMi and released into the cytoplasm. In the cytoplasm, the equilibrium constants for brain CK and CKBB (Quest et al., 1990; Boehm et al., 1996, 1998). Adapted from Brewer & Walliman, 2000; drawn from information on rat heart and chick brain.

1.6.3 Cr supplementation on neurodegenerative diseases

One potential mechanism of neuroprotection of Cr is buffering of intracellular energy stores by increasing PCr levels. CK is also coupled directly or indirectly to energetic processes required for Ca^{2+} homeostasis (Wallimann et al., 1992; Steeghs et al., 1997).

The interest in the Cr/PCr system and, consequently, Cr supplementation effects on the CNS is relatively recent. The fact that exogenous Cr is able to cross the blood–brain barrier (BBB) and increase the cerebral Cr concentration has generated interest in the scientific community, since several neurological diseases are associated with reduced cerebral Cr content. The promising effects of Cr supplementation on neurodegenerative diseases are notable (see Table 1.2).

Table 1.2-
Clinical trials investigating the effects of Cr supplementation on neurodegenerative diseases. Adapted from Andres et al., 2008.

Authors and year	Disease (n)	Study Design	Cr Protocol	Effect
Mazzini et al., 2001	ALS (28)	Open trial	20 g/day for 7days + 3 g/day for 6months	↑Isometric power
Shefner et al., 2004	ALS (104)	Randomized, double-blind, placebo-controlled	20 g/day for 5days + 5 g/day for 6months	None
Tabrizi et al., 2003	HD (13)	Open trial	10 g/days for 1year	Possible stabilization of signs[a]
Bender et al., 2005	HD (20)	Open trial	20 g/days for 5days + 6 g/days for 8–12weeks	↓[Glutamate]
Tabrizi et al., 2005	HD (13)	Open trial	10 g/days for 2years	Possible stabilization in some patients[a]
Hersch et al., 2006	HD (64)	Randomized, double-blind, placebo-controlled	8 g/days for 16weeks	↓Oxidative stress
Bender et al., 2006	PD (60)	Randomized, double-blind, placebo-controlled	20 g/days for 6days + 2 g/days for 6months + 4 g/days for 18months	↑Mood
NINDS NET-PD, 2006	PD (134)	Randomized, double-blind, placebo-controlled	10 g/days for 1year	Not considered futile[b]
Hass et al., 2007	PD (20)	Randomized, double-blind, placebo-controlled	20 g/days for 5days + 5 g/days for 12weeks	↑Muscle function ↑Strength

↑ = improved or increased, ↓ = decreased
[a]As inferred by the lack of disease deterioration
[b]A treatment is considered futile when it does not show good therapeutic value

There is ample evidence showing the presence of CK in pyramidal cells, which are involved in memory and learning processes (Kaldis et al., 1996). In light of this, some investigations have addressed the effects of Cr supplementation on the cognition process.

ALS is a severe neurodegenerative disease that occurs with progressive motor neuron loss (Andres et al., 2008). In transgenic rats mimicking this disease, reductions in ATP content (Browne et al., 2006) and CK activity were observed (Wendt et al., 2002). Moreover, Cr supplementation seems to present neuroprotective properties in the same model, likely due to its direct antioxidant capacity or via the energetic role of the Cr/PCr system (Dupuis et al., 2004). Oral supplementation with 1 and 2% Cr in the ALS mouse model starting at 70 days of age protected against the loss of neurons in both the motor cortex and the *substantia nigra*, reduced the level of oxidative damage, produced a dose-dependent improvement in motor performance and extended survival (Klivenyi et al., 1999). In spite of these promising findings in animal models, the first clinical trials did not reveal significant benefits in ALS patients (see Table 1.2).

AD is a common disease characterized by dementia. Cerebral CK expression seems to be reduced in patients with this disease (Aksenov et al., 1997). Appreciably, it has been demonstrated that Cr exerts neuroprotective effects on neurons cultivated in neurotoxic media, induced by glutamate and β-amyloid protein (Brewer & Wallimann, 2000). Nevertheless, clinical trials are needed to assess whether Cr supplementation can be an effective approach against AD.

As revealed before, HD is an autosomal dominantly inherited neurodegenerative disease that clinically occurs with progressive movement loss and important cognitive and emotional dysfunction (Andres et al., 2008). There is evidence linking the severity of this disease to increased lactate concentration, decreased muscle PCr content, and mitochondrial dysfunction (Grunewald & Beal, 1999). Accordingly, Cr supplementation was proposed to be an appropriate therapeutic approach to prevent the gradual neuronal loss. Ferrante and co-workers (2000) placed HD transgenic mice (line 6/2) and wild-type (WT) littermates on either unsupplemented diets or diets supplemented with 1, 2 or 3% Cr at 21 days of age. Cr supplementation significantly improved body weight and motor performance, delayed the onset of diabetes present in this particular model, and reduced the development of brain atrophy, loss of striatal neurons and formation of Htt-positive aggregates (Ferrante et al., 2000). Cr also exerted similar therapeutic effect in another transgenic mouse model of HD (N171-82Q) (Adreassen et al., 2001). Clinical studies in patients with HD have demonstrated that Cr supplementation reduces oxidative stress

(Hersch et al., 2006) and glutamate concentration (Bender et al., 2005); in addition to normalizing cerebral Cr content (Ryu et al., 2005).

PD is characterized by a predominant and progressive loss of dopaminergic neurons in the *substantia nigra*, resting tremor, bradykinesia, rigidity, and postural imbalance (Andres et al., 2008). Evidence indicates that a defect in electron transport chain might be involved in the physiopathology of PD (Schapira et al., 1990; Alam & Schmidt, 2002), which suggests that therapeutic strategies focused on improvement in mitochondrial function may be promising. *In vitro* experiments mimicking PD revealed that Cr has protective effects against neurotoxic insults. Systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) produces parkinsonism in experimental animals by a mechanism involving impaired energy production. MPTP is converted by monoamine oxidase B to 1-methyl-4-phenylpyridinium (MPP1), which blocks complex I of the electron transport Chain (Matthews et al., 1999). Matthews and co-workers (1999) found that oral supplementation with each 1% Cr or 1% cyclocreatine for 2 weeks produced significant protection against MPTP-induced dopamine depletion in mice. Using an experimental *in vitro* paradigm of PD, Andres and co-workers (2005) observed neuroprotective effects of Cr against toxic insults induced by 6-hydroxydopamine (6-OHDA) (Andres et al., 2005a) or 1-methyl-4-phenyl pyridinium (MPP+) (Andres et al., 2005b) exposure in ventral mesencephalic rat cultures. Nevertheless, the results of clinical studies in PD patients remain controversial (Bender et al., 2006; Hass et al., 2007).

1.7 Animal models for MJD

Appropriate disease model systems are an essential tool in understanding the molecular mechanism underlying the pathogenesis and in development in therapeutic approaches. Cell culture and mouse models continue to be mainstays to complement study of many diseases for their complexity and simplicity, respectively. In recent times, invertebrate models (flies and worms) have started been used to great advantage in the study of neurodegenerative disease (Link, 2001; Driscoll & Gerstbrein, 2003; Thompson & Marsh, 2003; Westlund et al., 2004).

Since the discovery of the disease-causing gene in MJD (the mutant *ATXN3* gene), several *in vitro* (Evert et al,. 1999; Yoshizawa et al., 2000; Yoshizawa et al., 2001) and *in*

vivo (Ikeda et al., 1996; Warrick et al., 1998; Cemal et al., 2002; Goti et al., 2004; Bichelmeier et al., 2007; Alves et al., 2008; Chou et al., 2008; Boy et al., 2009; Boy et al., 2010; Silva-Fernandes et al., 2010; Teixeira-Castro et al., 2011a) models have been developed, in order to investigate the underlying pathology, identify factors and pathways that modify the disease process, and test potential therapeutic approaches. These MJD models have been developed using mouse and rat, the fruitfly *Drosophila melanogaster* and the nematode *C. elegans*. The following is a succinct description of MJD animal models available to data.

1.7.1 Mouse models of MJD

The first MJD mouse model was generated expressing the complete and truncated cDNAs of *MJD1/ATXN3* gene specifically in the Purkinje cells of the cerebellum (slightly affected in MJD). The transgenic mice expressing the C-terminal ATXN3 with 79Q were ataxic, displayed cerebellar atrophy and Purkinje cells loss, four weeks after birth (Ikeda et al., 1996).

Since 1996, seven additional mouse models have been generated (Cemal et al., 2002; Goti et al., 2004; Bichelmeier et al., 2007; Chou et al., 2008; Boy et al., 2009; Boy et al., 2010; Silva-Fernandes et al., 2010). Cemal and co-workers (2002) generated transgenic mouse models using a yeast artificial chromosome (YAC) containing MJD1 gene with 64 and 84 CAG repeats (Cemal et al., 2002). Animals with the expanded alleles developed a very mild and slowly progressive cerebellar deficit, manifesting as early as 4 weeks of age. NIs and cell loss were observed in the pontine and dentate nuclei, with variable affection of the cerebellar cortex and of Purkinje cells. As the disease progressed, pelvic elevation became markedly flattened, and this was accompanied by hypotonia, and motor and sensory loss. This mouse was the first model described expressing the full length *MJD1* gene under the control of its own regulatory elements. Goti and co-workers (2004) had observed a much more severe phenotype in a mouse model expressing human expanded ATXN3 (variant MJD1a) with Q71 or normal ATXN3 (Q20) under the control of a mouse prion protein promoter (moPrP). Q71 transgenic mice expressing mutant ATXN3 above a critical level experienced a phenotype similar to MJD including progressive postural instability, gait and limb ataxia, weight loss, premature death, neuronal intranuclear inclusions, and decreased tyrosine hidroxylase -positive neurons in the *substantia nigra*. Q20 transgenic mice had normal behavior and pathology. Interestingly, a C-terminal cleavage fragment of the transgene product has been revealed in this model, which the

researchers also observed in human patients' post-mortem brains (Goti et al., 2004). The group of Olaf Riess (2007) developed another MJD transgenic mouse model expressing the full-length ATXN3 with 15, 70 or 148 CAG repeats under the control of the moPrP transgenic mice for ATXN3 with 15 CAG repeats were normal with no signs of neurodegeneration, while mice expressing mutant ATXN3 with (70 and 148 CAG repeats) displayed a neurological phenotype including reduced motor and exploratory activity, tremor and premature death by 3 to 6 months. ATXN3 and Ub-positive inclusions were observed in almost all brain with the exception of Purkinje cells. Q148 mice displayed a more severe neurological phenotype with more inclusions and earlier death when compared with mice expressing mutant ATXN3 with 70 CAG repeats. The authors proved that the nuclear localization of the mutant protein is crucial for pathogenesis to occur (Bichelmeier et al., 2007). In 2008, a new transgenic mouse model was generated expressing the human ATXN3 cDNA containing 79 CAG repeats, under the moPrP. These transgenic mice displayed motor dysfunction with an onset age of 5–6 months, and neurological symptoms deteriorated in the following months. A prominent neuronal loss was not found in the cerebellum of 10 to 11-month-old ATXN3-Q79 mice displaying pronounced ataxic symptoms, suggesting that instead of neuronal demise, ATXN3-Q79 causes neuronal dysfunction of the cerebellum and resulting ataxia (Chou et al., 2008). In order to analyze whether symptoms caused by ATXN3 with an expanded repeat are reversible *in vivo*, a conditional mouse model of MJD with the full-length human ATXN3 cDNA with 77 repeats was created using the Tet-Off system (Boy et al., 2009). The trangenic mice developed a progressive neurologic phenotype characterized by neuronal dysfunction in the cerebellum, reduced anxiety, hyperactivity, impaired rotarod performance and decreased gain of body weight. After turning off the expression of the expanded ATXN3 at an early symptomatic stage of the disease the observed motor symptoms regressed and after five months of treatment, transgenic MJD mice were indistinguishable from their controls suggesting that both neurological and physiological symptoms of MJD are reversible. The same group published another MJD transgenic mouse model expressing ATXN3 with 148 CAG repeats under the control of the Htt promoter, resulting in ubiquitous expression throughout the whole brain (Boy et al., 2010). The model resembles many features of the disease in humans, including a late onset of symptoms and CAG repeat instability in transmission to offspring. They observed a biphasic progression of the disease, with hyperactivity during the first months (not seen in

human patients) and a decline of motor coordination after about 1 year of age; however, NIs were not visible at this age, but only at the age of 18 months.

Recently, our lab generated two transgenic mouse lineages expressing the expanded human ATXN3 under the control of the cytomegalovirus promoter (pCMV): CMVMJD83 and CMVMJD94, carrying Q83 and Q94 stretches, respectively. Behavioral analysis revealed that the CMVMJD94 transgenic mice developed motor uncoordination, intergenerational instability of the CAG repeat and a tissue-specific increase in the somatic mosaicism of the repeat with aging. Histopathological analysis of MJD mice at early and late stages of the disease revealed neuronal atrophy and astrogliosis in several brain regions; however, they found no signs of microglial activation or neuroinflammatory response prior to the appearance of an overt phenotype. In this model, the appearance of MJD-like symptoms was also not associated with the presence of ATXN3 cleavage products or NIs. The transgenic CMVMJD94 mice as a useful model to study the early stages in the pathogenesis of MJD and to explore the molecular mechanisms involved in CAG repeat instability (Silva-Fernandes et al., 2010).

All these MJD transgenic mouse models suggest that despite the expression of mutant ATXN3 in the whole brain, there are considerable differences in the affected brain regions and cell types. However, no model has yet completely reproduced the pathological and clinical symptoms observed in MJD patients.

1.7.2 Rat model of MJD

Overexpression of mutant ATXN3 in the rat brain using lentiviral vectors (LV) led to neuropathological abnormalities: formation of ubiquitylated ATXN3 aggregates, alpha-synuclein immunoreactivity and loss of dopaminergic markers (TH and VMAT2). Alves and co-workers (2008) proposed striatal pathology as contributing factors for the dystonia and chorea observed in some MJD patients (Alves, et al., 2008).

1.7.3 *Drosophila melanogaster* models of MJD

The first transgenic *Drosophila melanogaster* model of a human neurodegenerative disease was obtained for MJD (Warrick et al., 1998) and demonstrated formation of neuronal inclusions and late-onset cell degeneration. Interestingly, different sensitivity to the expanded protein has been observed among different cell types, the neurons being particularly susceptible. More recently, the same group, using the *Drosophila melanogaster* model, has established a direct link between normal protein function and

disease pathogenesis, namely in relation to ubiquitin pathways in MJD. Their findings underline the importance of the normal function of normal ATXN3 in MJD pathogenesis and suggest a potential protective role of ATXN3 activity for polyQ diseases (Warrick et al., 2005). Thus, *Drosophila melanogaster* models have significantly contributed to our growing understanding of the molecular bases of MJD.

1.7.4 *C. elegans* models for MJD

As described in more detail later, *C. elegans* systems represent an attractive intermediate model system by combining sufficient complexity to allow investigation of both cellular and behavioral phenotypes with a simplicity that facilitates rapid, high-throughput testing of hypotheses.

Khan and co-workers (1996) described a study of the pathogenic effects of polyQ expanded tracts within the context of ATXN3 (Khan et al., 2006). Full-length ATXN3 (variant MJD1a) was expressed in *C. elegans* nervous system under the regulation of the *unc-119* promoter, which is first active at the pre-comma embryonic stage (Altun-Gultekin et al., 2001). Animals expressing full-length ATXN3 with 130 glutamines showed age-dependent aggregation and toxicity (only in animals 7 days-old or more). In contrast, expression of a truncated form of ATXN3 (containing only the polyQ domain and C-terminal region) caused polyQ length-dependent aggregation and toxicity. Additionally, expanded polyQ proteins caused interruption of the synaptic transmission and induced swelling and abnormal branching of neuronal processes (Khan et al., 2006). All neurons showed equal susceptibility to the expression of expanded polyQ proteins.

Teixeira-Castro and co-workers (2011) have established a new *C. elegans* model of MJD in which ATXN3 was expressed throughout the nervous system. In this model, expression of both full-length and truncated forms of ATXN3, with different Q-lengths, results in a consistent pattern of neuronal cell-type specific aggregation, with the ventral and dorsal nerve-cord neurons being highly affected, while some lateral interneuron cell bodies are resistant. Certain sensory processes in the head contain aggregated foci, but only when the polyQ stretch is within the ATXN3 protein flanking sequences and not when expressed alone. They have also studied the impact of aging and of reprogramming animals' survival in their model, and found that reducing 1-like signaling (IIS) and activating heat-shock factor 1 (HSF-1) pathways (genetically or pharmacologically) reduced pathogenesis, supporting the mechanistic links between the aging process and neuronal toxic-protein aggregation, which are common hallmarks of neurodegenerative

disorders (Teixeira-Casto et al, 2011). They found that full-length ATXN3 aggregates only at high Q-length (AT3q130), and not with a WT Q-length (AT3q14).The presence of aggregated protein was confirmed by biophysical approaches (FRAP). Interestingly, AT3q130 proteins did not form aggregates in all *C. elegans* neuronal cells. This data suggested that this *C. elegans* model recapitulates an important aspect of polyQ diseases - the neuronal subtype-specific inclusion body formation. Therefore, this AT3q130 *C. elegans* model mimics MJD in many aspects, namely by showing a polyQ-length dependent aggregation that directly correlates with a progressive neurological dysfunction (Teixeira-Casto et al., 2011a).

1.8. *C. elegans* as a model system

Due to the complexity of the vertebrate brain and the fact that mammalian model organisms have their own limitations (e.g. cost, laborious transgenesis, long life spans), invertebrate models including *C. elegans* have been used in the past decade to delineate the molecular mechanisms underlying neurodegeneration.

C. elegans is a small, bacteriovorous nematode (or roundworm) found commonly in many parts of the world, first described by Emile Maupas in 1900 (Maupas, 1900). In 1960s Sydney Brenner selected *C. elegans* as a simple model for his intensive studies of the genetics and development of the nervous system, due to its characteristics as a model organism (Brenner, 1974).

Caenorhabditis elegans (*Caeno*: recent; *rhabditis*: rod; *elegans*: nice) is a free-living, non-parasitic soil nematode that can be safely used in the laboratory. *C. elegans* is transparent, small (two sexes, hermaphrodites and males, are each about 1mm in length as an adult) but easy to visualize under the microscope. It thrives in simple Petri-dish cultures, can be cultivated in large number (about 10 000 worms/Petri-dish) and is cheap to maintain in the laboratory; it feeds primarily on bacteria such as *Escherichia coli* (*E.coli*).

C. elegans is an animal, and so has, like other animals, muscles, a nervous system, a digestive system, and skin. It is a simple organism, both anatomically and genetically. The adult hermaphrodite contains exactly 959 cells and their position is constant. Sulston and co-workers (1983) mapped the origins and fates of all these cells during the

development (Sulston et al., 1983). The nervous system is the most complex organ in *C. elegans* – a third of the cells in the body are neurons (302 out of the 959 cells), located in the pharynx, ganglia in the head and tail and also in the ventral cord – and all the synapses, connections and neuronal circuits are well characterized, making possible to detect even minor defects (White et al., 1976; Albertson et al., 1976). The nervous system mediates a rich variety of behaviors, despite the small number of component neurons: animals move forward and backward by propagating sinusoidal waves along their bodies; they perform exploratory movements with their heads as they feed; and they respond to a number of sensory stimuli, including mechanical stimulation, changes in the chemical environement, osmolarity, and temperature (White et al., 1986). As mentioned before, *C. elegans* are transparent throughout the life-cycle allowing live imaging and thus, it is simple to track specific cells and follow cell lineages by light microscopy.

While the nervous system is of particular interest, this model organism has a short life cycle, which is dependent on temperature (see Figure 1.6). *C. elegans* goes through a reproductive life cycle (from egg to reproductive adult) in about 5.5 days at 15ºC, 3 days at 20ºC and 2.5 days at 25ºC. The eggs are fertilized within the adult hermaphrodite and laid a few hours afterwards – at about the 40-cell stage. A single self-fertilized hermaphrodite is capable of producing 300 progeny. Each embryo will develop, hatch, and proceed through four larval stages (L1-L4) before becoming an adult (Nayak et al., 2005). Under food deprivation or stress, *C. elegans* can enter an alternative third larval stage called dauer state. Dauer larvae are stress-resistant and are long-lived; it can survive for months (Cassada & Russell, 1975). In a normal population, self-fertilizing hermaphrodites are more than 99%, leading to homozygosis of alleles and thus, making it possible to study large clonal populations. Males arise at a low frequency, due to meiotic chromosomal non-disjunction. In the laboratory, crossing males can be used to produce progeny with desired genotypes which is of particular interest for genetic studies.

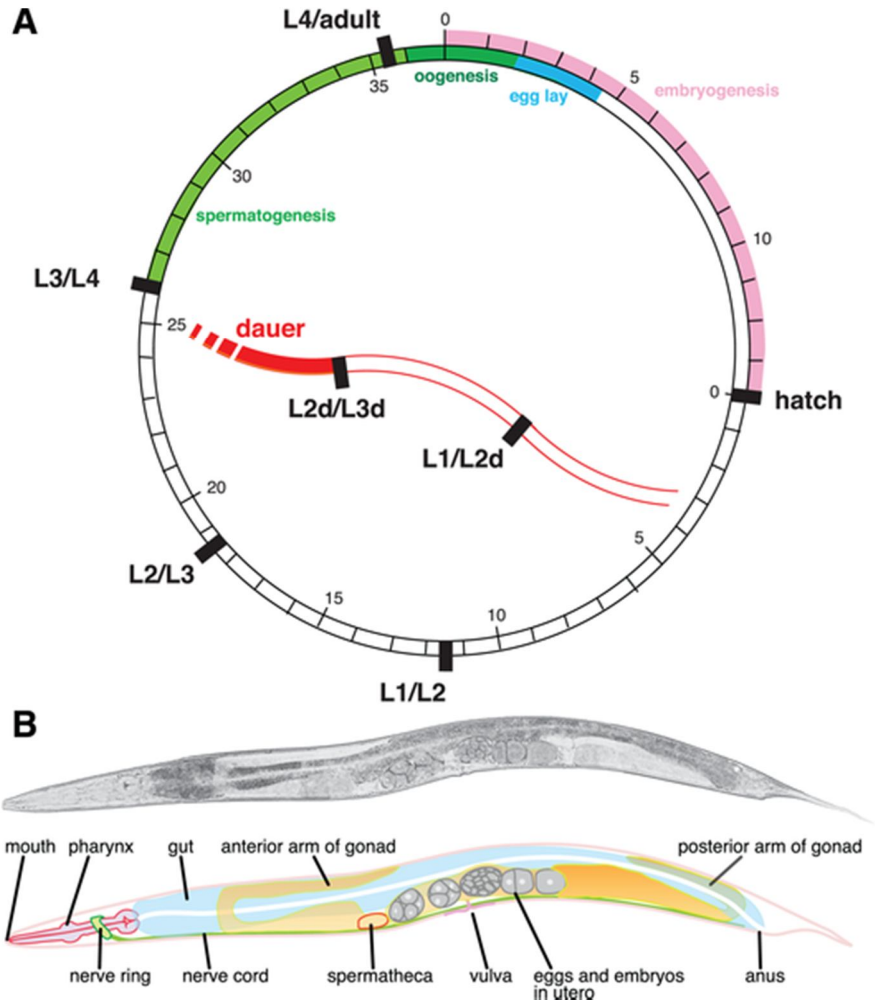


Figure 1.6- The simple life cycle and anatomy of *C. elegans*. (A) The larvae resemble the adults except in the lack of fully developed gonads, and their smaller size. The illustration shows the timing of developmental events at 25ºC, with hours since fertilization on the outside of the circle, and hours since hatching on the inside. Molts are indicated by solid black bars. (B) The adult hermaphrodite anatomy is simply observed under light microscopy. Above is an adult animal (length: 1mm). In the representation below the major organ systems are indicated. Adapted from www.plosbiology.org.

The importance of *C. elegans* for the study of human biology is twofold. One aspect is the startling finding that many of the genes in the *C. elegans* genome have close homologues in the human, and that many human disease genes are present in the worm (Gengyo-Ando et al., 1996; Wittenburg et al., 2000). The second is the ability to ask simple, direct questions of the *C. elegans* system and thus get simple, direct answers of universal significance. At least 42% of human disease-related genes have a *C. elegans* ortholog, suggesting that most biochemical pathways are conserved across evolution (Culetto & Sattelle, 2000). For these reasons, *C. elegans* has emerged as an attractive and powerful *in vivo* model system for studying pathological mechanisms in several major neurodegenerative disorders. The benefits of *C. elegans* as a model have led to the

development of multiple *C. elegans* systems designed for studying the effect of aggregation-prone proteins.

Nevertheless, it should be pointed that there are limitations to studying neurodegeneration in *C. elegans*. Worms do not have myelination or an active immune system, so they are presumably not appropriate for the study of some neurodegenerative conditions such as multiple sclerosis. In practice, worm neurons are small and difficult to patch clamp, so electrophysiology studies are somehow although recordings can be made from single identified neurons (Ramot et al., 2008).

1.8.1 *C. elegans* models of polyQ expansions

In agreement with the observations made in human disorders, polyQ proteins expressed in the *C. elegans* body wall muscle cells revealed a polyQ length-dependent aggregate formation and cellular toxicity (Satyal et al., 2000). Similar to human polyQ-associated diseases, which shows a progressive aggravation of the disease symptoms with age, the phenotypes observed in the animals expressing expanded polyQ tracts (and not normal polyQ-lengths) increased as the animals aged (Morley et al., 2002). A relation between aggregation onset and age-associated cellular changes was shown for the first time in polyQ *C. elegans* models (Morley et al., 2002; Hsu et al., 2003; Morley & Morimoto, 2004). In order to identify the protein factors that protect cells against the formation of protein aggregates, besides proteins involved in the aging pathway, Nollen and co-workers (2004) established transgenic *C. elegans* strains expressing Q35::YFP, the Q-length threshold associated with the age-dependent appearance of protein aggregation (Nollen et al., 2004). In order to investigate the effect of polyQ pathogenic motifs in neuronal cells and to determine if the expression of the same toxic species would affect differently distinct neuronal subtypes, Brignull and co-workers (2006) expressed polyQ expanded proteins throughout the *C. elegans* nervous system. By the expression of a range of polyQ lengths, they demonstrated, similarly to the polyQ *C. elegans* body wall muscle cells model, a polyQ length threshold of >Q40 for the formation of visible structures corresponding to immobile protein. Additionally, at the threshold Q-lengths, distinct neuronal subtypes were differently susceptible to the expanded polyQ protein (Brignull et al., 2006).

1.8.2 High-throughput screenings (HTS)

High-throughput screens (HTS) (genetic or pharmacological) using rodent models are very expensive and time-consuming and are limited to a small number of animals per treatment. A maintaining constant condition within each experiment, never mind between different research groups, is also very difficult with such complex organisms. Many research groups are recognizing the advantages of *in vivo* whole organism experiments using invertebrate animals. These advantages include rapid and inexpensive testing, as well as negligible animal welfare issues. Although *in vitro* cell-based HTS assays are commonly used, whole organism testing allows investigators to observe phenotypes that are well-characterized and biologically relevant.

Invertebrate models such as *C. elegans* are therefore attractive as they offer much greater experimental flexibility and control in a better defined genetic and physiological background. As mentioned before, *C. elegans* has a well-characterized and visually accessible nervous system. The morphology, location and connectivity of each of its 302 neurons are well defined. Combined with the wide array of genetic tools available to the *C. elegans* biologist and their capacity do grow in a 96-well plates in liquid culture, these properties make it amenable to HTS genetic and pharmacological (Lee et al., 2004). *C. elegans* neurodegeneration models have been used to test the effects of individual drugs (Parker et al., 2005; Locke et al., 2008) or relatively small collections of compounds (Wu et al., 2006; Voisine et al., 2007). Although large numbers of replicate *C. elegans* populations can be readily grown in small volumes, a previous hindrance in moving from low- to HTS was the requirement for manual manipulation and phenotypic scoring of worms. Advances have now overcome many of the practical limitations in using *C. elegans* models for HTS, most prominently the development of the Complex Object Parametric Analyzer and Sorter (a.k.a. the COPAS "worm sorter") (Pulak et al., 2006). The COPAS instrument was used in *C. elegans* by Burns and co-workers (2006) who demonstrated how this technology could be incorporated with a HTS digital imager and data management software for specifically scoring phenotypes (Burns et al., 2006). This instrument has been used for HTS of neurotoxins, suggesting that this instrument should be equally applicable for screening for neuroprotective compounds in many neurodegeneration models (Boyd et al., 2009).

In the absence of generally accepted cell and animal models, and of any compounds that produce positive effects in the clinical setting, a strategy could be to assess two

characteristics of MJD and focus on those drugs that work in several assays. Based on all this, this thesis will focus on the idea that the transgenic *C. elegans* model of MJD pathogenesis mentioned above (Teixeira-Castro et al, 2011a) - in which pan-neuronal expression of AT3q130 proteins cause neuron-specific aggregation and this aggregation phenotype correlates with locomotion impairment in the animal and both aggregation and behavior phenotypes aggravates with age, which mimic the human disease - can be used for the identification of compounds that recover mutant ATXN3 aggregation and its associated neurological dysfunction. We used the AT3q130 model for a therapeutic approach (i) focusing on mitochondria and (ii) for the screening of 1120 off-patent FDA-approved compounds from the Prestwick Chemical LibraryTM (Prestwick Chemical, France). Aggregation load and locomotion defects were used as readouts for the assessment of drug treatment efficacy.

1.9 Objectives

Little was known about therapeutic compounds that effectively prevent neurodegeneration in MJD patients when this experimental work was started. Bearing in mind the impact of neurodegenerative diseases in the society and that the mechanisms by which the expansion of the CAG-repeat of ATXN3 cause neurological dysfunction and disease are still unclear, our goals were:

Pharmacologic modulation of cellular pathways involved in polyQ pathogenesis - targeting mitochondria:

1. To define the toxicity threshold of Cr in *C. elegans* WT (N2);
2. To define the toxicity threshold of Cr in the transgenic *C. elegans* model of MJD pathogenesis (AT3q130);
3. To establish whether Cr treatment ameliorates the phenotype of aggregation of mutant ATXN3 in the transgenic *C. elegans* model of MJD pathogenesis (AT3q130);
4. To evaluate if Cr ameliorates the behavior and motor function in the transgenic *C. elegans* model of MJD pathogenesis (AT3q130).

Screening of a library of FDA-approved out-of-patent drug molecules – hypothesis-free approach:

1. To select non-toxic compounds from 1120 small molecules from the Prestwick Chemical LibraryTM (Prestwick Chemical, France) using a High-throughput pharmacological screening;
2. To identify drug compounds those improve the motor neuron-regulated locomotion defect in the transgenic *C. elegans* model of MJD pathogenesis (AT3q130).

Chapter II

Material & Methods

2.1. *C. elegans* strains culture and general methods

Nematodes were grown on NGM – nematode growth medium - plates seeded with *E. coli* OP50 strain at 20ºC, according to standard methods (unless otherwise noted) (Brenner, 1974). Populations were synchronized either by collecting embryos laid by adult animals within a 3-h period. All animals were scored at the same chronological age. Experiments were repeated three to four times.

2.2. Creatine treatment

Cr treatment was performed in regular NGM plates (Brenner, 1974). *E. coli* OP50 cultures were supplemented with Cr powder obtained from Sigma (C0780, St. Louis, MO, USA), at the indicated percentages. Plates were seeded with 100µL of OP50/Cr and left at room temperature to dry. We have also added Cr powder on standard solid media (before submitting the media to high temperature and pressure – 121ºC 20min). AT3q130 animals were always grown on Cr plates at least one generation prior to the beginning of the assays. For the toxicity screen, animals were observed every day and growth rates, fertility and survival parameters were evaluated. After determination of the safe toxicity range for Cr, synchronized day 4 animals (post-hatching) were scored for motility defects and alteration in the aggregation profile, using confocal imaging.

2.3. *C. elegans* treatment with compounds from the Prestwick Chemical LibraryTM

HTS were performed in 96-well plates in liquid culture (Voisine et al., 2007), with the following modifications: each well contained a final volume of 60 µL, including 20–25 animals in egg stage, drug at the appropriate concentration and OP50 bacteria to a final OD of 0.8 measured in the microplate reader (Bio-Rad microplate reader 680). Animals and bacteria were resuspended in S-medium supplemented with streptomycin, penicillin and nystatin (Sigma). Worms were grown with continuous shaking at 180 rpm at 20°C. The effect of compounds on *C. elegans* physiology is monitored by the rate at which the OP50

food suspension was consumed, as a read out for *C. elegans* growth, survival or fecundity. The absorbance (OD 595nm) was measured daily in the microplate reader. We used WT animals when determining compound concentration and AT3q130 animals for all the other assays. At day 4, AT3q130-drug-treated and control animals (N2) were imaged using a confocal microscope (Olympus FV1000 - Japan) and tested for motility defects.

2.4. Motility assays

All assays were performed at room temperature (~20ºC) using synchronized animals grown at 20ºC. Five 4-days old animals were placed simultaneously in the middle of a freshly seeded plate, equilibrated at 20ºC. Animals remaining inside a 1cm circle after 1min were scored as locomotion defective (Gidalevitz et al., 2006). Experiments were repeated until an n-value of at least 150 animals.

2.5. Confocal imaging

All images were obtained on an Olympus FV1000 (Japan) confocal microscope, under 60x oil objective. Animals four-days-old were immobilized with 1.5 mM levamisole and mounted on a 3% agarose pad. Z-series imaging was taken of all the *C. elegans* lines generated, using 514nm laser excitation for YFP fusion proteins. The pinhole was adjusted to 1.0 Airy unit of optical slice and a scan was taken every ~0.5 µm along the Z-axis.

2.6. Quantification of the aggregates

C. elegans fluorescent images were acquired using the Olympus FV1000 (Japan) confocal microscope. Confocal microscope parameters were set using Hi-Lo pallet, such that protein foci and not diffuse fluorescent areas of the animals nervous system presented pixel intensity higher than 255. The z-stack was collapsed and the aggregate load of each animal (per area unit) was calculated on an imaging processing application using MeVisLab as a platform (Teixeira-Castro et al., 2011b).

Chapter III

Results

3.1. Screening of therapeutic compounds in a *C. elegans* model of MJD: targeting mitochondria

There is considerable evidence for bioenergetic defects in MJD (Yu et al., 2009). We have tested the effect of a candidate compound targeting mitochondrial toxicity in our *C. elegans* model of MJD: Cr. We chose to test this compound mainly because Cr was shown to be neuroprotective in several *in vitro* and *in vivo* models of AD, HD, PD and of ALS (reviewed in Beal, 2011) and to target mitochondria and stabilize it.

We examined whether Cr, which may exert neuroprotective effects by increasing PCr levels or by stabilizing the mitochondrial toxicity, had beneficial effects in mutant ATXN3-mediated motor dysfunction and aggregation.

3.1.1. Determination of Cr safe concentration range in *C. elegans*

Cr showed very low solubility in any of the solvents tested (H_2O and HCl 0.02M, 0.05M - data not shown), so instead of growing the animals in liquid medium, we supplemented the food source (OP50 culture) with Cr powder and grew the WT animals on standard solid media. We have also added Cr powder to standard solid media (before submitting the media to high temperature and pressure – 121ºC 20min) and grew the WT animals. The final Cr concentrations tested were 1, 2 and 3%. We assessed survival, fecundity and animal growth to determine the highest non-toxic concentration of Cr that can be tolerated by *C. elegans*, when grown in standard conditions (see Figure 3.1). We found that the highest safe concentration was <2% for Cr-supplemented media. Animals grown in 3% Cr (food and medium-supplemented) showed a slight developmental delay and reduced brood size (Figure 3.1).

To further dissect the effects of Cr, we decided to grow mutant-ATXN3 animals also at intermediate concentrations (see Figure 3.2), using the same conditions. Also for AT3q130 animals <2% Cr was set as the highest safe concentration tolerated by the nematodes (Figure 3.2). Along with that, we decided to interrupt the addition of Cr powder to standard solid media because we found it hard to predict if Cr was resistant to the processing in the autoclave.

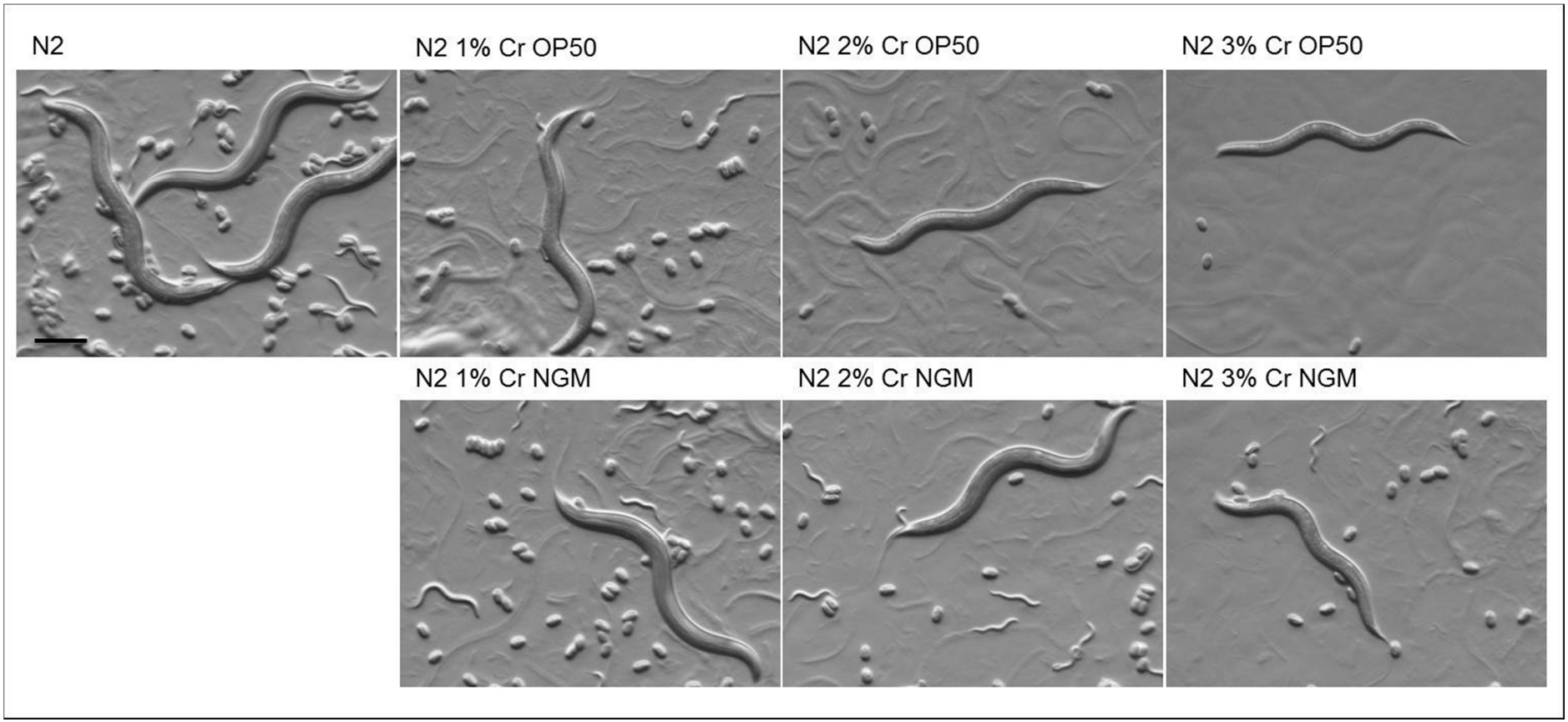


Figure 3.1- WT animals (N2) were grown in NGM media containing Cr or with NGM plates seeded with OP50 supplemented with Cr at the indicated concentrations. When treated with 3% Cr in both conditions, animals showed developmental delay (reduced length and thickness) and reduced progeny size (each photo is representative of the entire plate). The highest safe concentration was set as <2% for Creatine-supplemented media. Pictures show day 4 (post-hatching) age-synchronized animals. Scale bar, 200µm.

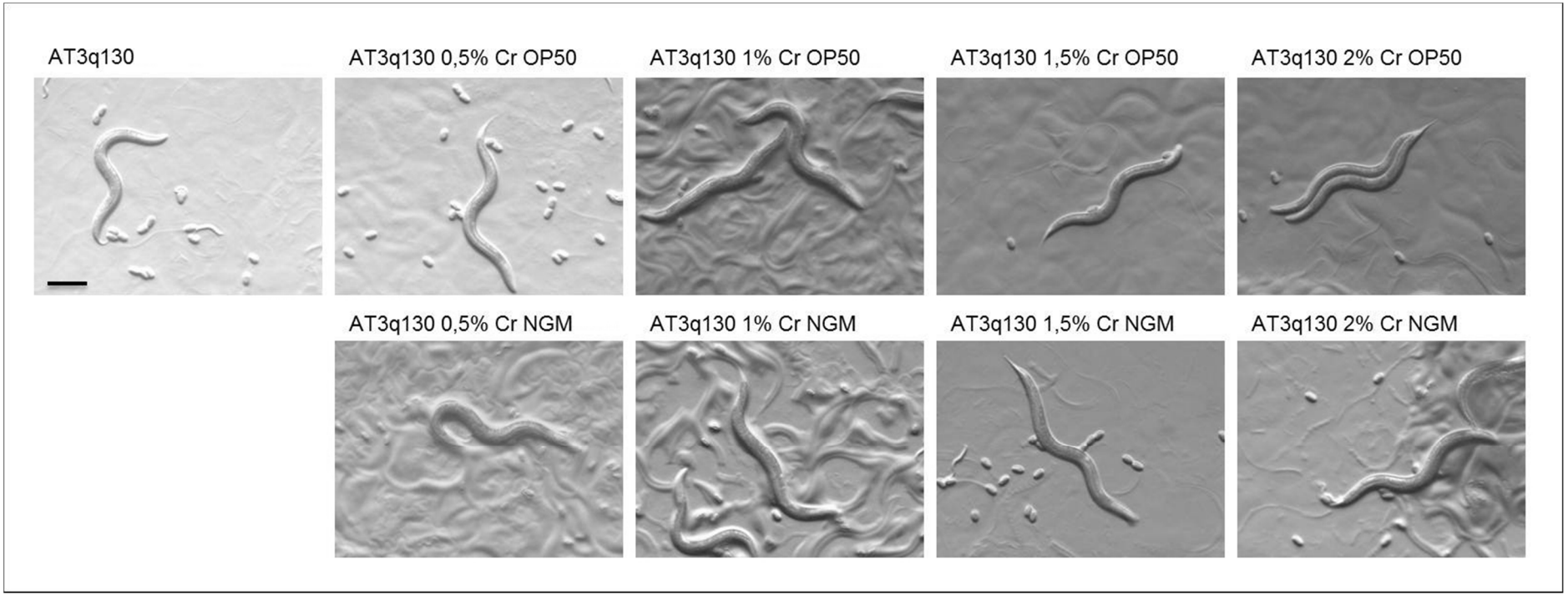


Figure 3.2- AT3q130 animals were grown in NGM media containing Cr or seeded with OP50 supplemented with Cr at the indicated concentrations. When treated with 2% Cr animals showed no considerable developmental delay (each photo is representative of the entire plate) The highest safe concentration was set as <2% for Cr-supplemented media. Pictures show day 4 (post-hatching) age-matched animals. Scale bar, 200μm.

3.1.2. Determining the effect of Cr in mutant ATXN3-mediated motor dysfunction in *C. elegans*

We evaluated Cr efficacy to rescue neuronal dysfunction in our *C. elegans* model of MJD pathogenesis. Expression of mutant ATXN3 (AT3q130) in all *C. elegans* neuronal cells resulted in neuronal cell-type-specific aggregation that correlated with toxicity (Teixeira-Castro et al., 2011a). AT3q130 animals were grown on standard solid media supplemented with or without Cr powder for 4 days, and then tested for defects in locomotion, by employing a motility assay (Gidalevitz et al., 2006).

In our *C. elegans* model of MJD pathogenesis, Cr food supplementation had limited effect in mutant ATXN3-mediated neuronal dysfunction at all concentrations tested (see Figure 3.3), not even after three generations (data not shown).

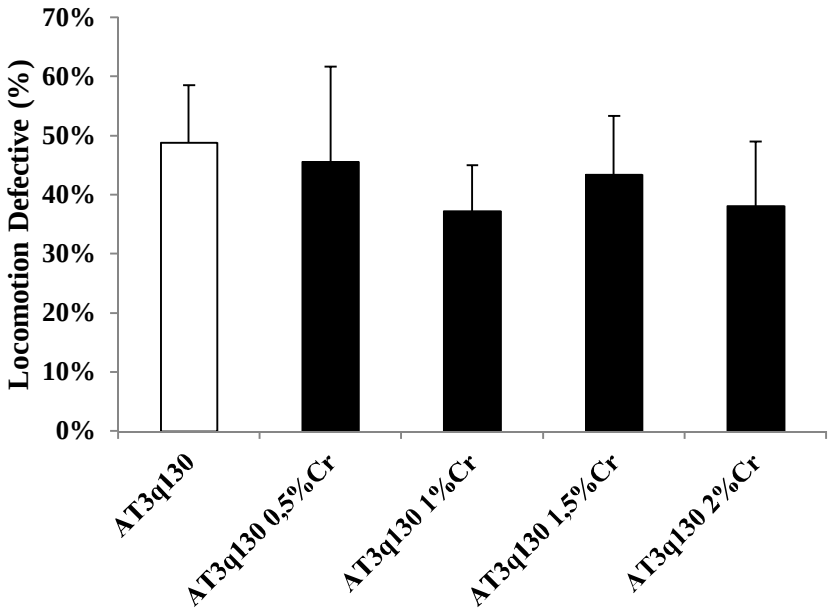
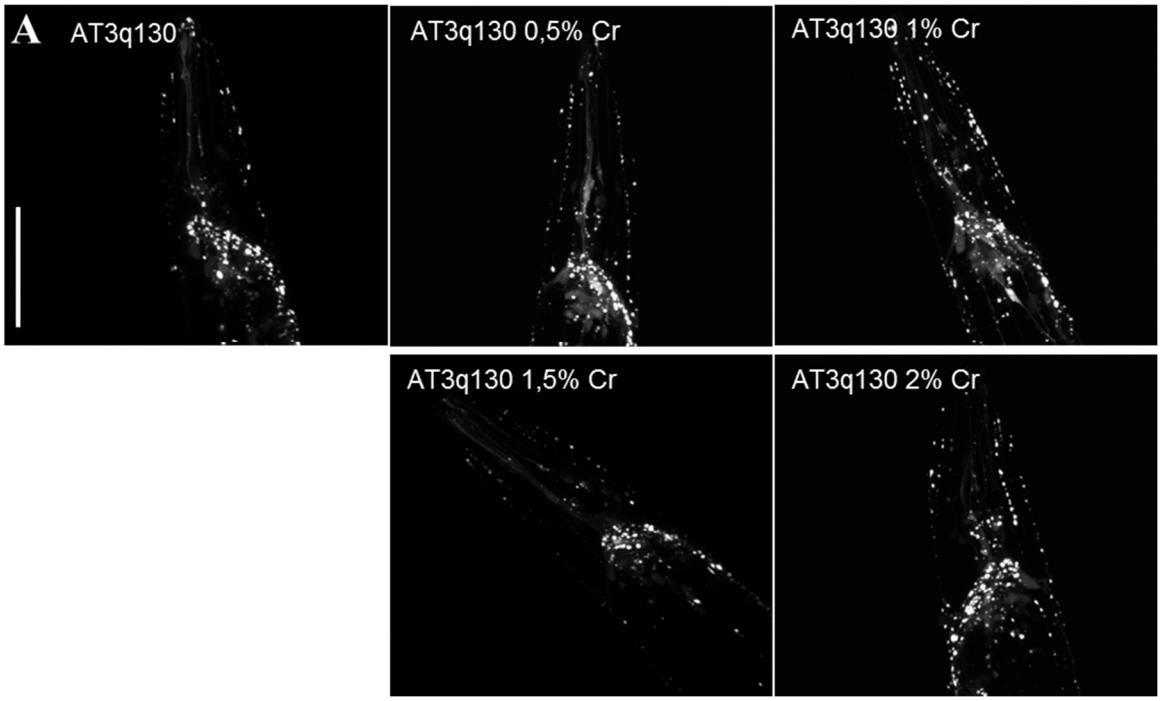


Figure 3.3- AT3q130 animals treatment with Cr targeting mitochondrial toxicity. Day 4 AT3q130 animals treated with Cr were subjected to the motility assay. Cr treatment showed no significant rescue of motor dysfunction. Motility data are the mean ± SD, at least 150 animals per data point.

3.1.3. Effect of Cr in mutant ATXN3-mediated aggregation

To determine if Cr produced potential alterations in mutant-ATXN3 aggregation profile in the animals, despite the absence of effect on motor dysfunction, we performed live confocal imaging assays in day 4 Cr-treated and vehicle-treated animals (Figure 3.4A).

Quantification of the number of aggregates showed that AT3q130 animals fed with bacteria supplemented with Cr did not show any significant differences in aggregation (see Figure 3.4B). However, Cr at 1.5 % seemed to have a tendency to a reduction in the aggregation load, which did not reach statistical significance.



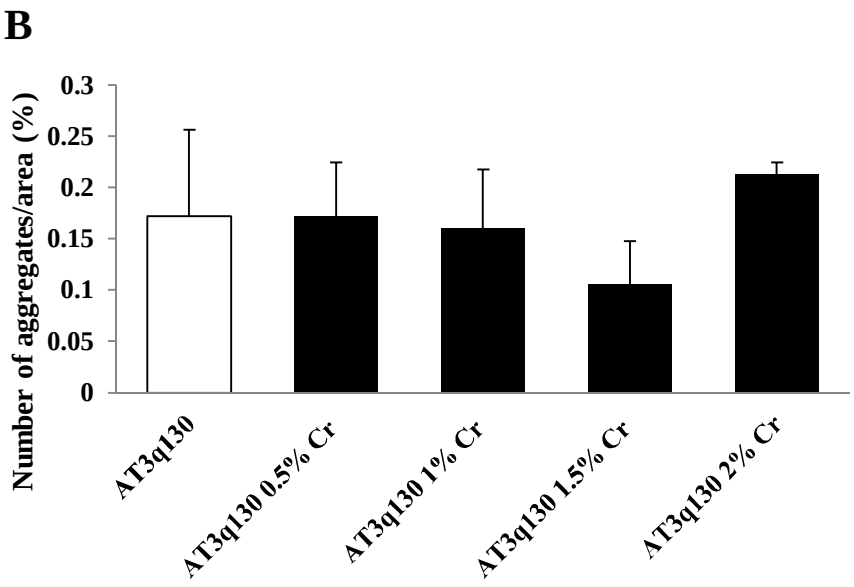


Figure 3.4-Creatine treatment of mutant ATXN3 animals showed no significant rescue of the number of aggregates per area unit. (B) Creatine at 1.5 % resulted in a tendency to a reduction in aggregation that did not reach statistical significance. Quantification of the number of aggregates per area was performed using a novel imaging processing application for aggregate quantification in *C. elegans* (Teixeira-Castro et al., 2011b), and values are the mean ± SD of 3 or more animals per group. (A) The images were obtained using on an Olympus FV1000 (Japan) confocal microscope. Scale bar, 50 μm.

3.2. Screening of therapeutic compounds in a *C. elegans* model of MJD: hypothesis-free approach

3.2.1. Determination of compound concentration range for drug screening in *C. elegans*

We have continued our search for small molecules that modulate mutant ATXN3 pathogenesis by using a hypothesis-free approach. In order to achieve that we have started testing a library of 1120 FDA-approved out-of-patent drug molecules. Figure 3.5 shows the flow chart of the screening. The first point of the screening was to identify and exclude small molecules that were toxic to the animals. One of the major disadvantages of using *C. elegans* in HTS could arise from the possible toxicity of the compounds in this animal. In

order to exclude the effects of high toxicity in our study, we used a systematic method, previously described by Voisine and co-workers (Voisine et al., 2007), for selecting optimal drug concentrations in *C. elegans* - the food clearance assay. This is a simple assay that uses a small amount of compound and is amenable to high-throughput format (96-well plates). Taking advantage of the short life cycle and the capacity of *C. elegans* to grow in liquid culture of *E. coli*, compounds were monitored by measuring the rate at which the food source (OP50 bacteria suspension) was consumed. Optical density was measured every day (from day 1 to day 8) at 595 nm (OD595) (see Figure 3.5). Each adult is capable of producing hundreds of progeny that rapidly eat the limited bacteria source in non-treated or vehicle-treated animals. At 20°C, after 4 days the optical density (OD) decreased significantly. A drug dosage was considered non-toxic to WT animals, if it did not affect growth, survival and fecundity. We have to take into account the fact that these drugs are solubilized in dimethyl sulfoxide (DMSO) and are very distinct chemically. Because of that, we decided to test two concentrations in which the highest corresponds to the highest DMSO concentration tolerated by the animals (1%) and the lowest concentration corresponds to half (0,5%). So, we tested 25µM and 50µM for each drug. Figure 3.6 shows, as examples, the toxicity data for 5 of the compounds tested, named after the library designation – Prestwick-211 to Prestwick-215. The graphs show that at both concentrations tested Prestw-212 was toxic to the animals, since the OD values did not significantly decrease over time. Visual inspection of the plates confirmed the OD results, since we observed that the drug caused strong developmental delay. In contrast, animals treated with Prestw-211, -213, -214 and -215 looked normal under the dissecting scope (data not shown).

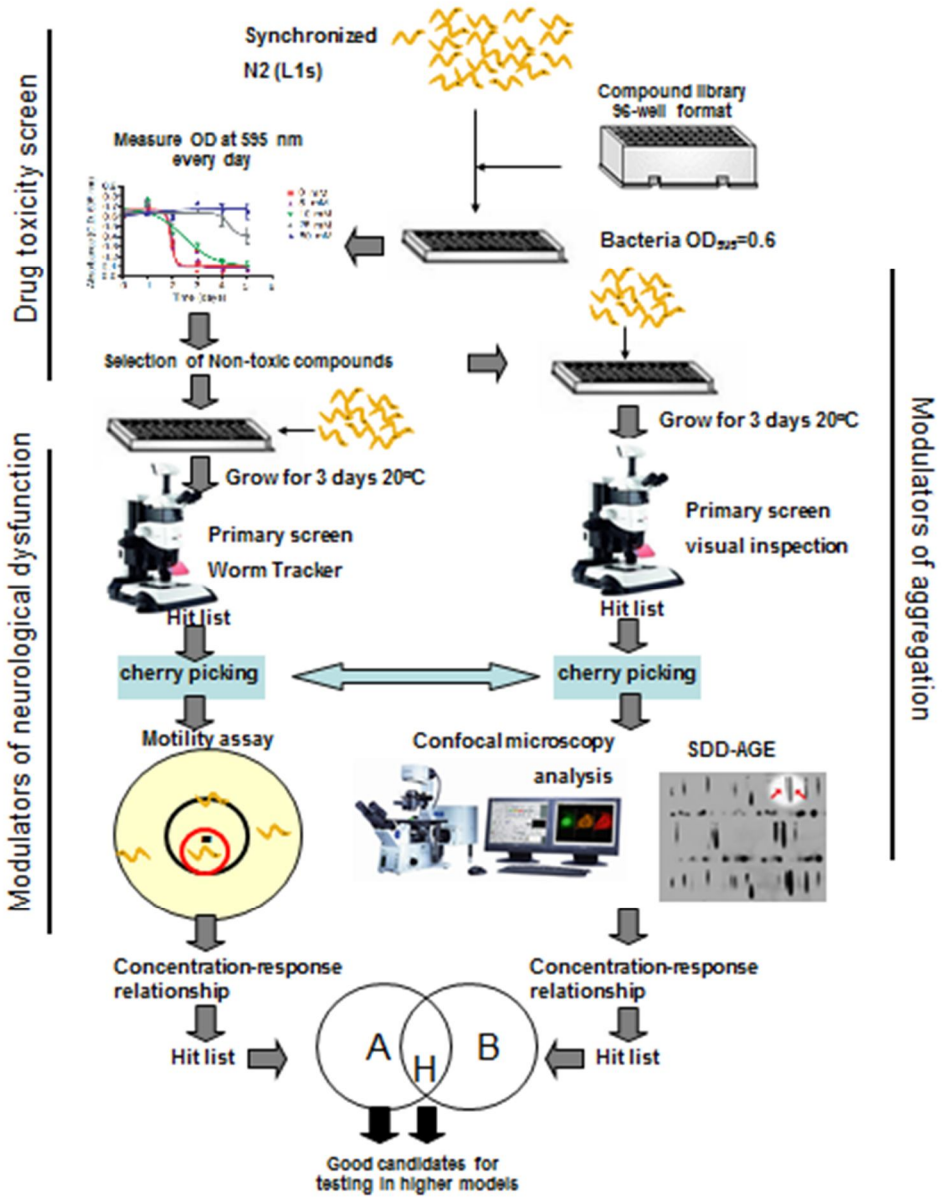


Figure 3.5- Experimental design for screening potential therapeutic compounds for MJD pathogenesis in *C. elegans*. The first step is to determine the range of safe concentrations for each compound, using the food clearance assay. The rate at which the food source is consumed by WT animals is a good indication of normal growth, survival and fecundity. Next, mutant ATXN3 animals are grown with different concentrations of each small molecule and assayed for locomotion defects and aggregation profile. Compounds that cause rescue of locomotion impairment and also a reduction of the aggregation load in AT3q130 animals are considered good candidates for testing in higher model organisms.

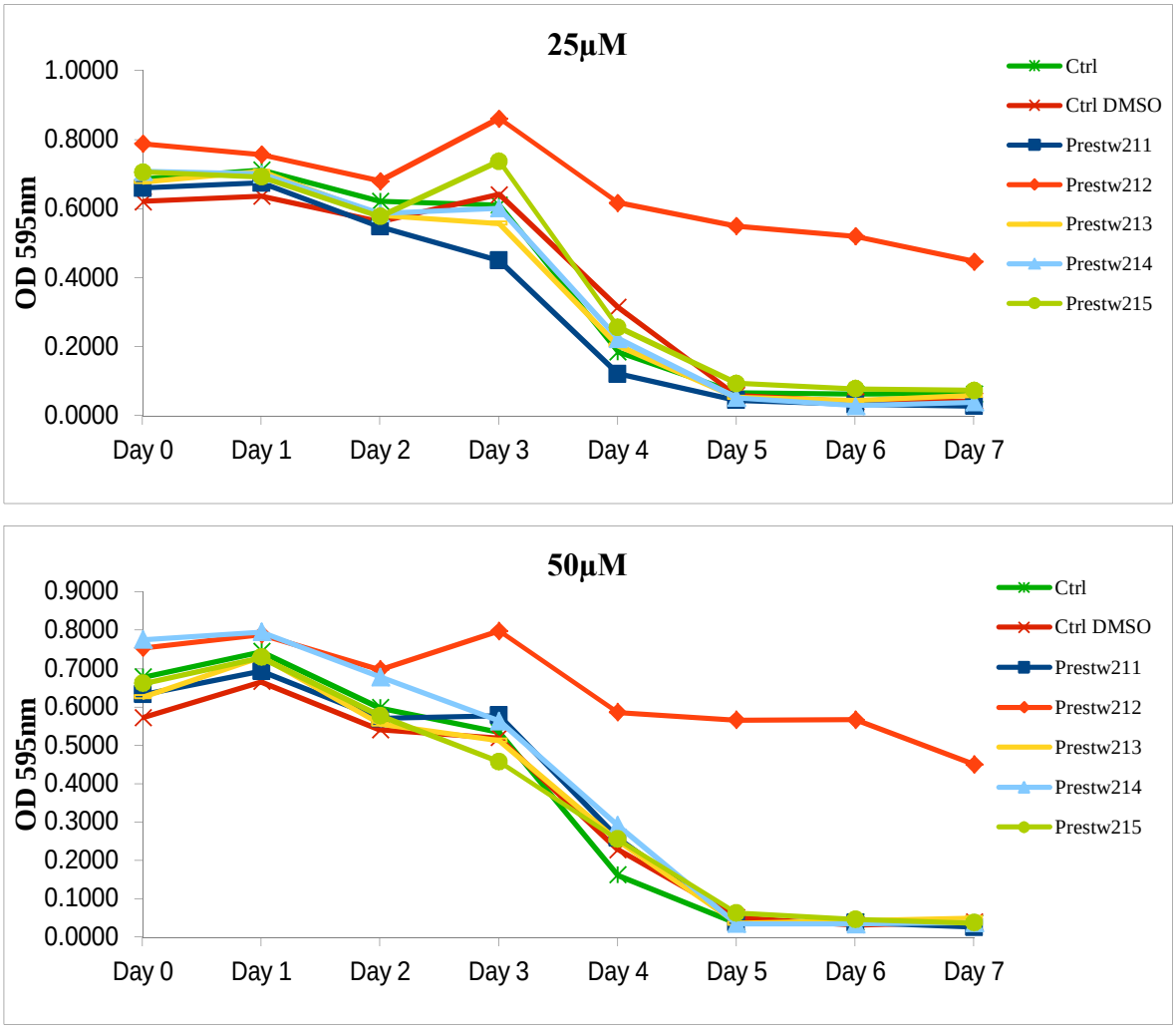


Figure 3.6- Toxicity evaluation of Prestwick library compounds using the food clearance assay. Example of 5 of the compounds tested is given here. At both concentrations tested Prestw-212 was toxic to the animals. The OD of *E. coli* is reported daily for each drug at 25 and 50 µM. The mean OD is calculated for each day from triplicate samples and plotted over time.

The main goal of the first step was to select non-toxic compounds from the small molecule library that will be subsequently used throughout the project. At this point we should be able to exclude the majority of the highly toxic compounds from the library. We will continue our study only with the compounds that fulfill this non-toxicity criterion. So far we already have tested 900 compounds, 264 of which were toxic at the concentrations tested and were excluded from further testing. A subset of the compounds was toxic when tested at 50µM and not at 25µM. Based on this, we selected 617 compounds safe at 50µM and 19 safe at 25µM that satisfied our non-toxicity criteria and continued for the next step: to determine the effect of the small molecules in the locomotion impairment of AT3q130

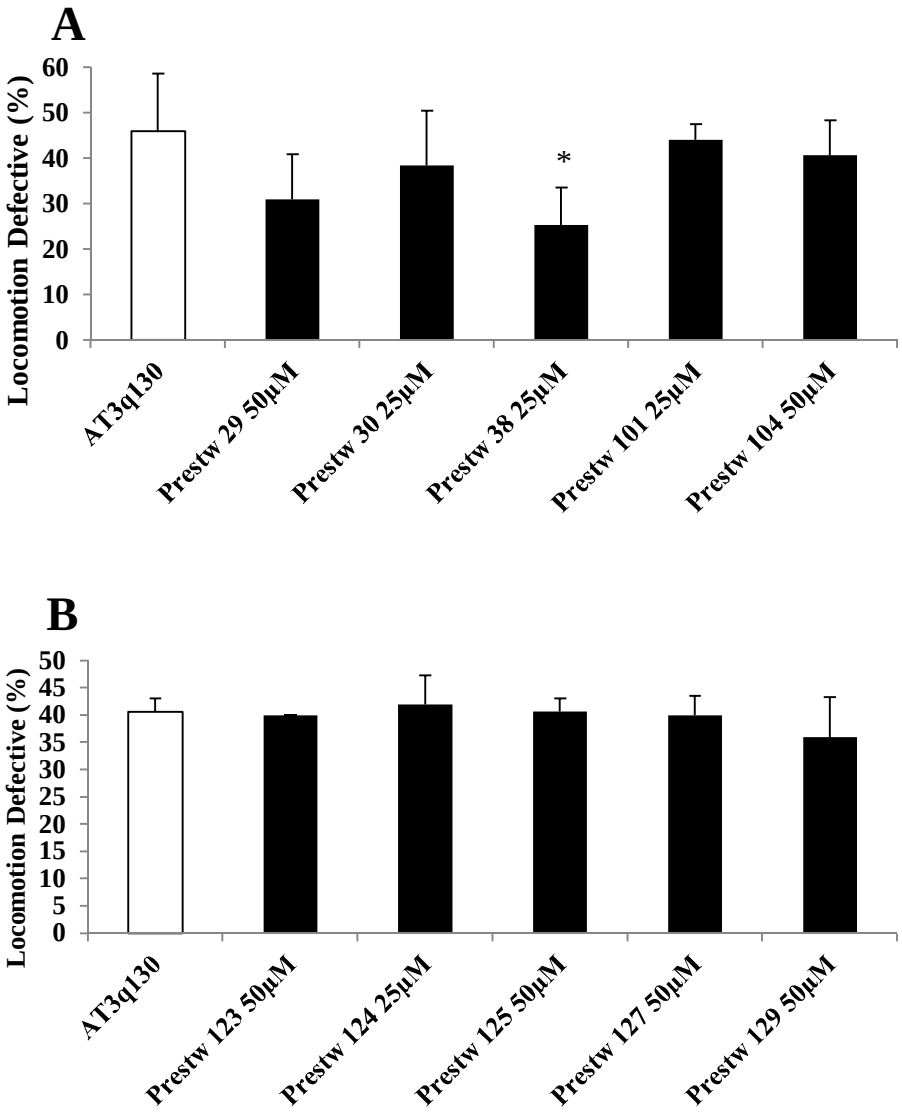
animals. Table 3.1 shows the toxicity data for compounds already tested. Drug toxicity was classified using the following criteria: Safe drug – all replicates at all days and both concentrations showed no toxicity; Toxic drug - all replicates at all days and both concentrations showed toxicity; Likely safe – at both concentrations and all days, at least 2 replicates showed no toxicity; Likely toxic - at both concentrations and all days, at least 2 replicates showed toxicity; Safe at 25µM - at 25µM only and on all days, all 3 replicates showed no toxicity; Likely Safe at 25µM – at 25µM only and on all days, at least 2 replicates showed no toxicity; Developmental delay – at both concentrations and at days 4/5 the drug is considered toxic, but all replicates showed no toxicity at day 6 at both concentrations; Developmental delay at 25µM – at both concentrations and at days 4/5 the drug is considered toxic, but all replicates showed no toxicity at day 6 at 25µM only; Unclear toxicity – all other situations – includes cases for instance where toxicity is higher at 50uM than 25uM. We are currently assessing the toxicity of the remaining 220 compounds.

Table 3.1- Toxicity data for the compounds tested.

Prestw Number	Therapeutic Group	Mechanism	Side Effect(s)	Veredict
Prestw-29	Analgesic; Antipyretic; Antiinflammatory	-	Nausea; Seizure; Drowsiness; Hemolytic anemia	Likely Safe
Prestw-30	Antipyrine metabolite	-	Irritant	Safe at 25 µM
Prestw-38	Antihypertensor	Ganglion blocking agent	-	Safe at 25 µM
Prestw-101	Emetic; Antiparkinson	D1 agonist; D2 agonist	Migraine; Asthenia; Tachycardia; Hypotensive; Cardivascular collapse	Safe at 25 µM
Prestw-104	Antiulcerative	H2 histaminic antagonist	-	Likely Safe
Prestw-123	Choleretic	-	-	Likely Safe
Prestw-124	-	P450 inhibitor	-	Likely Safe at 25 µM
Prestw-125	Antipsychotic; Tranquilizer	Dopamine antagonist	-	Likely Safe
Prestw-127	Antibacterial; Antifungal	Sterol 14-demethylase inhibitor	-	Likely Safe
Prestw-129	Antiulcerative	M1 antagonist; Anticholinergic	-	Likely Safe
Prestw-223	Nasal decongestant; Vasoconstrictor	Adrenergic alpha-agonists	-	Likely Safe
Prestw-224	Vasoconstrictor; Decongestant	Partial alpha2A agonist; 5-HT1A agonist; 5-HT1B agonist; 5-HT1D agonist; 5-HT1C mixed antagonist agonist	-	Likely Safe
Prestw-225	Analgesic; Antipyretic	Cyclooxygenase inhibitor	-	Likely Safe
Prestw-226	Antifungal; Antibacterial; Antiinflammatory	Enzymatic inductor	Headhache; Antabuse effect; Teratogenic (animal)	Likely Safe at 25 µM
Prestw-227	Antihistaminic; Local anesthesic; Antibacterial; Antifungal	H1 antagonist	Allergic	Likely Safe at 25 µM
Prestw-257	Keratolytic	-	-	Likely Safe
Prestw-258	Antihistaminic Sedative	H1 antagonist	Nausea, vomiting Thrombolytic anemia Sedation	Likely Safe
Prestw-259	Diuretic	Na^+ Cl^- uptake inhibitor	-	Safe at 25 µM
Prestw-260	Anthelmintic	Modulates cell membrane permeability	Bradycardia	Safe at 25 µM
Prestw-261	Progestogen	-	-	Likely Safe at 25 µM

3.2.2. Screening for compounds that rescued mutant ATXN3-mediated motor dysfunction

Secondly, we aimed to investigate the impact of the hit compounds on neuronal dysfunction caused by mutant ATXN3 expression (Figure 3.7). For this we perform motility assays in animals treated with the small molecules that passed the non-toxicity condition, looking for compounds that significantly improve the 40% locomotion defective phenotype observed in AT3q130 expressing animals. We have tested 20 compounds, two of which (Prestw-38 and Prestw-227) revealed to be modulators of mutant ATXN3-mediated motor impairment.



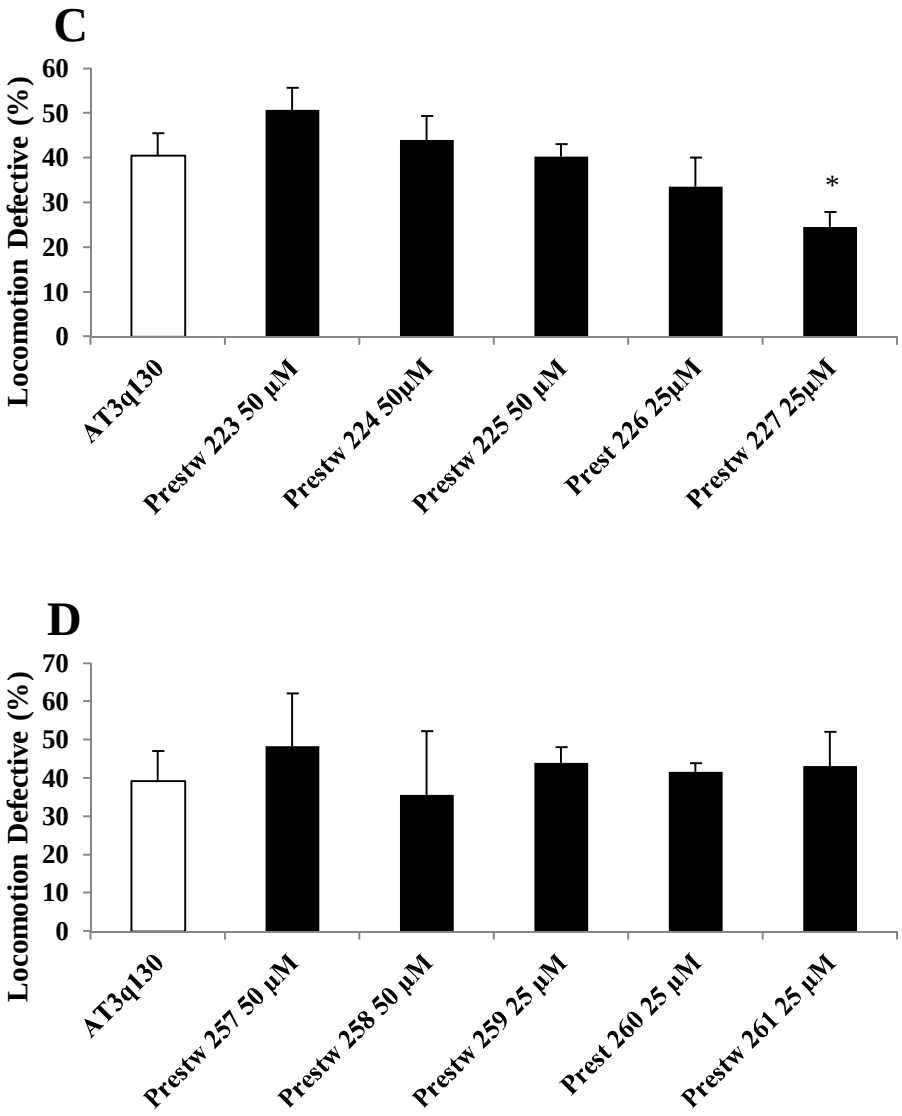
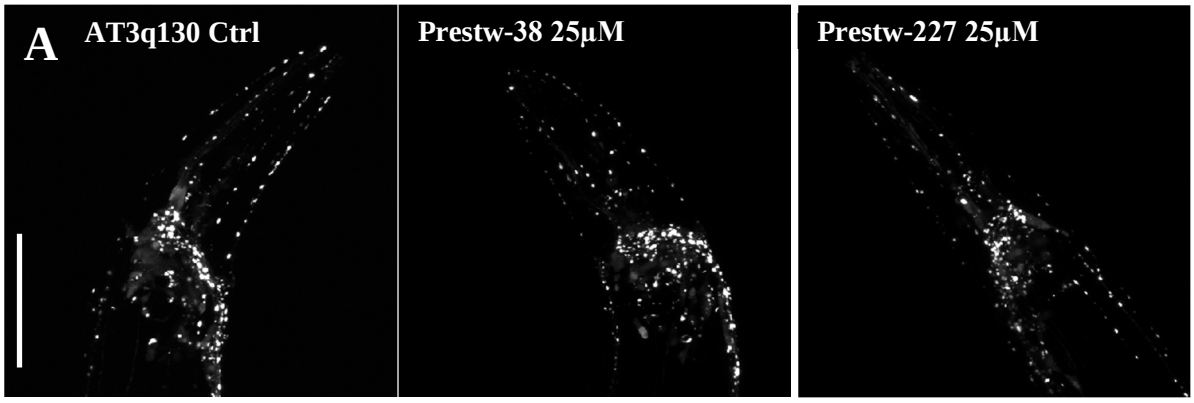


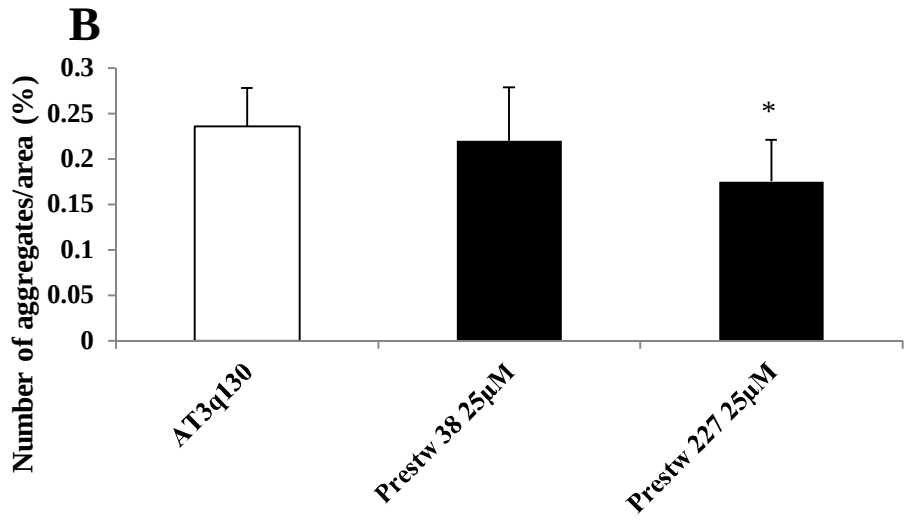
Figure 3.7- Treatment of AT3q130-expressing animals with the small molecule Prestwick-38 and Prestwick-227 showed an improvement of motor function (A and C). Day 4 mutant ATXN3 expressing animals treated with the small molecules Prestw-29, -30, -101, -104, -123, -124, -125, -127, -129 -223, -224, -225, -226, -257, -258, -259, -260, -261 showed no significant improvement in motility. Prestw-38and Prestw-227 were able to rescue the motor dysfunction of AT3q130 animals. Motility data are the mean ± SD, with at least 150 animals per data point. Student´s test, *, $p<0.05$

3.2.3. Assessment of the effect of phenotype-improving mutant ATXN3 aggregation

To determine if the effects of Prestw-38 and Prestw-227 on motor dysfunction were coupled with potential alterations in mutant ATXN3 aggregation profile in the animals, we performed live confocal imaging assays in day 4 treated and non-treated animals.

Aggregate quantification of animals treated with the small molecule Prestw38 revealed no major differences in the aggregation load (see Figure 3.8). However, Prestw-227 treated animals showed a significant reduction in the number of aggregates per area and in the area of aggregates per area (see Figure 3.8). The number of aggregates also decreased, however without reaching statistical significance. Ongoing experiments aim to determine the effect of these two different compounds in total mutant ATXN3-protein levels.





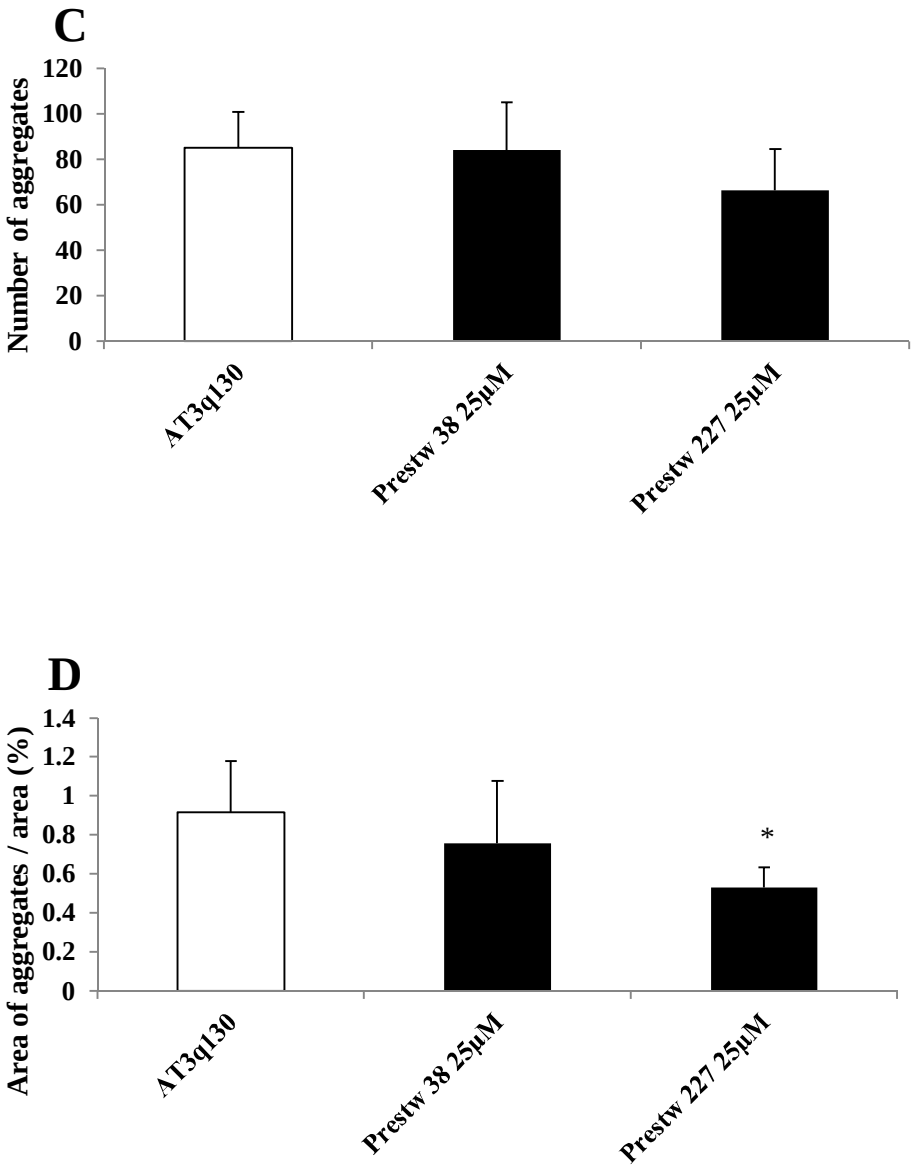


Figure 3.8- Animals treated with Prestw-38 revealed no major differences in the aggregation load and Prestw-227 rescue ataxin-3-mediated aggregation. AT3q130 animals treated with 25µM of Prestw-38 showed a mild reduction in the aggregation phenotype and Prestw227 treated animals showed a significant reduction in the number of aggregates per area and in the area of aggregates per area at day4 of adulthood. Scale bar, 50 µm (A). The images were obtained using an Olympus FV1000 confocal microscope. Quantifications of aggregates (B, C, D) were performed using our novel imaging processing application for aggregates quantification in *C. elegans* (Teixeira-Castro et al., 2011b), and values are the mean ± SD of seven or more animals per group. Student´s test, *, $p<0.05$.

Chapter IV

Discussion

In the last years, several human diseases, including neurodegenerative diseases like AD, PD, ALS and several polyQ disorders, have been shown to arise from the misfolding and aggregation of an underlying protein (Gatchel & Zoghbi, 2005). Inappropriate aggregation of proteins is normally prevented by complex cellular quality control mechanisms. Nevertheless, under certain circumstances, an unusual subset of proteins is able to aggregate within or around cells. A more thorough understanding of the factors that influence this balance is the key for determining how protein aggregation diseases arise and may provide novel opportunities for developing effective therapies against them.

With this work, we took advantage of our *C. elegans* model and approach for therapeutic value in MJD, using a candidate-drug strategy. Given the lack of positive effects, we initiated a screening to find new bioactive compounds using a hypothesis-free approach. These compounds may provide candidate drugs to test in higher organism models of the disease, but they may also provide clues concerning pathogenic mechanisms of MJD.

4.1 Targeting mitochondria as a therapeutic approach for MJD

As we already know, mitochondrial dysfunction has been implicated in ageing, which is a major risk factor for progressive neurodegenerative diseases. There is growing evidence that mitochondrial dysfunction may play an important role in neurodegeneration (for review see Knott et al., 2008). Therefore, targeting mitochondrial toxicity may be a valuable strategy for the treatment of many neurodegenerative disorders, like MJD. It is known that the brain also has cellular defense systems against oxidative stress. These systems include high levels of several antioxidant enzymes (e.g., catalase, SOD, GSH-px, and GSSG-R) (Lee et al., 2000). Because of the late onset of the MJD, Yu and co-workers (2009) hypothesized that the accumulated oxidative stress or/and defective antioxidant enzyme ability might be contributory factors in the pathogenesis of MJD. Their results demonstrated that mtDNA copy numbers are reduced in mutant ATXN3 expressing cells and MJD patient's leukocyte samples. Moreover, the amount of the common mtDNA 4,977-bp deletion is superior in MJD patients compared with that in normal individuals. So, mutant ATXN3 may influence the activity of enzymatic components to remove O_2^- and H_2O_2 successfully and promote mtDNA damage or depletion, which leads to mitochondria

dysfunction (Yu et al., 2009). There is therefore evidence of mitochondrial dysfunction in MJD. Although we did not address the presence of mitochondrial dysfunction in our *C. elegans* model of MJD, there is evidence of mtDNA dysfunction in a transgenic mouse model of MJD generated in our lab (results from a pilot-study – Nadya Kazachkova, personal communication, March 2011). These preliminary results support the hypothesis of an association between mtDNA dysfunction (depletion and deletion) with aging and neurodegeneration.

Since Cr had produced significant neuroprotection in the HD toxic rat model (Matthews et al., 1998), numerous studies have indicated that supplementation of Cr could promote strong neuroprotective effects. Cr administration also protects against glutamate and β-amyloid toxicity in rat hippocampal neurons (Brewer &Wallimann, 2000). Similar results were obtained in the R6/2 and the N-171-82Q transgenic mouse models of HD, even when Cr significantly improves survival, improves motor performance, slows the development of brain atrophy, increases brain ATP levels, and delays atrophy of striatal neurons and the formation of Htt-positive aggregates (Ferrante et al., 2000; Andreassen et al., 2001; Dedeoglu et al., 2003). Additionally, oral administration of Cr produces a dose-dependent improvement in motor performance and extends survival in G93A transgenic ALS mice, where it protects against loss of both motor neurons and *substantia nigra* neurons (Klivenyi et al., 1999) but Cr does not exert a beneficial effect on skeletal muscle function in the same transgenic mice (Derave et al., 2003).Based on these evidences, we decided to make use of our *C. elegans* model of. MJD to validate the approach of correcting mitochondrial dysfunction with Cr food supplementation as a therapeutic strategy for MJD.Unfortunately, Cr food supplementation had limited effect in MJD pathogenesis in mutant ATXN3-mediated neuronal dysfunction.This was an unexpected result given all the successful results in the previous research. On the other hand, it is important to notice that we are not sure if Cr was consumed or absorbed by the nematodes. This work opened several questions that we aim to answer in the near future. Assaying ATP and ADP levels provides a measure of the instantly available energy (Braeckman et al., 2002).We now propose to study mitochondrial dysfunction in our *C. elegans* model of MJD, measuring the number of mtDNA copies, the frequency of mtDNA deletions and the levels of ATP, because if Cr functions ATP levels in cells should increase (Tsang & Lemire, 2003).

The neuroprotective effects of Cr in HD and PD in transgenic mice were described to be potentiated when in combination with CoQ_{10} (Yang et al., 2009). The combination of the two compounds produced additive neuroprotective effects against dopamine depletion in the striatum and loss of tyrosine hydroxylase neurons in the *substantia nigra* pars compacta (SNpc). The combination treatment resulted in significant reduction in lipid peroxidation and pathologic α-synuclein accumulation in the SNpc neurons. In the R6/2 transgenic mouse model of HD, the combination of CoQ and Cr produced additive effects in terms of improving motor performance and extending survival. These results suggest that combination therapy using CoQ_{10} and Cr may be useful in the treatment of neurodegenerative diseases such as MJD. In addition, it was found that the combination of Cr and minocycline (a tetracycline antibiotic with neuroprotective properties) also provided additive neuroprotective effects in the G93A mouse model of ALS (Zhang et al., 2003). In the same way, additive neuroprotective effects of Cr and a COX-2 inhibitor were found in the MPTP mouse model of PD and the G93A transgenic mouse model of ALS (Klivenyi et al., 2003; Klivenyi et al., 2004). These findings also suggest that combinations of therapies targeting different disease mechanisms may be a useful strategy in the treatment of neurodegenerative diseases and may be a future approach for our *C elegans* model of MJD.

4.2 HTS for the identification of MJD therapeutic compounds

The development of *C. elegans* models for the study the pathogenesis of neurodegenerative diseases has contributed to the understanding of the disorders. They provide a major advance for testing or evaluating therapeutics. If therapeutics effects in *C. elegans* are shown to be predictive of beneficial effects in transgenic mouse models (mammalian systems), then this model will allow rapid screening of new therapies. One clear advantage of *C elegans* over other multicellular model organisms for neurodegeneration is its capacity to grow in liquid medium, in standard 96-well microtiter plates. Growth in a 96-well format permits application of HTS technologies developed for drug discovery.

In the present study, we have developed strategies for efficiently assessing therapeutic efficacy of compounds in our *C. elegans* model of MJD. We used the AT3q130

model for the screening of 1120 off-patent FDA-approved compounds from the Prestwick Chemical LibraryTM (Prestwick Chemical, France) and searched for small molecules that modulate mutant ATXN3 pathogenesis. The majority of the small molecules, 90%, are marketed drugs and the remaining 10% are bioactive alkaloids or related substances.The active compounds were selected for their high chemical and pharmacological diversity as well as for their known bioavailability and safety in humans.

The main goal of the first step was to select non-toxic compounds from the small molecule library that will be subsequently used throughout the project. We excluded the majority of the highly toxic compounds from the library and continued our study only with the compounds that fulfilled this non-toxicity criterion.

The main objective of the current project is of course to identify drug compounds that improve motor neuron-regulated coordination in our MJD pathogenesis model. In parallel with the toxicity analyses, we started performing motility assays of AT3q130 animals treated with the small molecules that passed the non-toxicity condition. Our study of the first set of compounds (Prestw-29, Prestw-30, Prestw-38, Prestw-101, Prestw-104, Prestw-123, Prestw-124, Prestw-125, Prestw-127, Prestw-129, Prestw-223, Prestw-224, Prestw-225, Prestw-226, Prestw-227, Prestw-257, Prestw-258, Prestw-259, Prestw-260 and Prestw-261) showed a significant reduction in the percentage of mutant ATXN3 animals that present locomotion defects, when treated with Prestw-38 and Prestw-227. Regarding Prestw-38, we simply know that it corresponds to the compound Hexamethonium Dibromide Dihydrate ($C_{12}H_{34}Br_2N_2O_2$) and that it belongs to the therapeutic group of antihypertensors. Prestw-227 corresponds to the compound Clemizole Hydrochloride ($C_{19}H_{21}C_{l2}N_3$) and it belongs to the therapeutic group of antihistaminic, local anesthetics, antibacterial and antifungal drugs, acting as an H1 antagonist.

One of the main biological markers of all polyQ disorders is the formation of protein aggregates that correlate with pathogenesis. Bearing this in mind, we determined whether these two compounds (Prestw-38 and Prestw-227) modulated mutant ATXN3 aggregation *in vivo*. Prestw-227 treated animals (and not Prestw-38) showed a significant reduction in the aggregation phenotype, suggesting that these two compounds may impact positively in mutant ATXN3-mediated pathogenesis through distinct cellular mechanisms/pathways.

Chapter V

Conclusion & Future Perspectives

In recent years, it has become progressively clear that many neurodegenerative conditions involve aggregation and deposition of misfolded proteins. PolyQ diseases, like MJD, comprise one of the most common groups of inherited neurodegenerative disorders. The etiopathology of these disorders is extremely complex and heterogeneous. The most outstanding evidence pointing to the complexity of these diseases is that to date no single drug has been developed that can prevent the degenerative process or restore viability of neurons that are dying.

Neuroprotective compounds targeting identified pathological mechanisms of diseases have the potential to delay the onset and to slow the progression of these diseases. Various experimental studies in models systems have validated energy insufficiency as a promising therapeutic approach. Compounds such as Cr and CoQ10 buffer neuronal energy demands and are attractive candidates for targeting this important disease mechanism. Considering the widespread effects of Cr supplementation, one may conclude that this compound could be one of the most promising supplements in the therapeutic field. However, we should be alert that several therapeutic effects attributed to this supplement are still speculative. In other terms, although *in vitro* and animal studies have provided strong reason for clinical Cr benefits, the number of randomized controlled trials is very limited.

Concerning our results, the main conclusions are:

- ✓ Cr treatment showed limited effects in MJD pathogenesis in our *C. elegans* model;
- ✓ 264 out of 1120 compounds from the Prestwick Chemical LibraryTM were toxic and were excluded from further testing;
- ✓ We selected 617 compounds safe at 50µM and 19 safe at 25µM that satisfy our non-toxicity criteria;
- ✓ We started a motility analysis with the safe compounds, having completed the analysis for 20 compounds;
- ✓ We have identified two compounds that reduced mutant ATXN3-mediated neuronal dysfunction;
- ✓ We have identified one small molecule that significantly reduced the aggregation load caused by pan-neuronal expression of mutant ATXN3;
- ✓ We have validated our *C. elegans* model of MJD for the screening of novel therapies for MJD.

This work opened several questions that we aim to answer in the near future. We will study mitochondrial dysfunction in our *C. elegans* model of MJD, measuring the mtDNA copy numbers, the frequency of mtDNA deletions and the levels of ATP. We believe that the measurement of ATP will provide a measure of energy available immediately after Cr supplementation and we will able to know the extent to which Cr is metabolized by our model. We will also analyze enzyme activities of MRC complexes. The MRC is composed of five electron transporting protein complexes (complex I to complex V) and the structure, metabolism, and bioenergetics of the nematode MRC are very similar to the mammalian counterpart, so many pathways of intermediary metabolism are conserved in *C. elegans* (Murfitt, et al., 1976; O'Riordan, et al., 1989; Wadsworth & Riddle, 1989).

With regard to combination therapies, many results suggest that combinations of therapies targeting distinct pathogenic mechanisms may be a useful strategy in the treatment of MJD. Considering this, whether Cr is actually at acceptable levels in our model or not, we can try a combination of drugs to a possible potentiation of effects.

Observing the identification of potential therapeutic compounds for testing in our *C elegans* model of MJD, our results are promising because two non-toxic compounds revealed to be modulators of mutant ataxin-3-mediated motor dysfunction which means that we are in the presence of compounds able to modulate the pathways involved in mutant ATXN3 pathogenesis. Regarding the effects of these compounds on ATXN3 aggregation, we will use FRAP to assess protein solubility states. This can be accomplished by photobleaching a region of a neuronal cell (region of interest) with a high-intensity laser pulse, and the movement of unbleached molecules from neighboring areas into the bleached area is recorded by time-lapse microscopy. We are also currently performing pharmacogenetic studies to determine drug target(s). This approach will allow us to complete the characterization of the hit compounds and establish aggregation-behavior correlates. Additionally we will study the impact of these drugs on mutant ATXN3-protein levels, we will exclude the drugs that may be acting at the level of the promoter by quantitative Real Time PCR (qRT-PCR) and perform Western-blot analysis to determine if the others small molecules modulates aggregation or/and neurological dysfunction by altering the steady-state levels of the mutant ATXN3-protein. Next, we will biochemically quantify the aggregate load of our *C. elegans* model, to establish a quantitative correlation between the presence of aggregates and ATXN3-induced

pathogenesis, and for that we will use a technique named Seprion (Sathasivam et al., 2009). We also will determine which intermediate species of aggregation are more toxic by comparing the profile of the aggregation-prone species, when run in agarose gels, with the impact on the animals' behavior, and for that we will perform NAGE (native agarose-gel electrophoresis) (vanHam et al., 2010) that will give us insights regarding native ATXN3 oligomeric species and SDD-AGE (Semi-Denaturating Detergent Agarose Gel Electrophoresis) (Halfmann and Lindquist, 2008) that is performed in denaturant conditions.

We expect to complete this study soon and should be able to identify several efficacious compounds that we will start to test in our MJD (CMVMJD135) mouse model (Silva-Fernandes et al., 2010) in the near future.

This HTS will also give us insight into the mechanisms of MJD pathogenesis. Additionally, the hit compound(s) identified in this screening may represent chemical "scaffolds" to be optimized by chemical modification and constitute strong lead compounds for the development of therapeutics for human polyQ diseases.

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