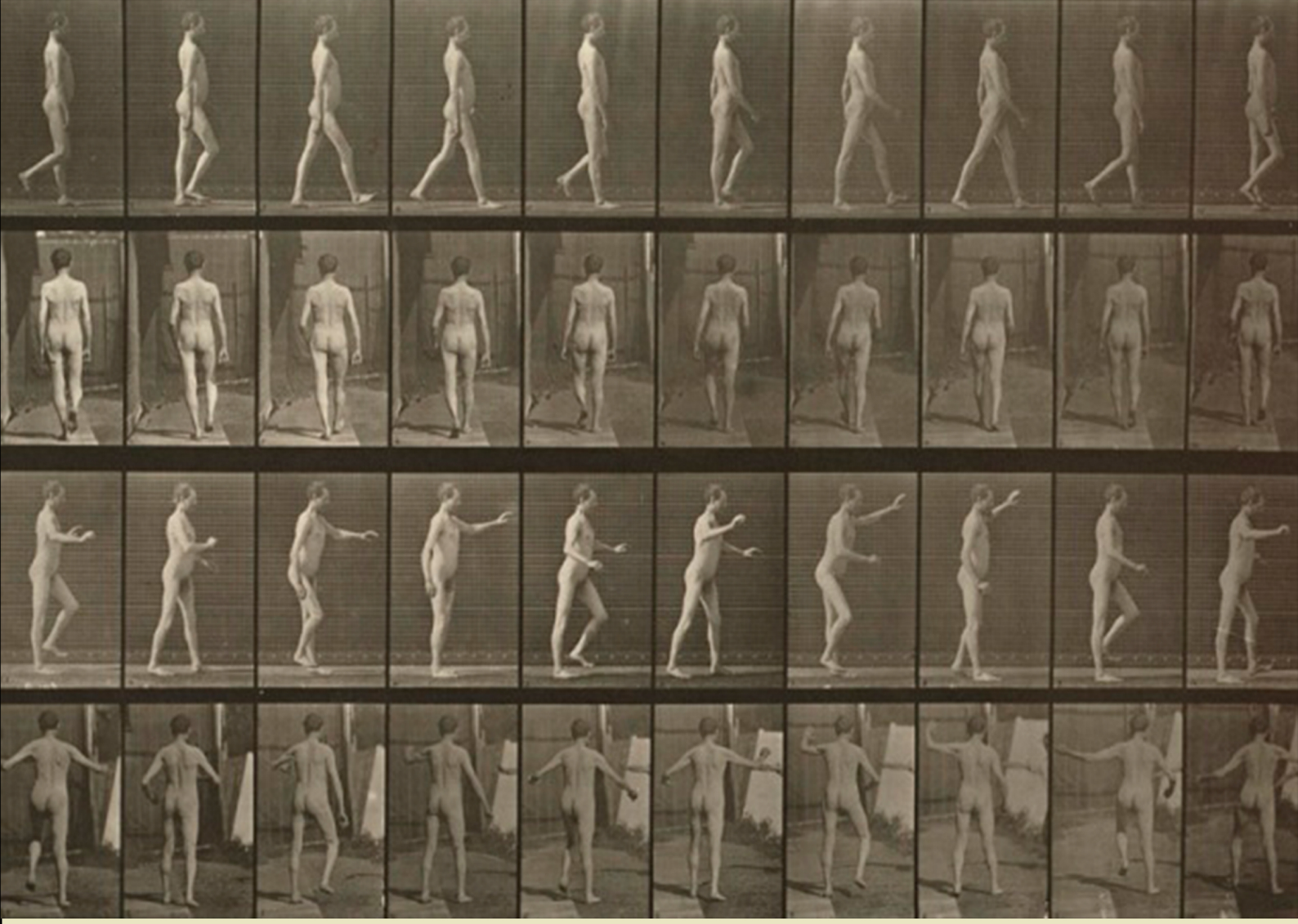

Ana Cristina Lopes Vasconcelos Ferreira

PHARMACOLOGICAL ACTIVATION OF AUTOPHAGY IN MACHADO JOSEPH DISEASE

Dissertação apresentada à Faculdade de Farmácia da Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia Farmacêutica, realizada sob a orientação científica do Professor Doutor Luís Pereira de Almeida (Universidade de Coimbra) e Clévio Nóbrega (Universidade de Coimbra).

Setembro 2012

· U C ·

Universidade de Coimbra

· U C ·
UNIVERSIDADE DE COIMBRA

Ana Cristina Lopes Vasconcelos Ferreira

Pharmacological Activation of Autophagy in Machado-Joseph disease

Dissertação apresentada à Faculdade de Farmácia da Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia Farmacêutica, realizada sob orientação científica do Professor Doutor Luís Pereira de Almeida (Universidade de Coimbra) e Doutor Clévio Nóbrega (Universidade de Coimbra)

Setembro 2012

AGRADECIMENTOS

Ao longo da realização da realização deste trabalho inúmeras pessoas estiveram envolvidas, apresentando um papel fundamental. A todas elas os meus sinceros agradecimentos:

Ao Professor Doutor Luís Almeida, pela oportunidade em integrar no seu grupo de trabalho. Pela disponibilidade e acessibilidade demonstradas. Pela orientação científica, conselhos e paciência no esclarecimento de qualquer dúvida.

Ao Doutor Clévio Nóbrega, que me acompanhou sempre ao longo do trabalho. Obrigada por toda a paciência, compreensão e disponibilidade demonstradas. Pelos bons ensinamentos, orientação fundamental na concretização desta dissertação.

A todos os meus colegas de laboratório pelas muitas horas de partilha e companhia no laboratório. Pelas dicas, ajuda e boa disposição.

À Sara um obrigada especial. Pela partilha dos cafezinhos, desabafos e experiências. Por ser a pessoa que acompanhou esta fase desde o início, e ter sempre uma palavra de ânimo.

À Gladys, que por serem já tantas as vivências partilhadas, parece-me conhece-la desde sempre. Pela amizade, cumplicidade, por se tornar o ponto de abrigo nos momentos de mais fragilidade.

Às minhas amigas de sempre, Andreia e Elisabete, por todas as palavras de incentivo e alento em nos momentos mais importantes. Que apesar de ausentes conseguem fazer-se sempre presentes. Obrigada por tudo.

Ao Francisco, pelo apoio incondicional. Pela força e coragem transmitidas nos momentos de maior cansaço e desânimo, pelo sorriso na hora certa. Por toda ajuda e até pelos sermões.

Ao meu irmão, que apesar de crescido será para mim o eterno miúdo. Pela companhia e compreensão, mesmo quando via nele a única forma de alívio do stress.

Aos meus pais, por todo o esforço e dedicação, pois sem eles não seria possível alcançar mais esta etapa. Por acreditarem e incentivarem a seguir sempre o meu caminho…

INDEX

ABBREVIATIONS

AD – Alzheimer's Disease

APP – Amyloid Precursor Protein

APP-CTF – APP-Derived Fragment

ATG – Authophagy-Related Genes

Atg1 – Autophagy Related Protein 1

ATP – Adenosine Triphosphate

Atx3- Ataxin-3

CACNA1A – Calcium Channel, Voltage Dependent P/Q Type, A1a Subunit

CAMP – Cyclic Adenosine Monophosphate

CBZ – Carbamazepine

CK-II – Casein Kinase II

CMA – Chaperone-Mediated Autophagy

Ct – Cycle Threshold

DAG – Diacylglycerol

DARPP32- cyclic AMP-regulated phosphoprotein, relative molecular mass 32,000

DMSO – Dimethyl Sulfoxide

DRPLA – Dentatorubral-Pollidoluysian Atrophy

DUB – Deubiquitylating Enzime

ECF – Enhanced Chemifluorescent Substract

EGFP- Enhanced Green Fuorescent Protein

ER – Endoplasmic Reticulum

ERAD – Endoplasmic Reticulum - Associated Degradation

FDA – Food And Drug Administration

FIP200 – Focal Adhesion Kinase Family-Interacting Protein Of 200 kDa

GABA- g -amino butyric acid

Gβl – G Protein B-Subunit-Like Protein

HA – Hemaglutinin

HD – Huntington's Disease

HIV-1 – Human Immunodeficiency Virus 1

HSPs- Heat-Shock Proteins

I1R – Imidazoline-1

Impase – Inositol Monophosphatase

IP3 – Inositol 3- Phosphate

IP3R – IP3 Receptor

Ippase – Inositol Polyphosphate 1-Phosphatase

Li – Lithium

LC3- Light Chain 3

MJD – Machado-Joseph Disease

mRNA – Messenger RNA

Mtor – Mammalian Target of Rapamycin

NES – Nuclear Export Signal

Niis – Neuronal Intranuclear Inclusions

NLS – Nuclear Localization Signal

NRB1- Neighbor of BRCA1 gene 1

OD – Optical Density

PBS – Phosphate Buffered Saline

PD – Parkinson's Disease

PE – Phosphatidylethenolamine

PFA – Paraphormaldehyde

PI3K – Phosphatidylinositol-3-Kinase

PI-3-P – Phosphatidylinositol-3-Phosphate

PLC – Phospholipase C

SBMA – Spinal And Bulbar Muscular Atrophy

Scas – Spinocerebellar Ataxias

SQSTM1 – Sequestosome 1

TBP – TATA Box Binding Protein

TBS – Tris-buffered Saline

Uims – Ubiquitin Interaction Motifs

ULK – Unc- 51- Like Kinase

UPS – Ubiquitin-Proteassome System

USA – United States of America

VCP – Valosin-Containing Protein

VPA – Valproic Acid

ABSTRACT

Machado Joseph disease, or spinocerebellar ataxia type 3, is a hereditary neurodegenerative disorder with severe clinical features, being the most common autossomal dominant spinocerebellar ataxia worldwide. This disease is caused by the expansion of the CAG repeat region in the *ATXN3/MJD1* gene, which translates into an expanded polyglutamine tract within the affected protein ataxin-3. Mutant ataxin-3 becomes prone to misfolding, accumulating as nuclear and intracellular inclusions and acquires toxic properties, which lead to neuronal dysfunction and cell death.

The lysosomal-macroautophagy pathway (here termed autophagy) is a major clearance pathway for the degradation of dysfunctional proteins and organelles and also essential in the removal of aggregate-prone intracytosolic proteins, such as mutant ataxin-3. Recently, our group provided evidence of the implication of autophagy in the accumulation of mutant ataxin-3 aggregates and neurodegeneration in Machado-Joseph disease, supporting the idea that modulation of autophagy might be a suitable strategy to mitigate the disease. The current pharmacological strategy for upregulating autophagy through inhibition of the mammalian target of rapamycin (mTor) may lead to complications associated to rapamycin immunosuppressant activity. Therefore, other pathways should be investigated particularly the mTor-independent autophagy induction. Recent evidence suggests that carbamazepine (CBZ), an anticonvulsant and mood-stabilizing drug, signals through phosphoinositol pathway, lowering inositol and IP3 levels and presumably enhancing the autophagy and the clearance of aggregate-prone proteins. Given these evidences the main goal of this study was to investigate the beneficial effects of CBZ in the alleviation of Machado Joseph disease, mediated by its autophagy-enhancing ability.

For this purpose we used a neuroblastoma cellular model, and two animal models, respectively a lentiviral-based MJD mouse model and a transgenic mouse model of disease. In neuroblastoma cells, we found that CBZ mediates a decrease of mutant ataxin-3 levels, by

enhancing autophagy, which was detected by increase of a) LC3-positive autophagic vesicles, b) the ratio of LC3II/LC3I protein levels and c) mRNA expression of LC3. In *in vivo* experiments, using the lentiviral-based model, treatment with CBZ resulted in an improvement of neuropathology indicated by a decrease of the number of mutant ataxin-3 inclusions and of the volume of the brain region depleted of the darpp-32 marker Moreover, evaluation of the effects of CBZ in transgenic mice, revealed a decrease of both aggregated and soluble mutant protein, accompanied by increased levels of mRNA for the autophagy marker LC3 in treated animals. To examine the effects of long-term use of CBZ on motor coordination and activity profile of transgenic mice behavioural tests were performed, using the rotarod and activity box tests. Treated mice presented better performance relative to control mice, travelled increased distances, spending less time resting and more time moving fast.

Altogether these results suggest that CBZ ability to activate autophagy might be a suitable therapeutic approach to mitigate Machado-Joseph disease and potentially to other diseases characterized by misfolding and toxicity of aggregate-prone proteins.

Keywords: MJD; Atx3MUT; CBZ; autophagy; mTor independent pathway

RESUMO

A doença de Machado Joseph ou ataxia espinocerebelosa do tipo 3, é uma doença neurodegenerativa hereditária com manifestações clínicas severas, sendo a ataxia espinocerebelosa dominante autossómica mais comum em todo o mundo. Esta doença é causada por uma repetição excessiva do trinucleótido CAG no gene *MJD1/ATXN3*, que se traduz numa cadeia de glutaminas expandida na proteína afetada, ataxina-3. Devido a esta mutação, a ataxina 3 mutante toma conformações alteradas e adquire propriedades tóxicas, que levam a acumulação de inclusões nucleares e citoplasmáticas, disfunção neuronal e a morte celular.

A via lisosomal da autofagia (aqui designada de autofagia) é um importante mecanismo de degradação de proteínas e organelos disfuncionais, e também fundamental na eliminação de proteínas insolúveis com tendência para agregar; como é característico da ataxina-3 mutada nesta doença. Recentemente o nossos grupo apresentou evidências da implicação da autofagia na doença de Machado Joseph, reforçando a ideia que a modulação da autofagia poderá ser uma estratégia viável para mitigar a doença. A estratégia farmacológica de ativação da autofagia recorrendo ao uso da rapamicina e seus análogos, que têm a capacidade de inibir o complexo mTor (mammalian target of rapamycin) tem contra-indicações para tratamentos a longo prazo devido à sua actividade imunossupressora. Desta forma, impõe-se o estudo de outras vias cuja ativação da autofagia seja independente do complexo mTor. Evidências recentes sugerem que a carbamazepina (CBZ), um anticonvulsivo e estabilizador de humor, atua na via de sinalização do fosfoinositol, diminuindo os níveis de inositol e IP3, o que presumivelmente leva à ativação da autofagia e à degradação de proteínas agregadas. Tendo em conta estas evidências, o principal objetivo deste estudo foi investigar os potenciais efeitos benéficos da carbamazepina na atenuação da doença de Machado Joseph.

Nesse sentido, recorremos a modelos celulares da doença, bem como ao modelo lentiviral e transgénico no roedor. Nas células de neuroblastoma verificámos que a carbamazepina levou a uma diminuição dos níveis da proteína mutada mediada por autofagia, o que foi detetado pelo

aumento do número de vesículas positivas para o marcador de autofagia LC3, do rácio de LC3II/LC3I e pelos níveis aumentados de expressão de LC3.

As experiências *in vivo*, usando o modelo lentiviral, mostraram que o tratamento com CBZ resultou numa melhoria da neuropatologia indicada pela diminuição do número de inclusões de ataxina-3 mutante e de perda de volume do marcador DARPP-32. Para além disso, a avaliação dos efeitos da CBZ em murganhos transgénicos revelou uma diminuição da proteína mutante agregada e solúvel, acompanhada por um aumento da expressão de LC3 nos animais tratados. De forma a avaliar o efeito a longo prazo do uso da CBZ na coordenação motora e perfil de movimento e atividade foram realizados testes de comportamento recorrendo à roda rotativa e caixa de atividade. Os animais tratados apresentaram melhor performance relativamente aos controlos, percorrendo distâncias superiores, permanecendo menos tempo imóveis e mais tempo em movimento rápido.

Em suma, estes resultados sugerem que a capacidade da CBZ em ativar a autofagia poderá ser uma abordagem terapêutica viável no tratamento da doença de Machado-Joseph e potencialmente de outras doenças caracterizadas pela acumulação de proteínas agregadas.

Palavras-chave: MJD; ATX3MUT; CBZ; autofagia; vias independentes de mTor

CHAPTER 1 – INTRODUCTION

1.1. Triplet Repeats Disease

Several human neurological disorders have been found to be associated with a type of genetic mutation: the expansion of trinucleotide repeats (Fu *et al.*, 1991; La Spada *et al.*, 1991). These repeats show moderate levels of length variation within the normal population. However, in disease conditions, the repeat expands beyond the normal range, altering the biological function of the gene. The unstable properties of triplet repeat DNA sequences explain the non-Mendelian inheritance patterns, as well as the increase in severity and the decrease in the age at onset of a disease in subsequent generations.

The expansion of triplet repeat may be found in coding regions, as in the spinal and bulbar muscular atrophy (SBMA) and huntington's disease (HD) or in non-conding regions (introns, 5'UTR, 3'UTR), as is the case of Fragile X syndrome (Tsuji, 1997). The mutation in noncoding-repeat regions leads to a loss of function of the respective proteins or, in some cases, an acquired function of a toxic triplet repeat transcript, which accounts the different mechanisms of pathology. Exonic CAG repeat expansions translate into a long polyglutamine tract, conferring a toxic function to the protein (Cummings and Zoghbi, 2000). This class of disorders is called as polyglutamine diseases.

1.1.1. Polyglutamine diseases

Polyglutamine diseases consist of a group of neurodegenerative disorders, including HD, dentatorubral-pollidoluysian atrophy (DRPLA), Kennedy's disease or spinal and bulbal muscular atrophy (SBMA, X-linked recessive disorder) and six forms of spinocerebellar ataxias (SCAs) (Orr and Zoghbi, 2007; Havel *et al.*, 2009; Wilburn *et al.*, 2011) (see table 1). Despite the wide range of neurological, psychiatric and motor symptoms present in these conditions, all disorders are progressive, typically striking in midlife and causing increasing neuronal dysfunction and eventual neuronal loss 10–20 years after onset of symptoms (Zoghbi and Orr, 2000; Gatchel and Zoghbi, 2005).

Table 1 Polyglutamine diseases

Disease	Protein	CAG repeat		Inclusions	Brain regions most affected
		Normal	Pathogenic		
HD	Huntingtin	6-34	36-121	Nuclear and cytoplasmatic	Striatum, cerebral cortex
SMBA	Androgen receptor	9-36	38-62	Nuclear and cytoplasmatic	Anterior horn and bulbar neurons, dorsal root ganglia
DRLPA	Atrophin 1	7-34	49-88	Nuclear	Cerebellum, cerebral cortex, basal ganglia, Luys body
SCA1	Ataxin 1	6-39	40-82	Nuclear	Cerebellar Purkinje cells, dentate nucleus, brainstem
SCA2	Ataxin 2	15-24	32-200	Nuclear	Cerebellar Purkinje cells, brain stem, frontotemporal lobes
SCA3/ MJD	Ataxin 3	13-36	61-84	Nuclear	Cerebellar dentate neurons, basal ganglia, brain stem, spinal cord
SCA6	CACNA1 A	4-20	20-29	Citoplasmatic	Cerebellar Purkinje cells, dentate nucleus, inferior olive
SCA7	Ataxin-7	4-35	37-306	nuclear	Cerebellum, brain stem, macula, visual cortex
SCA17	TBP	25-42	47-63	nuclear	Cerebelllar Purkinje cells, inferior olive

HD, Huntington's disease; DRPLA, dentatorubral-pallidoluysian atrophy; SBMA, spinal and bulbar muscular atrophy; SCA, spinocerebellar ataxia; MJD Machado-Joseph disease; CACNA1A, calcium channel, voltage dependent, P/Q type, a1A subunit; TBP, TATA box binding protein.

An important common feature to all polyQ diseases is the negative correlation between the age of onset and the number of CAG repeats, leading to an earlier development of the disease (Maciel *et al.*, 1995; Maruyama *et al.*, 1995; Ranum *et al.*, 1995; Zoghbi and Orr, 2000; Riess *et al.*, 2008).

The genetic similarities among all these diseases could indicate a possible common mechanism of pathogenesis, caused by the toxic properties of the polyglutamine tract.

Several mechanisms of pathogenesis have been proposed (Fig.1.1). In many polyglutamine diseases, such as HD, SBMA and SCA3/MJD, the pathogenesis is associated to proteolityc

cleavage that generates a toxic fragment. The expanded polyglutamine tract within a target protein facilitates transition to a novel, toxic conformation, causing alterations at different levels. Toxicity may be caused by the peptide as a monomer or it associated form: oligomers. The oligomers can assemble into larger aggregated species and eventually are deposited in intracellular inclusions. Proteolytic cleavage of full-length protein into toxic cleavage fragment may also contribute to this process.

The main toxic effects of the pathological protein include transcriptional changes, caused by interactions of the expanded protein with transcription factors; proteotoxic stress as a result of the disruption of the quality control systems of the cell and metabolism and mitochondrial dysfunction (Shao and Diamond, 2007) (Fig.1.1).

The majority of polyglutamine disorders are spinocerebellar ataxias.

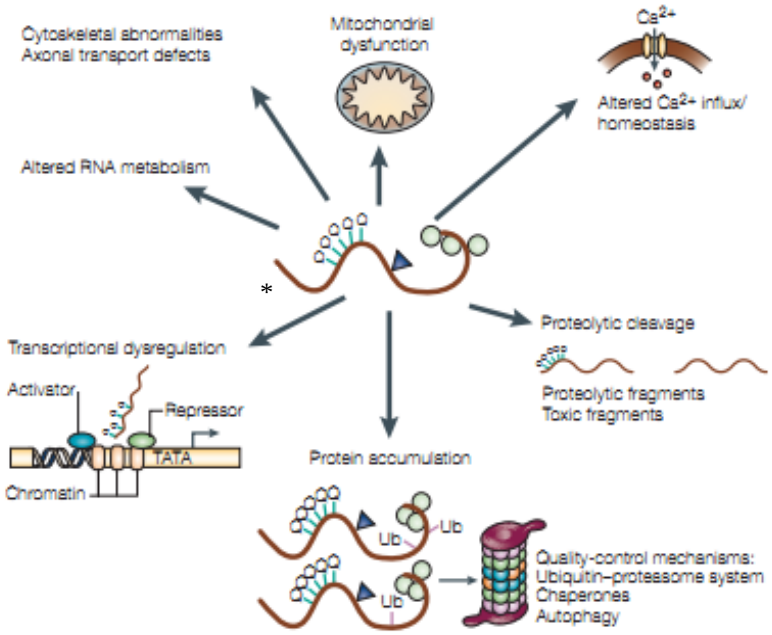

Figure 1.1. Mechanisms of pathogenesis of polyglutamine diseases(Gatchel and Zoghbi, 2005).

*mutant polyglutamine protein

1.1.2. Autossomal dominant spinocerebellar ataxias

SCAs are a heterogeneous group of neurologic disorders characterized by variable degrees of degeneration of the cerebellum, spinal tracts, and brain stem. The autosomal dominant spinocerebellar ataxias present progressive gait and limb ataxia as a core clinical feature of SCAs—in most cases the predominant, disabling feature of disease— but the ataxia is usually accompanied by signs and symptoms that reflect damage beyond the cerebellum and its afferent and efferent pathways (Paulson, 2007). The disease onset is usually between 30 and 50 years of age, progressively evolving to a fatal state (Zoghbi, 2000; Taroni and DiDonato, 2004; Duenas *et al.*, 2006; Carlson *et al.*, 2009).

1.2. Spinocerebellar ataxia type 3/Machado Joseph-disease

SCA3 or MJD is a clinically and genetically heterogeneous neurodegenerative disorder which leads to progressive cerebellar ataxia (Schols *et al.*, 1995). This neurodegenerative disease was first described among Portuguese immigrants living in New England, United States of America (U.S.A) as an autosomal dominant form of ataxia in the descendants of William Machado (Nakano *et al.*, 1972). A few years later, other cases in Thomas and Joseph families of Portuguese immigrants were described in the U.S.A. (Woods and Schaumburg, 1972; Rosenberg *et al.*, 1976). These reports led to different designations: Machado disease; Nigro-spino-dentatal-degeneration with nuclear ophthalmoplegia (Woods and Schaumburg, 1972), Joseph disease (Rosenberg *et al.*, 1976) and Azorean disease (Romanul *et al.*, 1977). Later on, Lima and Coutinho proposed the name of Machado-Joseph disease for the previously described clinical entities (1980). An autosomal dominant ataxia called spinocerebellar ataxia type 3 (SCA3) was also described, but after genetic studies, by identification of the *MJD1* gene, it was shown that both correspond to the same disease (Kawaguchi *et al.*, 1994; Stevanin *et al.*, 1994; Cancel *et al.*, 1995; Twist *et al.*, 1995).

Presently, MJD is considered the most common form of SCA worldwide (Schols *et al.*, 2004). It presents a large geographic distribution with higher frequency in countries such as Brazil and Portugal, which reaches highest values in the islands of the Azores (Lima *et al.*, 1997), China and Germany (Schols *et al.*, 1997; Jardim *et al.*, 2001; van de Warrenburg *et al.*, 2002; Jiang *et al.*, 2005; Vale *et al.*, 2010).

1.2.1. Clinical symptoms

MJD is a neurodegenerative disorder characterized by a wide range of clinical manifestations involving predominantly the cerebellar, pyramidal, extrapyramidal, motor neuron and oculomotor systems (Bettencourt and Lima, 2011). The phenotype is one of the most variable (Rosenberg *et al.*, 1976) among SCAs; typical signs include ataxia, progressive external

ophthalmoplegia, pyramidal and extra pyramidal signs such as dystonia with rigidity, distal muscular atrophies, facial and lingual fasciculation, nystagmus, eyelid retraction, as well as bulging eyes. Sleep disturbance are also common in SCA3 and mostly derive from restless legs syndrome and periodic leg movements in sleep responding to dopaminergic stimulation (Lima and Coutinho, 1980; Rosenberg, 1992; Sudarsky and Coutinho, 1995; Schols *et al.*, 1998).

Disease manifestations usually start during adulthood, with a mean age at onset of 37.4 years. The average survival time after onset is 20 years (Sequeiros and Coutinho, 1993). The wide of clinical features of MJD led to classification in various subtypes of disease (Cancel *et al.*, 1995; Maciel *et al.*, 1995; Durr *et al.*, 1996; Jardim *et al.*, 2001).

1.2.2. Neuropathology

The neuropathology of MJD consists of neuronal loss in many regions of the brain, specifically in the basal ganglia, thalamus, midbrain, pons, medulla oblongata and cerebellum (Fig. 1.2). In addition, there is loss of pontine neurons, neurons of the substantia nigra, anterior horn cells, and Clarke's column in the spinal cord, as well as neurons in many cranial motor nuclei and posterior root ganglia. Structures such as the cerebral and cerebellar cortex and inferior olives are relatively spared (Rosenberg *et al.*, 1976; Coutinho and Andrade, 1978; Coutinho *et al.*, 1982; Sakai *et al.*, 1983; Kanda *et al.*, 1989).

A hallmark of neurodegeneration in brains of patients with MJD is the presence of neuronal intranuclear inclusions (NIIs). These inclusions result from the nuclear accumulation of misfolded and toxic forms of mutant polyQ proteins (Paulson *et al.*, 1997; Schmidt *et al.*, 1998). NIIs are spherical and eosinophilic aggregates, varying size from 1 to 4 μm (Yamada *et al.*, 2008). It is known that these NIIs contain numerous other proteins such as proteasome subunits, ubiquitin (Chai *et al.*, 1999), and several transcription factors (Schaffar *et al.*, 2004). Therefore, neuronal intranuclear inclusions might be linked to the pathogenesis of polyQ diseases through mechanisms such as the impediment of transcription due to the sequestration of transcriptional activators (McCampbell *et al.*, 2000; Chai *et al.*, 2002) and the disturbance of the quality control systems, due to recruitment of proteasome components and chaperones (Paulson *et al.*, 1997; Chai *et al.*, 1999; Chai *et al.*, 1999; Warrick *et al.*, 1999; Ferrigno and Silver, 2000; Muchowski *et al.*, 2000; Schmidt *et al.*, 2002), causing cellular dysfunction.

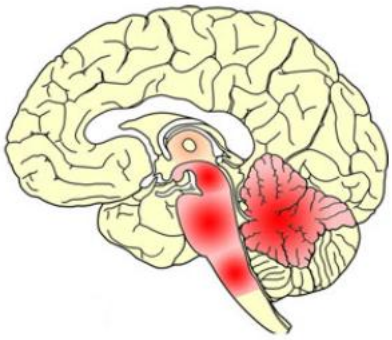

Figure 1.2. Distribution of neuronal loss in the brain of patients with Machado Joseph disease. Diagrammatic representation of midsagittal sections through the cerebrum, cerebellum and brainstem of the human brain. The degree of neuronal loss in the cerebral cortex, basal forebrain, thalamus, cerebellum and brainstem is indicated by red (severe) or light red (marked) (Seidel et al., 2012).

1.2.3. *MJD1/ATXN3* gene

The disease locus was first mapped in the long arm of chromosome 14q32.1 (Takiyama *et al.*, 1993) and in 1994 the *MJD1/ATXN3* gene was identified (Kawaguchi *et al.*, 1994). A few years later, the genomic structure of the MJD gene was described. The *MJD1* gene was found to span 48,240 bp and to contain 11exons, with the CAG tract located at the exon 10. Transcripts ubiquitously expressed in human tissues with different sizes- 1.4, 1.8, 4.5, and 7.5kb, probably resulting from differential splicing (exon 10 or 11) and polyadenylation (exon 11) were also detected (Ichikawa *et al.*, 2001). Recently two novel exons were described for the *MJD1* gene by Bettencourt and collaborators. Fifty-six alternative splicing variants, generated by four types of splicing events, were observed, of which 50 of those transcripts were not previously described and 26 were MJD-specific (Bettencourt *et al.*, 2010).

The normal *MJD1* gene encodes a polyQ-containing protein named ataxin-3 (atx3); in pathologic conditions the overrepetition of the CAG trinucleotide translates into an expanded polyglutamine tract at the C-terminus of ataxin-3 (Kawaguchi *et al.*, 1994; Durr *et al.*, 1996). The highly polymorphic CAG repeats are unstable and selectively expanded in MJD patients. The wild-type alleles of MJD1 range from 12 to 44 CAG repeats whereas MJD patients alleles range from 52 up to 87 repeats. Although rare, there is an intermediate size where alleles have between 45 and 51 CAG repeats (Kawaguchi *et al.*, 1994; Cancel *et al.*, 1995; Maciel *et al.*, 1995; Maciel *et al.*, 2001; Padiath *et al.*, 2005).

1.2.4. Ataxin-3 protein

1.2.4.1. Expression and subcellular localization

MJD1 gene encodes ataxin-3, an intracellular protein of unclear physiological role with a predicted molecular weight of 42 kDa, depending on the number of CAG repeats (Kawaguchi *et al.*, 1994). Wild-type ataxin-3, as well as the mutant ataxin-3, is expressed ubiquitously in different human tissues and cell types (Paulson *et al.*, 1997; Wang *et al.*, 1997; Trottier *et al.*, 1998; Ichikawa *et al.*, 2001; Costa *et al.*, 2004). This protein is widely expressed in the brain, with variable levels of expression depending of the region (Paulson *et al.*, 1997; Trottier *et al.*, 1998). As regional localization of ataxin-3 expression does not match completely with neurodegenerated regions in MJD, it was suggested that cellular ataxin-3 expression is not by itself sufficient to cause neurodegeneration, and other cell-specific factors must be involved to explain the pathology (Paulson *et al.*, 1997; Schmidt *et al.*, 1998).

The localization of ataxin-3 is predominantly cytoplasmatic (Paulson *et al.*, 1997; Schmidt *et al.*, 1998), although it is also detected in the nucleus where it accumulates upon polyglutamine expansion (Wang *et al.*, 1997; Tait *et al.*, 1998; Evert *et al.*, 1999).

1.2.4.2. Structure and domains

Ataxin-3 belongs to the family of cysteine proteases. Structurally, it is composed of 339 aminoacids and by a variable number of glutamines, which constitute the polymorphic polyglutamine tract (poly-Q tract) (Kawaguchi *et al.*, 1994). Ataxin-3 is composed of a globular N-terminal Josephin domain with a papain-like fold, combined with a flexible C-terminal tail that contains 2 or 3 ubiquitin interaction motifs (UIMs), that binds polyubiquitylated proteins with a strong preference for chains containing four or more ubiquitins, and the poly-Q tract (Burnett *et al.*, 2003; Masino *et al.*, 2003) (Fig.1.3). It has been reported that the 3UIM ataxin-3 protein is the predominant isoform in the central nervous system (Harris *et al.*, 2010).

The Josephin domain is an important functional region of ataxin-3 that plays a role both in the normal and in the pathological functions of the protein. There are evidences that

thermodynamic stability of ataxin-3 is driven by the properties of the Josephin domain, but the presence of an expanded polyQ tract increases drastically the protein's tendency to aggregate (Masino *et al.*, 2004; Tzvetkov and Breuer, 2007).

The josephin domain has two additional Ub binding surfaces, one is close to the active site (site 1) and the second Ub-binding site (site 2) coincides with the interaction surface of the Ub-like (Ubl) domain of hHR23B (Nicastro *et al.*, 2005). The Ub-binding site 1 is indispensible for the enzymatic activity of ataxin-3, while site 2 may be dedicated to the recognition of hHR23 proteins (Nicastro *et al.*, 2010).

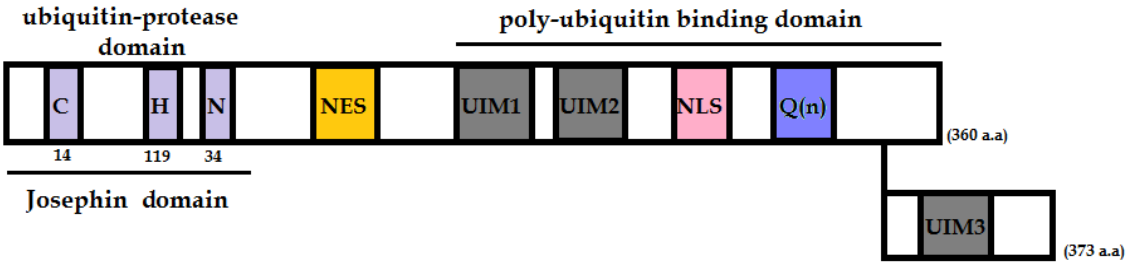

Figure 1.3. Atxaxin-3 protein structure. Shown are the three predicted UIM regions of ataxin-3, the polyQ domain, the conserved Josephin domain encoding a predicted ubiquitin-specific protease with catalytic triad (Cys14, His119, and Asn136), a nuclear export signal (NES) and nuclear localization signal (NLS).

The catalytic activity is mediated by a glutamine upstream of a cysteine in the JD N-terminal part and a histidine and an asparagine or aspartate in the JD C-terminal part. The cysteine, the histidine, and the asparagine constitute the catalytic triad characteristic of cysteine proteases (Albrecht *et al.*, 2004).

A highly conserved, putative nuclear localization signal (NLS), is found upstream of the polyQ stretch. This NLS follows a potential casein kinase II (CK-II) phosphorylation site, which may determine the rate of the observed ataxin-3 transport into the nucleus (Tait *et al.*, 1998). Ataxin-3 may also contain two nuclear export signals (NES 77 and NES 141) following the Josephin domain. The localization of the NES signals in the N-terminal region, predicted to be cut-off from the NLS- and polyglutamine-containing C-terminal part of ataxin-3 in its aberrant

form, could explain important aspects of the pathogenesis of SCA3 (la Cour *et al.*, 2003; Antony *et al.*, 2009).

1.2.4.3. Functions

Ataxin-3 is a deubiquitylating enzime (DUB), being part of the Josephin family, and initial insights suggest that it has both nuclear and cytoplasmic functions (Li *et al.*, 2002). The presence of UIMs in ataxin-3 raised the possibility that ataxin-3 is normally an ubiquitin-binding protein and that its colocalization to ubiquitin-conjugated protein inclusions is mediated by this property. Therefore, it was reported that ataxin-3 binds and hydrolyses polyubiquitin chains (Doss-Pepe *et al.*, 2003; Chai *et al.*, 2004; Berke *et al.*, 2005; Burnett and Pittman, 2005), interacts with ubiquitinated proteins and can associate with the proteasome through interactions with the ubiquitin- and proteasome-binding factors Rad23 and valosin-containing protein (VCP/p97) (Doss-Pepe *et al.*, 2003; Zhong and Pittman, 2006; Wang *et al.*, 2007), p45 (Wang *et al.*, 2007) and Ubxn-5 (Rodrigues *et al.*, 2009). Additionally, it was shown that the DUB activity and UIMs of Atx3 are required for aggresome formation (Burnett and Pittman, 2005). Polyglutamine expansion may affect the interactions of Atx3 with other proteins and/or affect its DUB activity. Although there are evidences that wild-type and pathological Atx3 have very similar DUB activity, pathological Atx3 cleaves short ubiquitin chains more efficiently than does wild-type Atx3 (Burnett *et al.*, 2003).

Ataxin-3 has been also implicated in the cytoskeletal network. *In vitro*, the absence of Atx3 leads to an overt cytoskeletal/adhesion defect. Cells display fewer projections and present evident cytoskeletal disorganization, loss of cell adhesion and increased cell death (Rodrigues *et al.*, 2010).

Ataxin-3 function was also associated to Endoplasmic reticulum (ER)- associated degradation (ERAD), which eliminates misfolded ER proteins by exporting them into the cytosol for degradation by the ubiquitin–proteasome system (Meusser *et al.*, 2005). Atx3 is suggested as a shuttling factor with an involvement of its deubiquitinating activity in ER-

associated protein degradation regulation (Kuhlbrodt *et al.*, 2005). There are evidences that ataxin-3 interacts with the retrotranslocation complex consisting of Derlin-1, VIMP, certain ER-specific ubiquitin ligases, and the ATPase p97 in the ER membrane. The expression of the poly-Q–expanded atx3 mutants may also impair ERAD and trigger ER stress, which may contribute to the pathogenesis of spinocerebellar ataxia (Wang *et al.*, 2006; Zhong and Pittman, 2006).

Ataxin-3 is also important for myogenesis through regulation of integrin subunit levels. Ataxin-3 was found to be strongly expressed in the early myotome of mouse embryos, and it depletion in cells perturbs the phenotypical response to differentiation. Moreover, levels of integrin subunits, as well as many other proteins implicated in the integrin signalling pathway, are altered in ataxin-3-depleted cells. These findings raise the possibility that ataxin-3 contributes to muscle differentiation by preventing the degradation of integrin subunits through the ubiquitin-proteassome system (UPS) (do Carmo Costa *et al.*, 2010).

Furthermore, ataxin-3 seems to play a role in agressome formation. The stability and aggregation properties of non-expanded Atx3 are determined by the properties of the Josephin domain, but the presence of an expanded polyQ tract increases the protein's tendency to aggregate. Polyglutamine expansion may affect the interactions of Atx3 with other proteins and affect its DUB activity (Masino *et al.*, 2004; Burnett and Pittman, 2005; Masino *et al.*, 2011).

Recently, was shown that the absence of ataxin-3 leads to an enhanced stress response in *C. elegans*. This phenotype was correlated with a distinct transcriptomic and proteomic profile with several molecular chaperones abnormally up-regulated during heat shock and recovery. These evidences are important in disease context. Long-term deregulation of heat-shock proteins (HSPs) can be detrimental for cell growth, division and viability (Feder *et al.*, 1992; Krebs and Feder, 1997) and, along with the proteotoxic stress; this may contribute to neuronal demise in the context of MJD (Rodrigues *et al.*, 2011).

1.3. Protein clearance mechanisms

A common cytopathological feature of the polyglutamine disorders, as well as other neurodegenerative disorders, is an intracellular deposition of aggregated and ubiquitinated poteins. The aggregated forms of these proteins and their intermediate forms, in most of cases, present toxic and pathological features, and may lead to the impairment of protein clearance mechanisms (Bence *et al.*, 2001; Bennett *et al.*, 2005; Rubinsztein, 2006).

Eukaryotic cells have two main pathways for protein degradation: the ubiquitin- proteasome system and the autophagy- lysosomal pathway, which constitute essential components of the cellular quality systems (Ciechanover, 2005).

1.3.1. Ubiquitin-Proteasome system

The UPS is responsible for a highly selective degradation of short-lived and misfolded proteins present in cytoplasm and nucleus. The system involves the targeting of susceptible unfolded proteins by ubiquitin and to be degraded by this system (Fig. 1.4).

The degradation of a protein via the ubiquitin-proteasome pathway involves two steps: tagging of the substrate by covalent attachment of multiple ubiquitin molecules and degradation of the tagged protein by the 26S proteasome complex with release of free and reusable ubiquitin. This process is mediated by ubiquitin recycling enzymes DUBs. Activation and conjugation of ubiquitin to the protein substrate proceeds via multi- step reactions, catalysed by E1 -ubiquitin-activating enzyme, E2 -ubiquitin-conjugating enzyme, and E3 -ubiquitin-protein ligase (reviewed by (Ciechanover, 2001; Glickman and Ciechanover, 2002; Ciechanover, 2005).

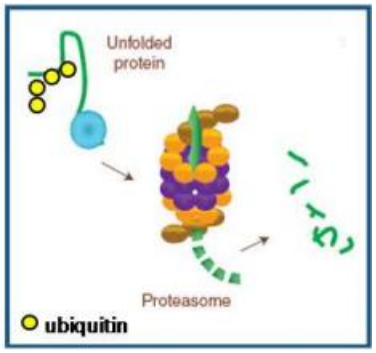

Figure 1.4. The ubiquitine-proteosome system. Adapted from Wong and Cuervo, 2010.

1.3.2. Autophagy

Autophagy, or cellular self-digestion, is a cellular pathway in which dysfunctional proteins and organelles are transported to lysosomes for degradation. These single membrane vesicles contain in their lumen the largest variety of cellular hydrolases including proteases, lipases, glycosilases and nucleases (De Duve and Wattiaux, 1966). Christian de Duve was awarded the Nobel Prize for the discovery of lysosome in 1974. However, only in the last years the autophagy pathway has been intensively explored and its mechanisms elucidated (Harding *et al.*, 1996; Klionsky *et al.*, 2003; Wang and Klionsky, 2003).

Depending on the type of cargo and the cellular conditions, cargo recognition and delivery occurs by different mechanisms, giving rise to different modalities of autophagy (Mizushima *et al.*, 1998; Yang *et al.*, 2002; Yang and Klionsky, 2009). The three major intracellular pathways in eukaryotic cells are macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) (Fig.1.5). In macroautophagy, a whole region of the cytosol is sequestered into a double-membrane vesicle that fuses with lysosomes for cargo delivery. By contrast, microautophagy involves the direct engulfment of cytoplasm that is internalized into the lysosomal lumen as a single membrane vesicle, whereas in CMA a targeting motif is recognized by chaperones that mediate the translocation of unfolded, soluble proteins directly across the limiting membrane of the lysosome (Yang and Klionsky, 2009; Wong and Cuervo, 2010).

Several cellular functions have been associated with autophagy. This clearance pathway is responsible to remove the damage long-lived protein and the organelles and contribute for normal turnover of these intracellular components, playing a key role in cellular quality control.

Furthermore, autophagy has also the main function of maintenance of the cellular energetic balance, providing a cellular adaptive response to stress conditions (Singh and Cuervo, 2011).

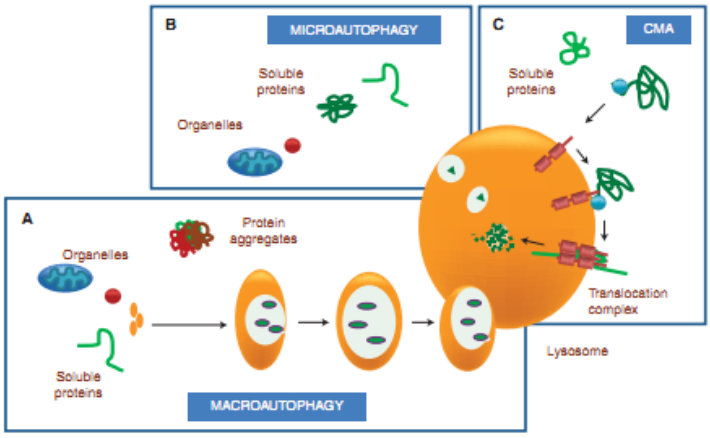

Figure 1.5. Autophagy pathways. (A) Macroautophagy (B) Microautophagy (C) Chaperone-mediated autophagy (CMA) Adapted from Wong and Cuervo, 2010.

There are more than 30 autophagy-identified genes in yeast and many of these have mammalians orthologs. These authophagy-related genes (ATG) can be grouped according to the functions of their products (ATG proteins), which are involved in regulation of different steps of the autophagy pathway: initiation, elongation, maturation, and fusion with the lysosome (Mizushima *et al.*, 1998; Mizushima *et al.*, 2002)

1.3.2.1. Autophagy pathway

Initiation

Autophagy occurs at basal levels under normal conditions, however this phenomenon can be up-regulated by specific stress factors such as starvation, hypoxia, oxidative stress, toxin exposure, among others (Kiffin *et al.*, 2004; Zhang *et al.*, 2008)

The induction of autophagy is mediated through the mammalian target of rapamycin (mTor)-dependent and independent pathways. The serine/threonine protein kinase mTOR is the central inhibitor of autophagy, which is activated upon nutrient rich conditions and inhibited by rapamycin administration or under starvation conditions, up-regulating autophagy. The mTOR kinase integrates input information from several upstream transduction pathways, such as

insulin, class I Phosphatidylinositol-3-kinase (PI3K) and Akt (Laplante and Sabatini, 2009; Sengupta *et al.*, 2010) and also negatively regulates autophagy related protein 1 (Atg1), and other serine/threonine kinase proteins like (Unc- 51- Like Kinase1 and 2) (ULK-1 and 2) which are the mammalians homologs (Chan and Tooze, 2009; Ganley *et al.*, 2009). The ULK-1 and 2 are in a large complex that includes the mammalian homolog of Atg17, focal adhesion kinase family-interacting protein of 200 Kda (FIP 200), and the mammalian Atg13. The complex formed has a key role in the induction of autophagy. A decrease in mTOR activity leads to dephosphorylation of ULK1, ULK2 and Atg13, activating ULK1/2, which phosphorylates FIP200 and to induce autophagosome formation (Chang and Neufeld, 2009; Ganley *et al.*, 2009; Hara and Mizushima, 2009; Hosokawa *et al.*, 2009; Jung *et al.*, 2009).

The formation of the phagophores, the precursors of autophagosomes, is the inicial step of macroautophagy pathway (Fig.1.6). The membrane source from which phagophores arise is still an issue of debate. There are studies that support different hypothesis: a) autophagosomes can be generated *de novo* from preexisting intracellular precursors (Hirsimaki *et al.*, 1982; Baba *et al.*, 1995); several organelles can contribute for autophagosomes formation such as b) ER (Dunn, 1990; Kim and Klionsky, 2000; Axe *et al.*, 2008; Hayashi-Nishino *et al.*, 2009; Yla-Anttila *et al.*, 2009; Matsunaga *et al.*, 2010); c) Golgi complex, mainly in Yeast (Reggiori *et al.*, 2004; Geng *et al.*, 2010; van der Vaart *et al.*, 2010); d) mitochondria, which may be a membrane source during starvation (Hailey *et al.*, 2010; Tooze and Yoshimori, 2010) or e) plasma membrane (Ravikumar *et al.*, 2010).

The autophagosome formation start with the interaction of ULK complex with another kinase complex, the autophagy-regulating macromolecular complex (PI3K complex) to induce the formation of the phagophore, nevertheless autophagy can be induced in an mTOR-independent way by direct activation of the class III PI3K complex or Beclin-1.

The class III PI3K is a crucial protein regulating the nucleation of the phagophore. Vps34, which is part of the PI3K complex is responsible for the formation of phosphatidylinositol-3-phosphate (PI-3-P), which has been implicated in the recruitment of Atg proteins, essential for the formation of the phagohore (Petiot *et al.*, 2000; Axe *et al.*, 2008). Other autophagy-related

proteins are found in the same complex including Beclin-1/ Atg6, Atg14/barkor, and p150/Vps15, UVRAG (UltraViolet irradiation Resistant-Associated Gene) and Ambra 1 (Volinia *et al.*, 1995; Kihara *et al.*, 2001; Fimia *et al.*, 2007; Itakura *et al.*, 2008; Liang *et al.*, 2008; Sun *et al.*, 2008). Beclin-1 has been correlated with autophagy activity since heterozygous deletion of beclin-1 leads to neurodegeneration (Pickford *et al.*, 2008) and the overexpression of this protein reflects an increase of autophagy in response to pathogenic proteins (Erlich *et al.*, 2006; Nascimento-Ferreira *et al.*, 2011). Beclin-1 binding partners like ambra-1, UVRAG, and bif-1 have been identified as essential to autophagosome formation. On the other hand, anti-apoptotic proteins Bcl-2 or Bcl-X negatively regulate this pathway (Pattingre *et al.*, 2005).

Elongation

The step following the nucleation of the phagofore is the expansion and elongation of the membrane. Therefore, two ubquitin-like reactions are required. First, the ubiquitin-like protein Atg12 is conjugated to Atg5, reaction mediated by Atg7 (E1 ubiquitin activating enzyme-like) and Atg10 (E2 ubiquitin conjugating enzyme-like) (Mizushima *et al.*, 1998). Then, Atg12-Atg5 is conjugated with Atg16L, resulting in a large multimeric complex with 800-kDa called Atg16L complex, localized in the isolation membrane.

In the other ubiquitin-like reaction, occurring in parallel, the mammalian homologue of Atg8, LC3 is synthesized as precursor form (pro-LC3) and cleaved by the cystein protease Atg4 to generate LC3-I, a cytosolic form of the protein (Hemelaar *et al.*, 2003). Next, LC3-I is activated by E1 Atg7 (Tanida *et al.*, 2001) and transferred to the active-site cystein of the E2 Atg3, which then conjugates to phosphatidylethenolamine (PE) to form LC3-II (Noda *et al.*, 2008; Oh-oka *et al.*, 2008). LC3-II and the Agt16L complex are essential for growth and expansion of phagophore. Atg16L determines where the autophagosome membrane expansion starts, indicating the sites of LC3 lipidation (Mizushima *et al.*, 2002; Fujita *et al.*, 2008) and LC3-II, in turn, will lead to elongation of the isolation membrane (Nakatogawa *et al.*, 2007; Weidberg *et al.*, 2011).

Since LC3-II associates specifically with autophagosomes membranes and remains bound even after fusion with lysosomes, LC3-II levels can be correlated with the number of autophagosomes present in the cell, which is the basis of many assays to measure autophagic flux (Kabeya *et al.*, 2000; Klionsky *et al.*, 2008; Rubinsztein *et al.*, 2009).

Cargo sequestration

Although autophagy was previously considered a nonselective bulk degradation pathway, in the last few years, several evidences support the selectivity of the sequestration of autophagic cargo (Kraft *et al.*, 2009). Selective autophagy may be possibly involved in the recognition of post-translational modification such as ubiquitination or acetylation (Jeong *et al.*, 2009; Kirkin *et al.*, 2009).

The first cargo-recognizing molecule described was the sequestosome 1/p62 (SQSTM1/p62), which was reported to bind ubiquinated protein aggregates and through it interaction with LC3 brings autophagosome formation to these aggregates (Bjorkoy *et al.*, 2005; Tan *et al.*, 2008). Later on, other molecules were reported to be able to recognize signals from ubiquitin, which is the case of neighbor of BRCA1 gene 1(NBR1) (Pankiv *et al.*, 2007; Kirkin *et al.*, 2009; Lamark *et al.*, 2009).

Changes in substrates as well as in the autophagic system could lead to inefficient cargo recognition and consequently to autophagic failure (Martinez-Vicente *et al.*, 2010).

Maturation and Fusion

In the final steps of the autophagic pathway, the autophagosome vesicules may fuse with lysosomes, forming autolysosomes (Fig.1.6). For that, autophagosome are transported along microtubules towards lysosomes, in the perinuclear region, using the dynein-dynactin complex (Ravikumar *et al.*, 2005; Jahreiss *et al.*, 2008; Kimura *et al.*, 2008).

The machinery involved in the fusion of autophagosomes to the lysosome include proteins such as ESCRT, SNAREs, Rab7, and the class C Vps proteins (Atlashkin *et al.*, 2003; Gutierrez

et al., 2004; Rusten *et al.*, 2007; Furuta *et al.*, 2010). Also the UVRAG protein, a Beclin 1 interacting protein, is involved in this process through the recruitment of class C Vps complex. This interaction stimulates Rab7 GTPase activity and autophagosome fusion with late endosomes/ lysosomes, consequently increasing delivery and degradation of the autophagic cargo (Gutierrez *et al.*, 2004; Liang *et al.*, 2008).

Furthermore, also lysosome activity is crucial to the efficient cargo degradation. Studies using bafilomycin A1 or chloroquine, which inhibit the intra-lysosomal acidification, have described a blockage of the fusion of autophagosomes with lysosomes (Yamamoto *et al.*, 1998; Jahreiss *et al.*, 2008). Due to their ability to inhibit the acidification and the consequent accumulation of autophagosomes, these molecules are often used to measure the autophagic flux in the cells (Perry *et al.*, 2009; Zois *et al.*, 2011).

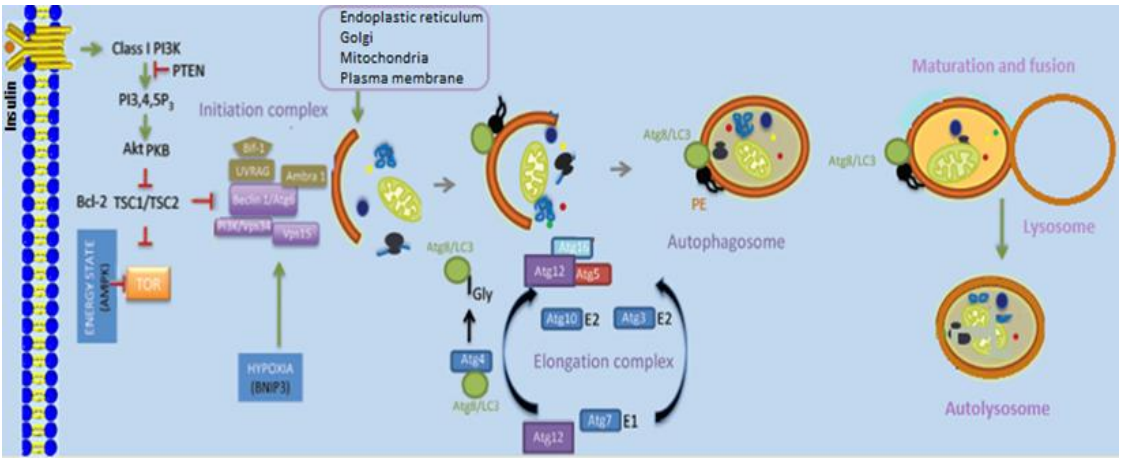

Figure 1.6. Autophagy pathway and machinery. Several regulatory signals activate the recruitment of the autophagy initiation complex. Current evidence suggests that multiple compartments, including the endoplasmic reticulum, Golgi, mitochondria, and the plasma membrane may act as lipid donors to the growing phagophore. Beclin-vps34complex regulates the formation of phagophore. Two ubiquitin-like conjugation systems are involved in the elongation step. The Atg5-Atg12 congugation involves Atg7 (E1-like) and Atg10 (E2-like), while Atg7 and Atg3 act as the E1-like and E2-like, respectively, in LC3-PE conjugation. Once the membrane seals to form the autophagosome, this double membrane vesicle is delivered to lysosomes where, upon membrane fusion, lysosomal hydrolases gain access to cargo. Adapted from Sridhar *et al*., 2012

1.3.2.2. Autophagy and neurodegeneration

Several evidences reveal that one of the areas where autophagy appears to be crucial is to prevent neurodegeneration and therefore, alterations in the different steps of this clearance

process might be intimately linked to many human diseases (Fig. 1.7). The first indication of altered autophagy in different neurodegenerative conditions is the presence of an abnormal number of autophagosomes in the affected neurons (Roizin *et al.*, 1974; Anglade *et al.*, 1997; Nixon *et al.*, 2005). Another proof of the importance of autophagy was demonstrated in studies using knockout mice for Atg5 or Atg7, where it was observed that the impaired autophagy function led to the accumulation of ubiquitin-positive inclusions and to the development of characteristic symptoms of neurodegeneration in these mice (Hara *et al.*, 2006; Komatsu *et al.*, 2006).

Although basal autophagy has an important function in healthy individuals, the requirement for autophagy is even more evident under disease conditions, supporting the idea that autophagy induction might be a suitable therapeutic strategy in a range of adult-onset neurodegenerative diseases.

Intracellular protein aggregates and dysfunctional organelles are common features of neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), and polyglutamine diseases like HD and spinocerebellar ataxias (SCAs). In these disorders, the pathology is associated with the protein propensity to aggregate and accumulate in specific types of neurons, leading to neuronal dysfunction (Rubinsztein, 2006; Imarisio *et al.*, 2008). Therefore, in the presence of toxic proteins, autophagy upregulation has also been shown to be beneficial. In Alzheimer's disease, for example, the amyloid beta (Aβ) peptide and the amyloid precursor protein (APP)-derived fragment (APP-CTF) are cleared upon autophagy induction (Tian *et al.*, 2011). In Parkinson's disease, when a lentivirus expressing beclin-1 was delivered to the brain of a α-synuclein transgenic mouse, the synaptic and dendritic pathology was ameliorated and the accumulation of α-synuclein in the limbic system was reduced (Spencer *et al.*, 2009). The same strategy was performed in MJD models, resulting in the clearance of mutant ataxin-3 and the mitigation of neuropathology (Nascimento-Ferreira *et al.*, 2011). In addition, in HD it was demonstrated that rapamycin attenuates huntingtin accumulation and cell death in cell models of HD (Ravikumar *et al.*, 2004) and later, others studies probed the

therapeutic potential of autophagy induction in various models of HD (Ravikumar *et al.*, 2003; Sarkar *et al.*, 2007).

One important factor in polyglutamine diseases when considering up-regulation of autophagy is the cellular distribution of polyQ inclusions, since in diseases like SCA3/MJD, SC7, SCA17, SBMA, HD, Dentatorubral-Pollidoluysian Atrophy (DRPLA) these aggregates can be found in the nucleus. This is relevant because autophagy is described as a cytoplasmic mechanism, in view of the fact that there are not lysosomes in the cell nucleus (Klionsky and Emr, 2000; Cuervo, 2004; Montie *et al.*, 2009). Autophagy has been shown to eliminate cytoplasmic polyglutamine-containing aggregates more efficiently than nuclear aggregates (Iwata *et al.*, 2005) and this differential efficiency of degradation could explain the greater toxicity associated with intranuclear inclusions in the diseases where both cytoplasmic and nuclear aggregates are present (Yang *et al.*, 2002).

If and how nuclear aggregates are degraded by autophagy is currently debated. Degradation of aggregate precursors may occur at the cytoplasm before their translocation to the nucleus (Rubinsztein, 2006; Li *et al.*, 2008); or there may be shuttling of nuclear aggregates to the cytoplasm, regulated by acetylation and phosphorylation (Jeong *et al.*, 2009; Thompson *et al.*, 2009).

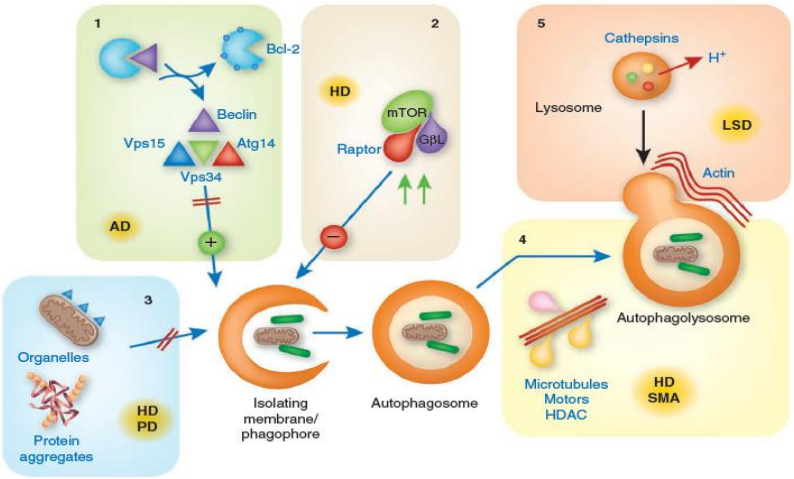

Figure 1.7. Possible steps of autophagy altered in neurodegeneration. (1) reduced autophagy induction; (2) enhanced autophagy repression; (3) altered cargo recognition; (4) inefficient autophagosome/lysosome fusion, and (5) inefficient degradation of the autophagic cargo in lysosomes. Examples of neurodegenerative diseases for which alterations in each autophagic step have been described are shown. Atg, autophagy-related proteins; Vps, vesicular protein secretion protein; GβL, G protein beta protein subunit-like; HDAC, histone deacetylase; AD, Alzheimer's disease; HD, Huntington's disease; PD, Parkinson's disease; LSD, lysosomal storage disorders; SMA, spinal muscular atrophy. (Wong and Cuervo, 2010).

1.3.2.3. Strategies for modulation of autophagy

There are several pathways and small molecules that regulate autophagy, via mTor and mTor-independent mechanisms. The knowledge of these mechanisms allows their modulation in order to inhibit or to activate this clearance pathway.

In the sections below we will focus in strategies to activate autophagy, with special interest on therapeutic approaches for polyglutamine diseases.

Activation of autophagy through mTor-dependent pathways

The mTOr is the most studied pathway regulating mammalian autophagy (Codogno and Meijer, 2005). Many factors such as growth factors, nutrient signals and energy status affect the mTor pathway (Fig.1.8) (Loewith *et al.*, 2002; Sarbassov *et al.*, 2005). The mTor exists in two functional complexes: mTor complex 1 (mTorC1), sensitive to rapamycin, and mTor complex 2 (mTorC2); both containing mTor catalytic subunit and G protein β-subunit-like protein, or mLST8 (GβL) but differing in their other subunits (Sarbassov *et al.*, 2005). The activity of mTorC1 can be inhibited by rapamycin and its analogs, activating autophagy both in vitro and in vivo (Ravikumar *et al.*, 2002; Ravikumar *et al.*, 2004). First isolated from *Streptomyces hygroscopicus* (Montgomery *et al.*, 1998) rapamycin until recently was the only drug known to upregulate autophagy (Guertin and Sabatini, 2009). Later, other inhibitors of mTor have been described. In a study screening a library of 3500 chemicals, perhexiline, niclosamide, amiodarone, and rottlerin were identified as stimulators of autophagy by inhibiting mTorC1 signiling; three of those are drugs already approved for other therapeutic approaches (Balgi *et al.*, 2009). Also, Adenosine triphosphate (ATP)-competitive inhibitors of mTOR such as Torin1 (Thoreen *et al.*, 2009) and PP242 (Feldman *et al.*, 2009) show high inhibitory activity of mTOR.

Nevertheless, as mTor is a central regulator of many cellular processes, the long term treatment of diseases like MJD with mTor inhibitors could lead to several complications, since these molecules are not specific inducers of autophagy.

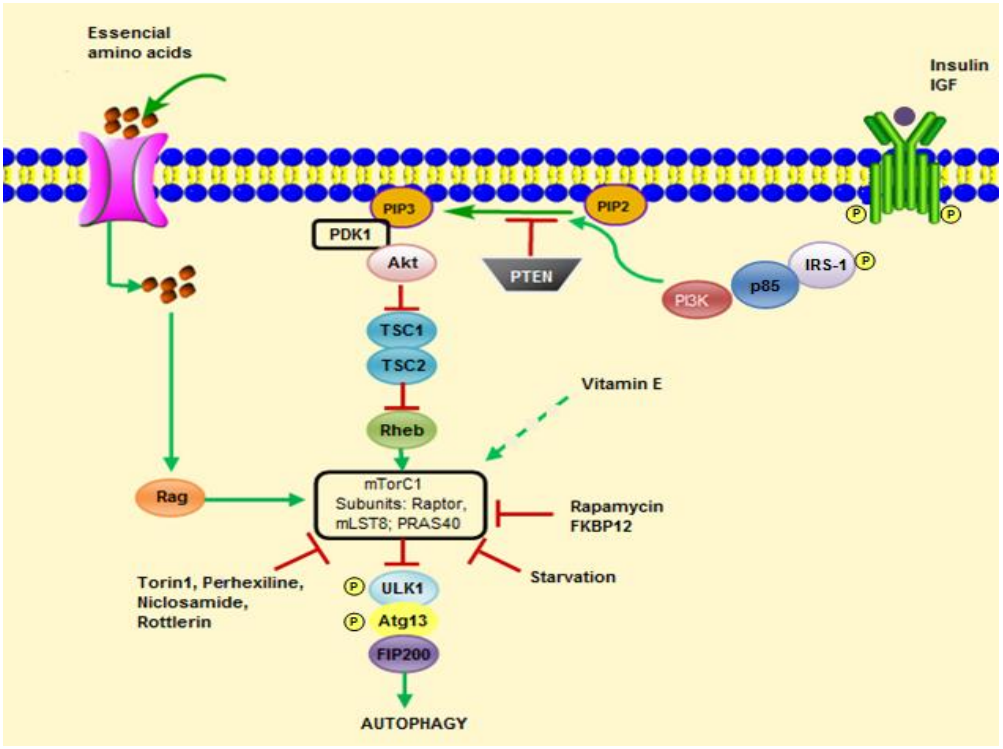

Figure 1.8 mTor-dependent pathways. The PI3K pathway is triggered by the binding of insulin (or growth factors) to insulin receptor (IR), thereby activating PI3K. Activated PI3K converts PIP2 to PIP3, which then recruits phosphoinositide-dependent kinase 1 (PDK1) and Akt to the cell membrane. Akt is then phosphorylated and activated which, in turn, inhibits the tuberous sclerosis complex (TSC)1/2, and activates the smallGTPase Rheb, resulting in mTORC1 activation. The ULK1-Atg13-FIP200 complex acts as an integrator of the autophagy signals downstream of mTORC1. Under starvation conditions or rapamycin treatment, mTOR dissociates from this complex, resulting in dephosphorylation-dependent activation of ULK1 and ULK1-mediated phosphorylations of Atg13, FIP200, and ULK1 itself, which triggers autophagy. Torin1, perhexiline, niclosamide and rottlerin also inhibit mTORC1 activity, either directly or indirectly. The vitamin E antioxidant activates the mTOR pathway and inhibits autophagy. Adapted from Ravikumar *et al*., 2010.

Activation of autophagy through mTor-independent pathways

In order to overcome the limitation of mTor-dependent autophagy inducers, it is important to explore novel targets aiming different autophagy inducing pathways. Accordingly, a study using image-based assays identified eight compounds that induce autophagy and promote long-lived protein degradation in expanded polyglutamine cell model (Zhang *et al.*, 2007). The compounds analysed can be divide in two classes: the class 1 consist of three Food and Drug Administration (FDA)-approved antipsychotic drugs, fluspirilene, trifluoperazine, and pimozide and the class 2 compounds consist of five compounds, including three FDA-approved drugs for cardiovascular indications, niguldipine, nicardipine and amiodarone, that inhibit intracellular Ca^{2+} currents, loperamide, a FDA approved drug for diarrhea, and penitrem A. The phosphorylation status of

mTOR in cells treated with these eight compounds was not altered compared with rapamycin, which permit to conclude that these compounds induce autophagy through mechanisms distinct from that of rapamycin. Furthermore, seven of these molecules present lower cytotoxicity than rapamycin, suggesting that it should be possible to use these drugs in the treatment of human diseases characterized by accumulation of misfolded proteins (Zhang *et al.*, 2007).

Later on, another study screening 253 FDA approved drugs was performed to identify novel autophagy-inducing pathways and to evaluate their effects in the clearance of huntingtin. The drugs analysed exhibit a cyclical mTOR-independent pathway regulating autophagy, where cyclic adenosine monophosphate (cAMP) regulates inositol (IP3) levels, influencing calpain activity, which completes the cycle by cleaving and activating Gsα, which regulates cAMP levels (Fig. 1.9) (Williams *et al.*, 2008).

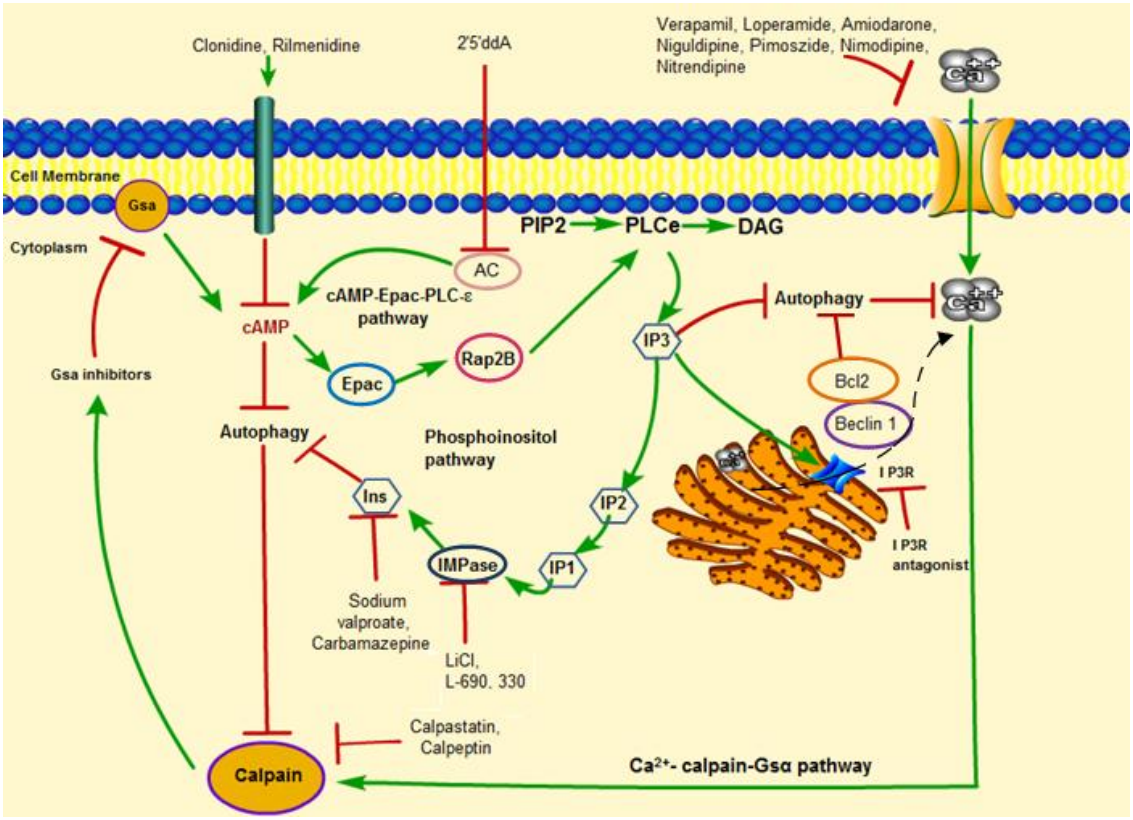

Figure 1.9. mTor-independent pathways. The cyclical mTOR-independent pathway consists of the cAMP-Epac-PLC-e, phosphoinositol and Ca^{2+}-calpain-GSα pathways. Intracellular cAMP levels are increased by adenylyl cyclase (AC), which activates Epac. Epac in turn activates a small G protein Rap2B, which activates phospholipase C (PLC), resulting in the production of IP3 and consequently releasing Ca^{2+} from the endoplasmic reticulum (ER) stores. Intracytosolic Ca^{2+} levels are also increased by Ca^{2+} influx due to L-type Ca^{2+} channel agonist binding. Increase in intracytosolic Ca^{2+} activates the cysteine protease called calpains, which cleave and activate GSα. GSα activation results in an increase in

AC activity elevating cAMP levels, therefore as part of a loop. Activation of this pathway inhibits autophagy. Drugs targets acting at distinct stages within the cAMP-Epac-PLC-e, phosphoinositol, and Ca^{2+}-calpains-Gsα pathways induce autophagy, such as imidazoline-1 receptors (I1R) agonists and adenylyl cyclase inhibitors which decrease intracellular cAMP levels, inositol-lowering agents (lithium, L-690,330, sodium valproate and carbamazepine), L-type Ca^{2+} channel antagonists, calpain inhibitors (calpastatin and calpeptin) , and Gsα inhibitors. Adapted from Ravikumar *et al*., 2010.

Regulating the cAMP-Epac-PLC-e pathway

cAMP negatively regulates autophagy through Epac-PLCe signalling, culminating on the modulation of IP3 levels. It was observed that pharmacological treatment with imidazoline-1 (I1R) receptor agonists, such as clonidine and rilmenidine, or the adenylyl cyclase inhibitor 20,50- dideoxyadenosine, induce autophagy by reducing cAMP levels (Williams et al., 2008). These drugs revealed the ability to induce autophagy in several models of HD, like cell culture models, zebrafish, flies and mouse models (Williams *et al.*, 2008; Rose *et al.*, 2010). Furthermore, these studies indicate the possibility of using cAMP modulators to treat polyglutamine diseases, since some of them are already used in other therapeutic indications.

Regulating the Ca^{2+}-calpain-GSa pathway

In the same study carried out by William and collaborators (2008), it was shown that L-type Ca^{2+} channel antagonists, such as verapamil, loperamide, amiodarone and nitrendipine induce autophagy. These drugs prevent the influx of extracellular Ca^{2+} through the inhibiting of L-type Ca^{2+} channels on the plasma membrane and thereby decreasing intracytosolic Ca^{2+} levels, which result on up-regulated autophagy. Some of these Ca^{2+} channel blockers were also reported in the study of Zhang and collaborators (Zhang *et al.*, 2007). On the other hand, elevated intracytosolic Ca^{2+} levels activate a family of cystein proteases, calpains, leading to inhibition of autophagy. Nevertheless this effect can be reverted with chemical inhibition of calpain by calpastatin and calpeptin; which has also been confirmed in this screen (Williams *et al.*, 2008).

Regulating PhosphoInositol or IP3 pathway

The phosphoinositol pathway was the first alternative mechanism described as increasing autophagy independently of mTor, by reducing intracellular levels of IP3 (Sarkar *et al.*, 2005). This pathway is stimulated by G protein coupled receptor-mediated activation of phospholipase C (PLC), which hydrolyses PIP2 to form IP3 and diacylglycerol (DAG) (Berridge, 1987). IP3 is a second messenger and acts on the IP3 receptor (IP3R), a mostly ER Ca^{2+} release channel that integrates a variety of cell signals (Berridge *et al.*, 2003). 5'-phosphatase and inositol polyphosphate 1-phosphatase (IPPase), and Inositol monophosphatase (IMPase) are required first to form inositol monophosphate (IP1) and second to catalyze the hydrolysis of IP1 into free inositol, essential for the phosphoinositol signaling (Maeda and Eisenberg, 1980; Majerus, 1992).

Mood-stabilizing drugs such as lithium (Li), valproic acid (VA) and Carbamazepine (CBZ) act in this pathway by reducing inositol levels and increasing autophagy (Williams *et al.*, 2002). Therefore, treatment with these drugs has been shown to promote an enhanced clearance of misfolded proteins such as mutant huntigtin in various HD models, and the A53T and in A30P mutants of α-synuclein (Sarkar *et al.*, 2005; Sarkar *et al.*, 2008; Williams *et al.*, 2008). The autophagy-inducing ability of lithium is attributed to its IMPase-inhibitory effect in the phosphoinositol signaling pathway, lowering inositol and IP3 levels. A specific IMPase inhibitor (L-690,330) and mood-stabilizing drugs such as CBZ and valproic acid have similar effects on activation of autophagy and on the clearance of aggregate-prone proteins. Furthermore, consistent with a role of IP3 on autophagy, a study has shown that autophagy can also be enhanced by genetic knockdown of IP3Rs (Criollo *et al.*, 2007).

In this work we are focus in the activation of autophagy through the phosphoinositol pathway, in particular using CBZ as an autophagy-enhancing drug.

Carbamazepine

CBZ belongs to the therapeutic class of antiepileptic, neurotropic and psycotropic agents. Discovered in 1953 by Walter Schindler, CBZ was introduced in the market in 1962 first indicated as anticonvulsant and for treatment of trigeminal neuralgia, and later as antiepileptic.

The mechanism of action of CBZ is still unclear. Nevertheless, it is known that CBZ acts on the stabilization of hiperexcited neuronal membranes and limits repetitive firing of action potentials, by blockage of voltage-dependent neuronal sodium channels. It also affects calcium channels, g -amino butyric acid (GABA) receptors, and adenosine receptors, and increases concentrations of serotonin and other neurotransmitters. While the reduction of glutamate release and the stabilization of neuronal membranes can contribute primarily to the antiepileptic effects, the depressing effect on the "turnover" of dopamine and norepinephrine can be responsible for the anti-manic properties of CBZ (Ambrosio *et al.*, 2002).

CBZ is almost completely absorbed, and the plasma levels of CBZ in the steady state are reached after about 1 to 2 weeks. The half-life of CBZ is approximately 36 hours after a single oral dose, whereas after repeated administration is, on average, only 16-24 hours, depending on the duration of the treatment. This drug is metabolized in the liver, by cytochrome P450 3A4 (Bertilsson, 1978).

In addition to the therapeutic indications currently used in the clinic, recent evidences reveal a role of this drug in activation of autophagy (Hidvegi *et al.*, 2010). As described above, mood stabilizing drugs have shown capacity to up-regulate autophagy by lowering IP3 levels, which might be a viable alternative to mTor inhibitors (as rapamycin) in the treatment of diseases characterized by accumulation of aggregate-prone proteins. Thus, besides the evidences of lithium potential in the clearance of huntingtin (Sarkar *et al.*, 2005; Williams *et al.*, 2008), CBZ appears as an autophagy enhancing drug, promoting degradation of Mutant α1-antitrypsin Z and reducing hepatic Fibrosis (Hidvegi *et al.*, 2010). Based on these findings this drug might be promising also in neurodegenerative disorders such as Machado –Joseph disease.

OBJECTIVES

The general objective of this work was to study the carbamazepine (CBZ) effect in Machado Joseph disease, concerning the described ability to enhance autophagy and promote clearance of the aggregate prone proteins.

The specific objectives were as follows:

- To investigate the activation of autophagy mediated by CBZ in a cellular model.
- To evaluate the best conditions of CBZ treatment in neuroblastoma cells, regarding the removal of all forms of mutant ataxin-3.
- To analyze the effects of CBZ treatment at neuropathological level in a lentiviral-based mouse model.
- To investigate the effect of a short-term CBZ treatment in a transgenic mouse model, regarding autophagy activation and clearance of mutant ataxin-3.
- To evaluate motor coordination and activity profile by motor behavior tests upon moderate/long-term administration of CBZ in a transgenic mouse model.

CHAPTER 2–MATERIAL AND METHODS

2.1. Lentiviral vectors

The complementary DNAs encoding for rat light chain 3 (LC3) (Kabeya *et al.*, 2000) fused to the enhanced green fluorescent protein (EGFP) were inserted downstream from the mouse phosphoglycerate kinase 1 promoter in a self-inactivating lentiviral transfer vector (de Almeida *et al.*, 2001). Viral vectors encoding a) enhanced green fluorescent protein-light chain 3 (EGFP-LC3) and b) human full length mutant ataxin-3 with 72 glutamines (Alves *et al.*, 2008), were produced in human embryonic kidney 293 T cells using a four-plasmid system as previously described (de Almeida *et al.*, 2002). The lentiviral particles were produced and resuspended in phosphate-buffered saline with 0.5% bovine serum albumin (PBS-BSA) and samples were matched for particle concentration by measuring human immunodeficiency virus 1 (HIV-1) p24 antigen content (RETROtek, Gentaur, France). Viral stocks were stored at -80°C until use.

2.2. In vivo experiments

The animals were housed in a temperature-controlled room and maintained on a 12 h light/dark cycle. Food and water were available *ad libitum*. The experiments were carried out in accordance with the European Community Council directive (86/609/EEC) for the care and use of laboratory animals.

2.2.1. Lentiviral MJD mouse model

Experiments with the lentiviral MJD model involved injection of lentiviral vectors encoding the full-length mutant ataxin-3 with 72 glutamine repeats in the right hemisphere of the brain, in the striatum. Three weeks after treatment with CBZ or equivalent volume of dimethyl sulfoxide (DMSO), the animals were killed by sodium pentobarbital overdose, transcardially perfused with 0.1M PBS and a 4% paraformaldehyde fixative solution (PFA 4%, Fluka, Sigma, St. Louis, USA) followed by brain removal. The tissue was cryoprotected in 25% sucrose- 0.1M PBS solution for 48h , dry ice- frozen and cut at a cryostat-microtome (Leica CM3050S, Leica Microsystems Nussloch Gmbh, Nussloch, Germany) in 20µm coronal sections. Slices of each animal were collected and stored in 48-well trays (Corning Inc., NY, USA), free-floating in

0.1M PBS supplemented with 0.12 µmol/L sodium azide. The sections were stored at 4°C until immunohistochemical processing.

2.2.1.1. Stereotaxic Surgery

Mice with 3 weeks of age were anesthetized by intraperitoneal injection of a mixture of ketamine (100 mg/kg) with xylazine (10 mg/kg). Particle content of lentiviral vectors was matched to 200 000 ng of p24/ml (concentrated viral stocks were thawed on ice and resuspended by vortexing). The animals received a single injection of 2µl of lentiviral vectors at a rate of 0.25 µl/min by means of an automatic injector (Stoelting Co., Wood Dale, IL, USA), at the following coordinates: +0.6 mm rostral to lambda, -1.8 mm midline, and -3.3 mm ventral to the skull surface, with the mouth bar set at -3.3. After the injection, the syringe needle was left in place for an additional 5 min to minimize backflow.

2.2.1.2. Carbamazepine treatment

CBZ was administered at 200 mg^{-1} kg^{-1} day^{-1} for 3 weeks by *gavage* and the equivalent volume of DMSO was given to the control group.

2.2.2. Transgenic mouse model

The Machado Joseph's disease transgenic mouse model expressing the N-terminal-truncated ataxin-3 with 69 glutamine repeats and an N-terminal hemaglutinin (HA) epitope driven by Purkinje-cell-specific L7 promoter (Tg-MUT) has been previously described (Torashima *et al.*, 2008; Oue *et al.*, 2009). A colony of these transgenic mice was established in the Center for Neurosciences and Cell Biology of the University of Coimbra and the line was maintained by backcrossing heterozygous transgenic males with C57BL/6 females. Genotyping was performed by PCR.

Transgenic mice with 17 weeks old, were killed by sodium pentobarbital overdose, transcardially perfused with 0.1M PBS and a 4% paraformaldehyde fixate solution (PFA 4%,

Fluka, Sigma, St. Louis, USA) followed by brain removal. The brain was cryoprotected in 25% sucrose- 0.1M PBS solution for 48h, and stored at -80ºC.

2.2.2.1. Carbamazepine treatment

In one experiment CBZ (SIGMA) was administered at 250 mg^{-1} kg^{-1} day^{-1} for one week by gavage, in a group of animals. The equivalent volume of DMSO (SIGMA) was administered to control group, also by gavage. In another experiment, the same dose of CBZ was administered for 12 weeks orally in water solution, after solubilization in DMSO. Every two weeks the animals were subjected to a behavioural assessment. The equivalent volume of DMSO was given to control groups. Animals at 3 weeks of age were used in both experiments.

2.2.2.2. Behaviour tests

Rotarod. Rotarod tests were carried out to measure the motor coordinative abilities and balance of mice. Mice were tested using fix and accelerating rotarod, respectively (Letica Scientific Instruments, Panlab, Barcelona, Spain) starting at 4 rpm and accelerating to 40 rpm during a period of 300 sec (5 min). The latency to fall off the rotating rod was recorded in four trials per time-point, with 15 min rest between trials. The tests were repeated every two weeks. Statistical analysis of obtained data was performed calculating the mean values of three trials for each group (the trial with the most distant value from average was eliminated from the analysis). To evaluate statistical significance two-way ANOVA tests were performed in GraphPad software (La Jolla, USA). Data are represented as mean ± SEM.

Open field analysis. For the assessment of mice explorative behavior, and locomotor horizontal activity, open field tests were performed. Mice were placed in a 50x50 cm arena with 50 cm high walls and their movement activity was recorded for 40 min using Acti-Track System (Panlab, Barcelona, Spain). The collected data was analyzed for the first 10 min in the arena, and for the last 30 minutes. Mean values for each measure was calculated. The statistical

analysis was performed by Two-way ANOVA, using the GraphPad software (La Jolla, USA). Data are represented as mean±SEM.

2.3. Cell cultures

2.3.1. Neuroblastoma cell culture

Mouse neuroblastoma cell line (Neuro-2a cells) obtained from the ATCC cell biology bank (CCL-131) were incubated in DMEM medium (SIGMA) supplemented with 10% FBS, 100U/ml penicillin and 100µg/ml streptomycin (Gibco, Paisley, Scotland, UK) (complete medium) at 37°C in 5% CO_2/air atmosphere.

2.3.1.1. N2A infection

Cells were plated in 12 well plates; 2 x 10^5 cells per well in 1000µl of medium and 24h later were simultaneously infected with lentiviral vectors expressing EGFP-LC3 and Atx3 MUT, or with only Atx3 MUT, at the ratio of 10 ng of p24 antigen/10^5 cells. For the infection, we replaced the medium with 400µl of new medium and 1.6µl of hexadimethrin bromid (Aldrich) (8µg/ml) and added the corresponding lentivirus. The plates were then incubated at 37°C in 5% CO2/air atmosphere. After 6h of infection, we added 600µl of new fresh medium to the wells. At two weeks post-infection cells were treated with CBZ and lysed for western bloting analysis or fixed for immunocitochemistry analysis.

2.4. mRNA expression analysis

2.4.1. RNA extraction, Quality and Concentration analysis

RNA of cerebellum lysates of transgenic mice and cell lysates were isolated using RNeasy Mini Kit, Qiagen and resuspended in 30µl of Rnase-free water. RNA concentration and purity was determined using the NanoDrop 2 000 spectrophotometer (Thermo). The RNA samples were stored at -80ºC until further use.

2.4.2. Reverse Transcription

For complementary DNA (cDNA) synthesis 500 ng of isolated RNA was used mixed with 4 µL of 5x iScript Select Reaction Mix, 1 µL of Reverse Transcriptase, 2 µL random primer and Nuclease-free water for a total volume of 20 µL per experimental condition (iScript™ Select cDNA Synthesis Kit, Bio-Rad). The reaction was performed at 25 ºC for 5 min for primer annealing to the template, followed by 30 min at 42 ºC for cDNA synthesis. The reverse transcriptase was then denatured and inactivated for 5 min at 85 ºC. The sample was cooled to 4 ºC, before storage at -20 ºC until further use.

2.4.3. Quantitive Real-Time PCR

PCR was carried out in a 15 µl reaction volume containing 1,5 µl of diluted cDNA, 7,5 µl of SSoAdvanced SYBR Green Supermix (BIO-RAD) and 1,5 µl of 10x Map1lc3b QuantiTect Primer Assay (Qiagen). Quantitative real-time PCR was performed in a iQ5 Optical System Software, which was initiated with enzyme activation by heating at 95 ºC during 30 sec, followed by 45 cycles of two steps, first step of 7 sec at 95ºC, second step of 15 sec at 55ºC.

The melting step was performed with slow heating, starting at 65 ºC and with a rate of 0.5 ºC per 10sec, up to 95 ºC. A melting curve was always built to demonstrate a single amplification product reaction per well.

The assay included a non-template control and a standard curve of cDNA. The amplification rate for each target was evaluated from the cycle threshold (Ct) numbers obtained with cDNA dilutions.

2.4.4. Data Analysis

Data analysis was performed by iQ5 Optical System Software for real-time PCR expression profile. To each gene, and each experiment, a standard curve was performed for assessing the efficiency of each set of primers. The reaction efficiencies were between 90% and 110%, and $r^2 > 0.99$, thus allowing the use of the comparative method for messenger RNA (mRNA) level

quantification, according to the following formula: $2^{-\Delta\Delta Ct}$x100 (Livak and Schmittgen, 2001). As reference gene were used hprt gene (Hprt QuantiTect Primer Assay, Qiagen). Statistical analysis was performed by the one-way ANOVA and student t-test. Data are represented as mean ± SEM.

2.5. Imunochemical procedures

2.5.1. Free-floating Immunohistochemistry

Free-floating immunochemistry was initiated incubating brain sections on phenylhidrazine diluted in Phosphate buffered saline (PBS) (1:1000; 30min, 37°C) for peroxidase inhibition, followed by three washes with PBS (5'; 5'; 10'). The sections were blocked 1h at room temperature in PBS 0.1% Triton solution with 10% NGS and incubated with the primary antibodies diluted in blocking solution: mouse monoclonal anti-ataxin-3 (1H9, Chemicon, Temecula, CA, USA; 1:5000, overnight 4°C) and rabbit polyclonal anti-DARPP-32 (Chemicon,Temecula, CA, USA; 1:2000, overnight 4°C). After the overnight incubation, the sections were washed three times with PBS. For light imaging, the secondary antibody used was biotinylated and followed a reaction with the vectastain elite avidin-biotin-peroxidase kit and by 3,3'-diaminobenzidine substrate (both from Vector Laboratories, Burlingame, CA).

2.5.2. Immunohistochemistry quantitative analysis

The quantification of ataxin-3 positive inclusions was performed by scanning 8–10 coronal sections spread over the anterior-posterior extent of the striatum (inter-section distance: 200 μm), using a 20× objective on Axioscope 2 Plus upright microscope (Zeiss, Jena, Germany). For each animal, the absolute number of inclusions in the striatum was calculated as described elsewhere (Alves et al., 2008a), using an image analysis software (ImageJ, National Institute of Health, USA). For the quantification of dopamine and cyclic AMP-regulated phosphoprotein, relative molecular mass 32,000 (DARPP-32) depletion volume, scanning of 8–10 coronal sections spread over the anterior-posterior extent of the striatum (inter-section distance: 200 μm), using a 20× objective on Axioscope 2 Plus upright microscope (Zeiss, Jena, Germany).

and the same image analysis software (ImageJ, National Institute of Health, USA). The volume was estimated as described elsewhere (Alves et al., 2008a) and data were expressed as the evaluated DARPP-32 depleted volume (mm^3).

2.5.3. Immunocitochemistry of N2a

Cells expressing EGFP-LC3 and mutant ataxin-3 were washed with PBS. The sections were counterstained with DAPI, washed three times and mounted with Fluorsave (Calbiochem, Germany). Light images were acquired with confocal microscope (LSM 510 Meta/Zeiss).

2.6. Protein extraction and Western blot

For the protein extraction protocol, brain tissue and cells were lysed in RIPA buffer solution (50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing proteases inhibitors (Roche diagnostics GmbH, Mannheim, Germany) followed by a 4s ultra-sound chase (1pulse/s). Pprotein concentration was determined using the Bradford protein assay (BioRad, Munich, Germany). Depending on the analysis, twenty (Neuro-2a), thirty (striatal primary cells) and sixty micrograms of protein extract were resolved in SDS-polyacrylamide gels (4% stacking, 12% running; 20-80%) with exception of striatal primary cells analysis that were resolved in different SDS-polyacrylamide gels (4% stacking, 6% running; 50-50%). The proteins were transferred onto PVDF membranes (GE Healthcare, Amersham, UK) according to standards protocols. The membranes were blocked by incubation in 5% nonfat milk powder in 0.1% Tween 20 in Trisbuffered saline (TBS-T) for 1 h at room temperature, and then incubated overnight with the following primary antibodies diluted in blocking buffer: anti-ataxin 3 (1H9; 1:5000; Chemicon, Temecula, CA, USA); LC3b (1:1000; Cell signaling, MA, USA); β-actin (1:10000; SIGMA); β-tubulin (1:10000; SIGMA). Blots were washed three times in TBS-T, for 15min each, and incubated with the secondary antibody goat anti-mouse (1:10 000; Vector Laboratories, Burlingame, CA) for 2h at room temperature. After washing, bands were visualized with Enhanced Chemifluorescent substract (ECF) (GE

Healthcare, Amersham, UK) and chemifluorescence imaging (Versadoc Imaging System Model 3000, Bio-Rad, Munich, Germany. Semi quantitative analysis was carried out based on the optical density (OD) of scanned films (Quantity One 1-D image analysis software version 4.4; Biorad, Hercules, CA, USA). Specific ODs were normalized with respect to those for actin or tubulin for experiments on total homogenates. The specific OD was then normalized with respect to the amount of actin or tubulin loaded in the corresponding lane of the same gel. A partition ratio was calculated and expressed as a percentage. The western blotting analyses were performed in triplicate for each experimental set.

CHAPTER 3 - RESULTS

3.1. Carbamazepine increases autophagosome formation in N2a cells

Mood stabilizing drugs, such as CBZ, have been associated to an increase of autophagy by an mTor independent pathway (Sarkar *et al.*, 2007) Therefore, these drugs could play an important role in the clearance of misfolded proteins, which are at the origin of several neurodegenerative diseases.

We first investigated whether CBZ would increase the autophagic flux in cellular models of MJD. Mouse neuroblastoma N2a cell lines stably expressing ataxin-3 MUT and EGFP-LC3 were generated by infection with lentiviral vectors. These lines allow the observation of autophagosome formation after treatment with CBZ as compared with untreated cells (Fig.3.1A). Increased EGFP-LC3 puncta formation was observed in cells treated with CBZ, a profile that was similar to the one observed in cells treated with rapamycin, used as positive control. Using of a lysosomotropic reagent, chloroquine, which blocks degradation of LC3-II and allows enhanced detection of the amount of LC3 that is delivered to lysosomes for degradation (Mizushima and Yoshimori, 2007; Klionsky *et al.*, 2008; Rubinsztein *et al.*, 2009), leads to the clear detection of autophagosomes.

To investigate whether the autophagosome formation observed by immunocytochemistry would correspond to autophagy activation¸ we performed western blot experiments to evaluate LC3 protein levels (Fig.3.1B-C) and mRNA expression (Fig.3.1D). Significant increase both in LC3II/LC3I ratio and in LC3 mRNA expression were observed in cells treated with CBZ when compared with controls ($P<0.05$). We also found high levels of LC3-II even under normal conditions (Fig.3.1B-C). Due to the highly sensitive nature of LC3 conversion and turnover assay, a high flux can be detected even during basal conditions, which makes it difficult to detect additional changes in LC3 turnover upon autophagy upregulation and for that to obtain meaningful results. Probably this explains the non-significant results of LC3-II/LC3I protein levels in rapamycin conditions (Fig.3.1C). Therefore, other assays should be performed in order to measure the autophagic flux.

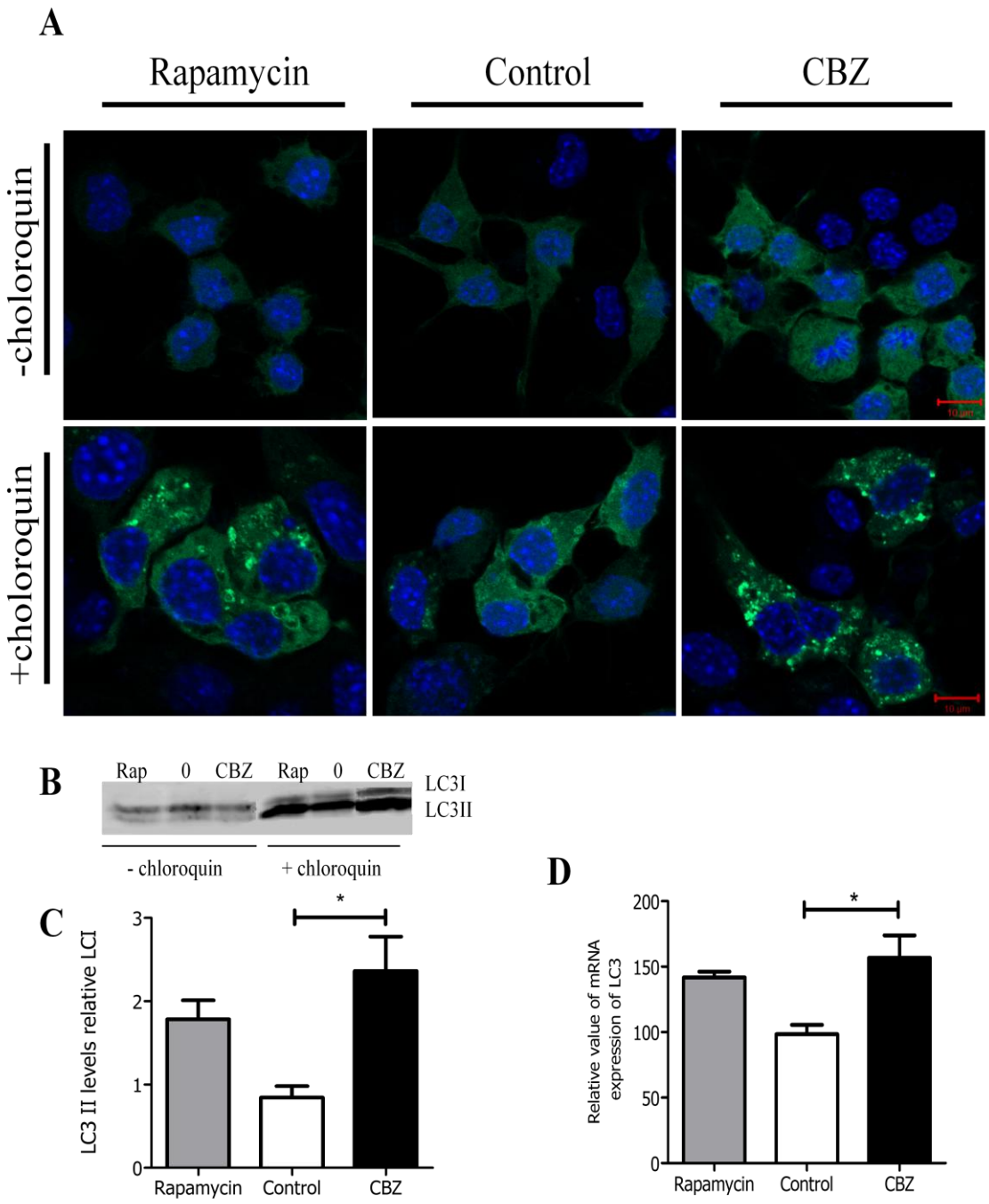

Figure 3.1. Carbamazepine increases autophagosome formation in N2a cells. (A) Confocal microscopy analysis of cells expressing EGFP-LC3 and mutant ataxin-3. Carbamazepine increases the number of EGFP-LC3 puncta formation an effect that is apparent under chloroquine effect. Scale bar: 10μm (B-C) Western blotting analysis of Atx3MUT cell lysates. (B) Representative western blot probed for LC3. Note the presence of LC3II (MW: 14KDa) and LC3I (MW: 17KDa) (C) Optical densitometry analysis. The conversion of LC3I to LC3II is significantly higher in cells treated with 50μm of CBZ, as compared with the control. (D) Quantitative real-time PCR analysis of N2a cells expressing mutant ataxin-3. Relative mRNA expression of LC3 is significantly higher in cells treated with CBZ, comparative with the control. Values are expressed as mean ± SEM of 3 independent experiments. *P<0.05 (One-Way Anova test, Bonferroni post-test).

3.2. Carbamazepine promotes clearance of aggregated and soluble mutant Atx3 in N2a cells

Having found evidences of CBZ-mediated activation of autophagy, we then evaluated whether this effect would reduce the levels of mutant ataxin-3 in this cellular model of disease. Therefore, we performed a dose-response curve with different concentrations of CBZ in order to identify the optimal range of concentrations that would promote decrease of the misfolded protein. Again, rapamycin was used as positive control (Fig.3.2). A robust around 50% decrease in the levels of aggregates for the tested concentrations of CBZ, suggesting that CBZ mediated an important effect in the clearance of mutant ataxin-3, being 50µM of CBZ the concentration that led to the most significant decrease of aggregate and soluble mutant ataxin-3 ($P<0.01$; Fig.3.2 B-C).

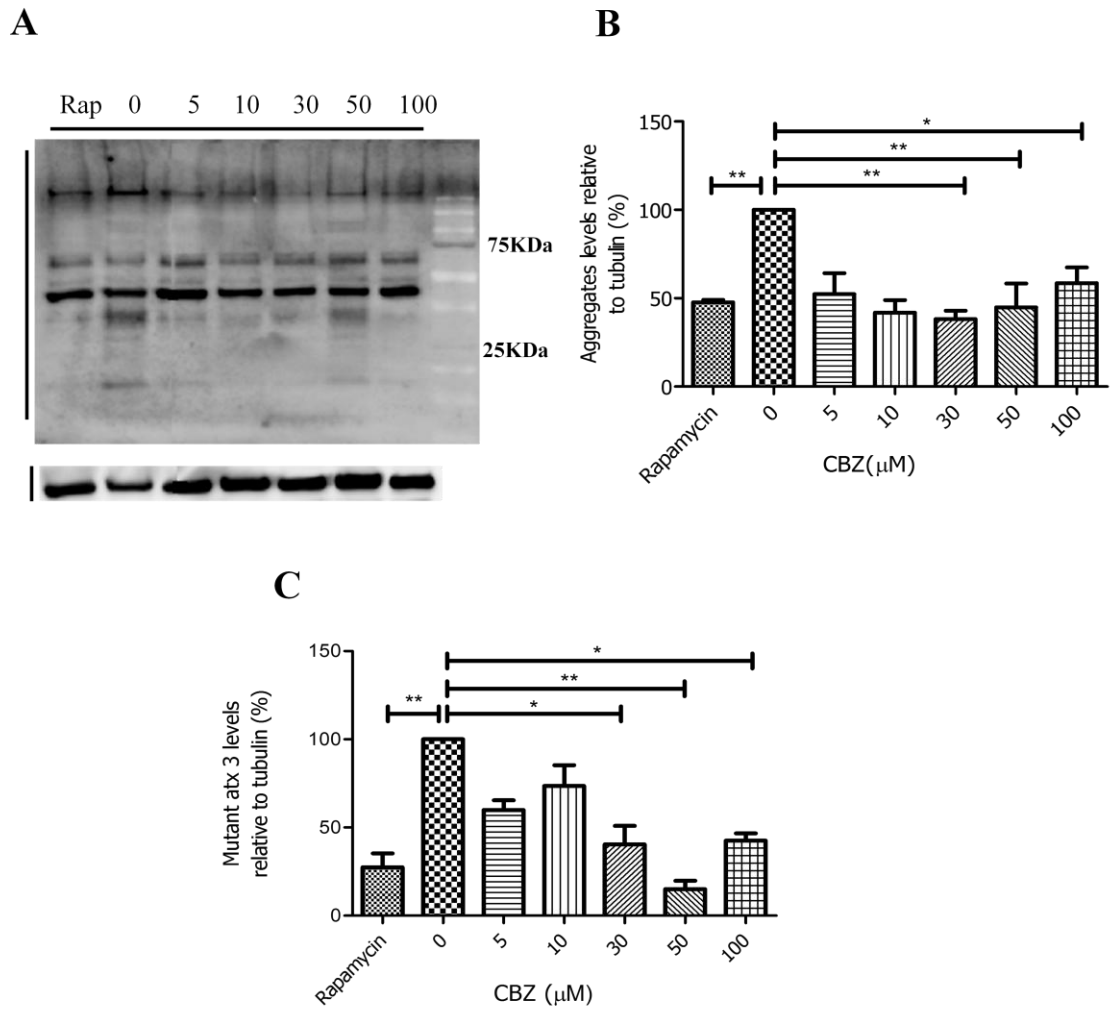

Figure 3.2 Carbamazepine promotes clearance of aggregated and soluble mutant Atx3 in N2a cells. (A-B) Western blotting analysis of Atx3MUT cell lysates. (A) Representative Western Blot probed to ataxin-3 and tubulin. Note the presence of endogenous Atx3 (MW: 42kDa); Atx3 MUT (MW: 67kDa); tubulin (MW: 55kDa) and aggregates (MW: 250kDa). (B) Optical densitometry analysis for ataxin-3 aggregates. (C) Optical densitometry analysis for Atx3 MUT. Significant decrease of atx3MUT and aggregates was detected in cells treated with CBZ especially with for the 30-50µM concentration of CBZ. Each Atx3 lane was normalized according to the tubulin loading control band. Results were expressed as ratio Ataxin-3/Tubulin. Values are expressed as mean ± SEM of 3-6 independent experiments. *P< 0.05; **P<0.01 (One-Way Anova test).

In the same experiment, it was evaluated if the tested concentrations of CBZ could affect the cell viability. Using the resazurin assay, it was verified that the tested concentrations of CBZ were not toxic to cells, suggesting that the observed effects were not due to modification of cell viability (Fig.3.3)

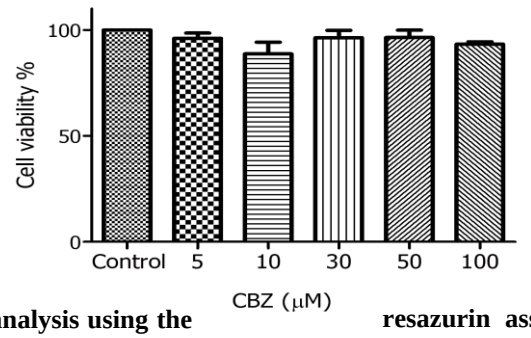

Figure 3.3. Cell viability analysis using the resazurin assay. CBZ treatment within the tested concentrations had no effect in cell viability.

3.3. CBZ treatment alleviates neuropathology in a lentiviral-based mouse model of MJD

To investigate the effect of CBZ *in vivo* we generated a lentiviral MJD model (Alves *et al.*, 2008), where mice expressing At3MUT (72CAG) were treated during 3 weeks with 200mg/kg/day of CBZ by *gavage* and after this time sacrificed and the brains processed for imunohistochemical analysis.

Since Machado-Joseph disease is characterized by the presence of mutant ataxin-3 nuclear inclusions (Paulson *et al.*, 1997), we investigated whether CBZ would clear mutant ataxin-3 aggregates formed in this model. Staining with the 1H9 antibody revealed a significant decrease in the relative number of atx3MUT inclusions in CBZ treated animals, as compared to control mice (n=5, $P<0.001$; Fig.3.4 B-C).

We further investigated whether neuroprotective effects accompanied the clearance of ataxin-3 inclusions. Given that expression of mutant ataxin-3 produces a depletion of neuronal DARPP-32 marker (Alves *et al.*, 2008), we performed immunohistochemical staining and quantitative analysis of the tissue volume depleted of DARPP-32 immunoreactivity (Fig.3.4 D). A decrease of the DARPP-32 depleted volume in CBZ-treated mice compared to the control group was observed (n=5; Fig.3.4 E), that despite not reaching significance ($p=0.0738$), was suggestive that CBZ might reduce neuronal dysfunction mediated by mutant ataxin-3.

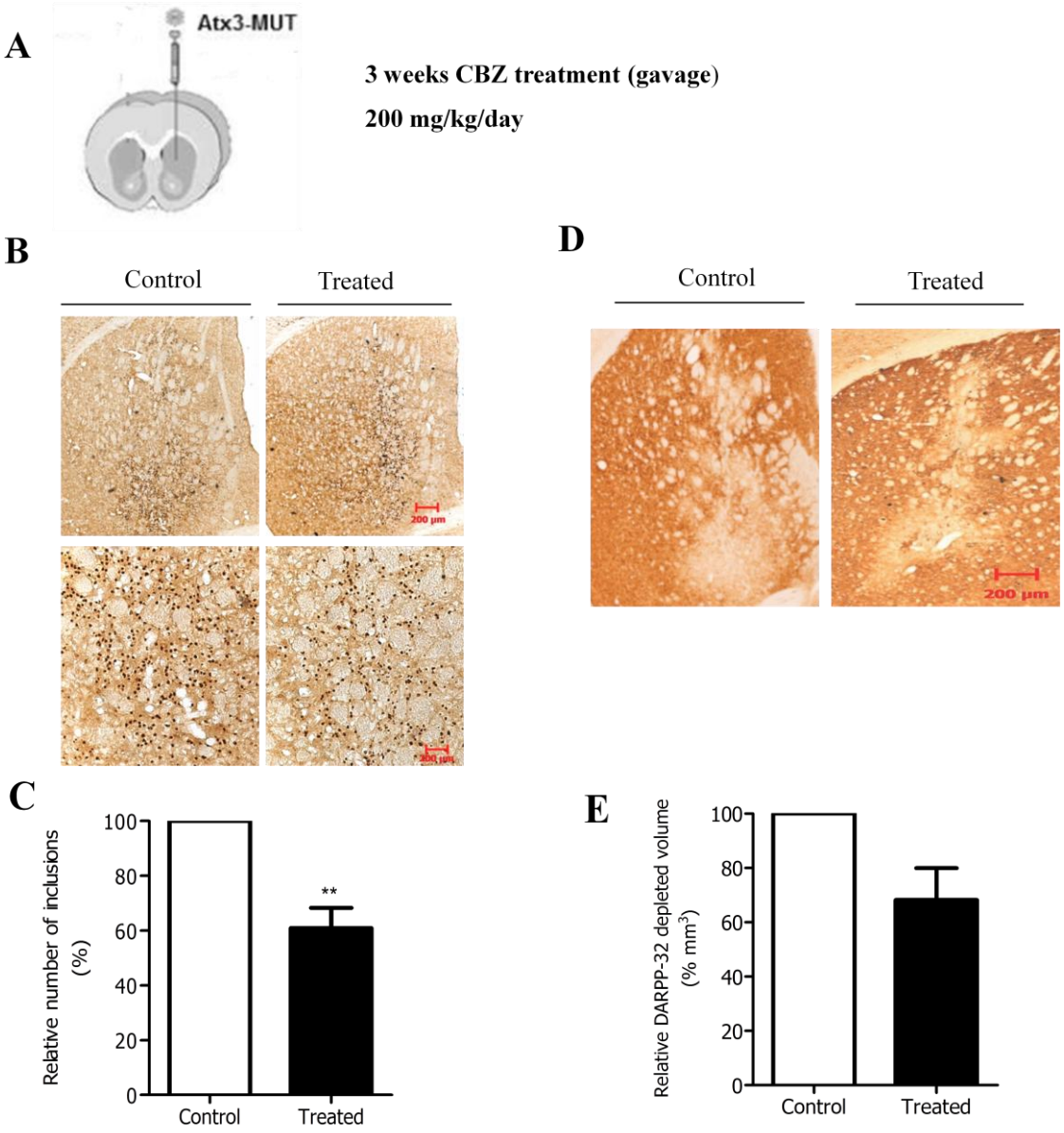

Figure 3.4. Lentiviral-based MJD mice model treated with CBZ presents an improvement of neuropathology. (A-E) Schematic representation and analysis of lentiviral-based MJD mice model treated during 3 weeks with 200 mg/Kg/day of CBZ. (A) Schematic representation of stereotaxic injection of lentiviral vectors in mice striata. Lentiviral vectors encoding human mutant ataxin-3 with 72 glutamines (Atx3 MUT) were injected in the striata of 3 weeks old C57/Bl6 mice. (B-E) Immunohistochemical analysis of mice striatal sections. (B) Immunohistochemical staining for ataxin-3-positive inclusions. (C) Quantification of relative number of mutant ataxin-3-positive inclusions. Significant decrease of relative number of atx3 inclusions were detected in treated mice comparative with control group. (D) Immunohistochemical staining for the neuronal marker, dopamine- and cyclic AMP-regulated phosphoprotein of 32 kDa (DARPP-32). (E) Quantification of relative DARPP-32 depleted volume (%mm^3). A decrease of relative DARPP-32 depleted volume (%mm3) was detected in treated mice as compared to control group. Values are expressed as mean ± SEM. **P<0.01 (paired Student's t-test) of n=5 for each experimental set. Scale bar: 200µm.

3.4. Carbamazepine promotes clearance of aggregated and soluble mutant Atx3 in transgenic mice, by enhancing autophagy

To further investigate the protective effects of CBZ at neuropathological and motor behaviour levels, at an advanced stage of disease, we then used a transgenic MJD mouse model, which expresses a truncated fragment of mutant ataxin-3 transcript with 69 glutamines (Torashima *et al.*, 2008). First we performed a treatment of one week of a daily dose of 250 mg/kg/day by *gavage* for biochemical analysis of brain homogenates. The lysates of the cerebellum were analyzed by western blot for the HA tag present in the transgenic mutant ataxin-3 (Fig.3.5 A). Robust and significant decreases in both aggregated protein (n=4, P<0.01; Fig.3.5 B) and soluble protein levels were observed (n=4, P<0.05; Fig.3.5 C) after one week of treatment when compared with the control group.

In order to evaluate carbamazepine-mediated activation of autophagy we then analyzed the mRNA expression of LC3 by quantitive-real time PCR. A significant increase of mRNA expression of this autophagy protein marker was observed in treated mice as compared to the control group (n=6, P<0.001; Fig.3.5 D).

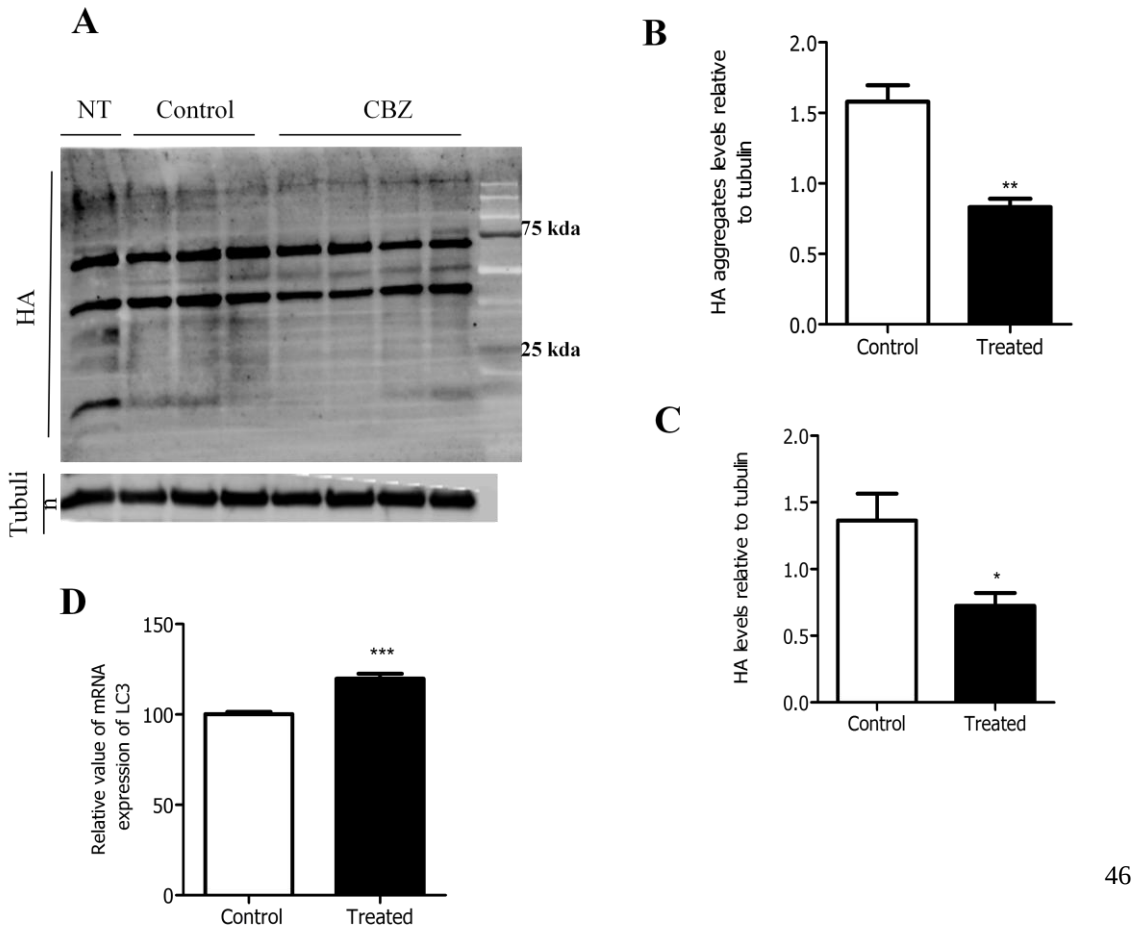

Figure 3.5. Carbamazepine promotes clearance of aggregated and soluble mutant Atx3 in transgenic mice, by enhancing autophagy (A-C) Cerebellum lysates western blotting of transgenic mice treated during one week with 250 mg/kg/day of CBZ. (A) Representative Western Blot probed to HA tag of transgenic mutant ataxin-3 and tubulin. Note the presence of truncated ataxin-3 detected with the HA antibody (MW: 40kDa); aggregates (MW: <250kDa) and tubulin (MW: 55kDa). (B) Optical densitometry analysis for aggregate levels. (C) Optical densitometry analysis for HA levels. Significant decrease of HA and aggregates levels was detected in transgenic mice treated comparative to control group. Each Atx3 lane was normalized according to the tubulin band. Tubulin was used as a loading control. Results were expressed as ratio HA/ Tubulin (n=4 for each experimental set). Values are expressed as mean ± SEM. *P< 0.05; **P<0.01 (Student's t-test). (D) Quantitive real-time analysis of cerebellum lysates. Relative of mRNA expression of LC3 is significantly higher in transgenic mice treated with CBZ, comparative with the control group. Values are expressed as mean ± SEM of n=6 for each experimental set. ***P<0.001 (Student's t-test).

3.5. Motor behaviour of transgenic mice treated with CBZ

We next evaluated the long-term effect of CBZ in behavioral performance of transgenic mice. While in the lentiviral model we evaluated the neuroprotective role of CBZ, since CBZ treatment was initiated one day after the injection of lentivirus, the study in this transgenic model allowed an evaluation of the disease rescue because the CBZ treatment was initiated when mice already presented a profound cerebellar atrophy and severe motor deficits.

CBZ administration was initiated at 3 weeks of age with a daily dose of 250 mg/kg in the drinking water, during 12 weeks. Every two weeks behavioural assessment was performed. In the rotarod test, mice were forced to walk on a rotarod apparatus accelerating from 4 to 40 rpm in 5 minutes and on a constant speed rotarod. In both tests a tendency for improvement of performance of treated mice was observed over time (Fig.3.6 A-B).

In the activity box, despite not reaching significance, robust differences were observed at the 10 and 12 weeks time-points between the treated and control groups (Fig.3.6 C). Transgenic mice treated with CBZ travelled higher distances (Fig. 3.6 D), spending less time resting (Fig.3.6 E) and more time moving fast (Fig.3.6 F).

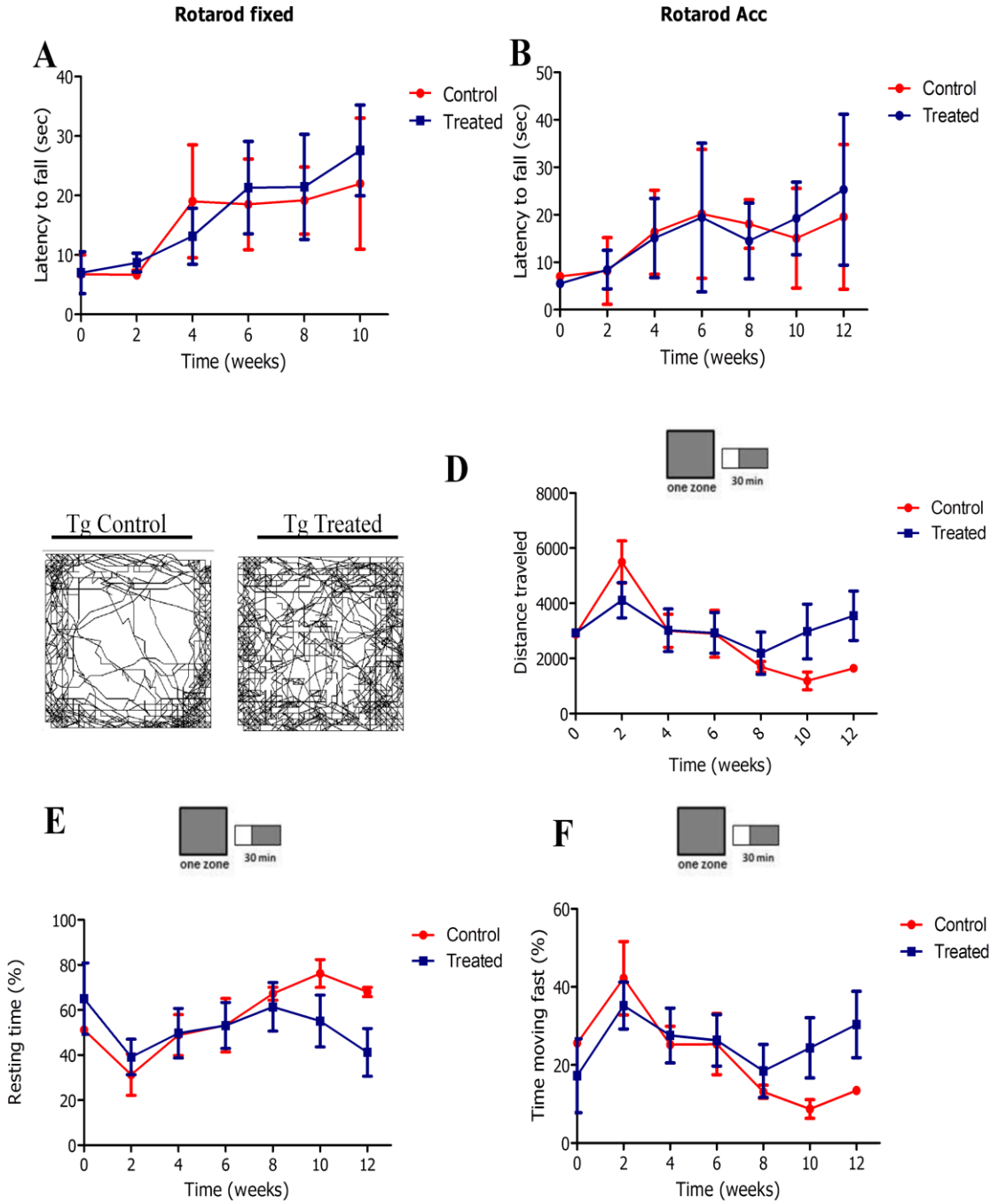

Figure 3.6. Transgenic mice treated with CBZ present improved motor performance. (A-F) Behavioral assessment of transgenic mice treated during 12 weeks with 250mg/Kg/day of CBZ (A-B) Rotarod test. (A) Mice were forced to walk on constant speed rotarod at 5rpm. (B) Mice were forced to walk on a rotarod apparatus accelerating from 4 to 40 rpm in 5 minutes. Latency to fall was measured for each time point. (C-F) Activity box analysis of Tg model treated with CBZ. (C) Activity profile of the distance travelled by mice during the 40 minutes of analysis. (D-F) Analysis of the last 30 minutes, which assesses the mice general activity. (D) Distance travelled by mice. Tg mice treated with CBZ travel longer distance compared with the control group. (E) Time spent resting (no movement). CBZ mediated a decrease in the time spent resting of Tg mice relative to the control group. (F) Time spending moving fast. CBZ promoted and increase in the time moving fast relative to control group. n=4-5 for each experimental set.

CHAPTER 4 - DISCUSSION

In this work we explored a novel therapeutic approach to treat Machado Joseph disease by using a potential autophagy enhancer, CBZ. In accordance with previous reports that mood stabilizing drugs act in a m-Tor independent way to activate autophagy (Sarkar and Rubinsztein, 2006), we provide the first *in vivo* evidence that CBZ increases autophagy and thereby promotes the clearance of soluble and aggregated mutant ataxin-3 in three models of MJD. Thus, these results should be further explored as a possible therapeutic approach to MJD.

Increasing evidence reported in the last years, underline the importance of autophagy in the degradation of misfolded proteins causative of several human diseases (Ravikumar *et al.*, 2002; Menzies *et al.*, 2006; Pickford *et al.*, 2008), Furthermore, the impairment of this mechanism is associated development of a neurologicaly-impaired phenotype or with the aggravation of pathology (Hara *et al.*, 2006; Khan *et al.*, 2008), which makes of autophagy a potential therapeutic target to attenuate neurodegenerative diseases such as AD, PD, ALS, MJD and HD (Webb *et al.*, 2003; Ravikumar *et al.*, 2004; Berger *et al.*, 2006; Nascimento-Ferreira *et al.*, 2011; Zhang *et al.*, 2011; Schaeffer *et al.*, 2012). Many studies have been developed to identify molecules with the ability to activate autophagy through different pathways, being rapamycin the first effective treatment in activating autophagy by inhibiting mTor (Zhang *et al.*, 2007; Williams *et al.*, 2008). In a MJD transgenic mouse model, the beneficial effect of a rapamycin ester (cell cycle inhibitor-779, temsirolimus) was demonstrated, by improving its motor performance (Menzies *et al.*, 2010). Nevertheless, as the mTOR pathway integrates diverse signals to regulate protein translation and cell growth, proliferation, and survival (Wullschleger *et al.*, 2006), its permanent inhibition could lead to severe side effects. Moreover rapamycin is an effective immunosuppressant (Cruzado, 2008), which may cause undesirable effects for long-term treatment. Growing evidence points to an important role of mTor-independent pathways for autophagy activation. Accordingly, our group recently showed that lentiviral-mediated overexpression of beclin-1, an m-TOR-independent autophagy activator, alleviates MJD neuropathology (Nascimento-Ferreira *et al* 2011). Nevertheless the approach involved viral gene transfer and may therefore take long to reach the clinics. An alternative could be the use of approved low-molecular weight drugs known to activate the autophagy pathway. Mood-

stabilizing drugs such as valproic acid (VPA), carbamazepine (CBZ) and lithium (Li) have been associated to the induction of autophagy in glioma cells (Fu *et al.*, 2010); hepatic fibrosis (Hidvegi *et al.*, 2010), HD (Sarkar *et al.*, 2005), and AD (Gomez-Sintes and Lucas, 2010), among others. Following these evidences, from a list of drugs that enhance autophagic degradation of misfolded proteins with polyglutamine repeats, we selected CBZ for this study because of its extensive safety profile in humans and its ability to cross the blood-brain-barrier.

In the first part of this study we minitore EGFP-LC3 puncta formation, LC3 turnover, and mRNA expression of LC3. Previously, other pharmacological agents with ability to activate autophagy have been identified based on a GFP-LC3 transfected cell-based screen (Zhang *et al.*, 2007). Furthermore, this GFP-LC3 labeling method has been successfully applied to *in vivo* mammalian autophagy research, by generating transgenic GFP-LC3 transgenic mice (Mizushima *et al.*, 2004), also in model organisms, including Drosophila (Rusten *et al.*, 2004; Scott *et al.*, 2004), nematodes (Melendez *et al.*, 2003), plants (Yoshimoto *et al.*, 2004) and zebrafish (He *et al.*, 2009). In neuroblastoma cells overexpressing mutant ataxin-3 we observed that CBZ mediated the activation of autophagy. An increased EGFP-LC3 puncta formation, representing autophagosomes, was observed in cells treated with CBZ comparatively to control conditions (Fig.3.1 A).

The activation of autophagy mediated by CBZ was confirmed using western blot assay (Fig.3.1 B-C) and by real-time PCR (Fig.3.1 D) that also showed increased levels of LC3. These results clearly indicate that autophagy flux is increased upon CBZ treatment comparatively to control conditions and are concordant with a previous study using CBZ to treat hepatic fibrosis, suggesting that CBZ plays an important role in autophagy enhancement (Hidvegi *et al.*, 2010).

As a consequence of the activation of autophagy, clearance of soluble and aggregated mutant ataxin-3 was observed in neuroblastoma MJD model, consistent with data obtained in other studies (Hidvegi *et al.*, 2010; Nascimento-Ferreira *et al.*, 2011). In order to identify the best condition to promote degradation of mutant protein different concentrations of CBZ were evaluated (Hidvegi *et al.*, 2010; Xiong *et al.*, 2011). Interestingly, even the lowest concentration of CBZ was able to promote extensive decrease of mutant ataxin-3 aggregates and soluble

protein levels, suggesting that lower concentrations of CBZ, regarding those previously described, could activate the autophagy pathway and lead to the clearance of mutant protein with similar efficacy. However, the efficiency of this process should be evaluated for longer times of treatment. As none of the tested concentrations affected cell viability, we chose to use 50μM to the following experiments, since it promoted the most efficient reduction of mutant ataxin-3 levels.

In the second part of this study we observed a neuroprotective effect of CBZ in the lentiviral-based MJD mouse model. In the last years, lentiviral vectors have been used successfully to create in vivo models of disease by overexpression of mutant proteins involved in the corresponding pathologies, either in the striatum, substantia nigra, amygdala or cortex (de Almeida *et al.*, 2002; Lo Bianco *et al.*, 2002; Kirik *et al.*, 2003; Lauwers *et al.*, 2003; Alves *et al.*, 2008). Lentiviral-mediated expression of mutant ataxin-3 in the rodent brain induces behavioral and neuropathological abnormalities associated with MJD, which may help to elucidate the molecular mechanism of mutant ataxin-3 toxicity and facilitate the evaluation of new therapeutic strategies (Alves *et al*., 2008; 2010; Nascimento-Ferreira *et al* 2011; Simoes *et al.*, 2012). Animals injected with lentiviral vectors encoding for atx3MUT in the striatum, were submitted to the treatment with CBZ during 3 weeks. The duration and dose were similar to the previously described regimen for *in vivo* treatment of hepatic fibrosis (Hidvegi *et al.*, 2010). This study provides evidence that simultaneous induction of MJD and stimulation of autophagy through CBZ treatment was able to hamper the disease progression in the lentiviral model of MJD, indicated by the decreased of the number ataxin-3MUT inclusions and the reduced neuronal dysfunction evaluated on darpp-32 stained sections, compared to control mice. This is concordant with the results obtained in the same MJD model when autophagy was promoted by overexpression of beclin-1 (Nascimento-Ferreira *et al.*, 2011), indicating that autophagy has a main role in the clearance of ataxin-3MUT in this model of MJD. Therefore, these data suggest a possible neuroprotective effect mediated by CBZ, preventing the pathological symptoms when initiated at an early stage of the disease. Furthermore, as polyglutamine inclusions are not removed by proteasome function, which may even become progressively impaired upon the

disease (Bence *et al.*, 2001; Venkatraman *et al.*, 2004), autophagy should be responsible for the ability of cells to clear the mutant protein and to retard the pathology.

To further investigate whether CBZ would alleviate neuropathology and motor behaviour impairments associated to MJD, when treatment was initiated after the disease phenotype became apparent, we used a transgenic mouse model of MJD (Torashima *et al.*, 2008) This transgenic mouse model has a predominant expression of mutant ataxin-3 in the cerebellum, which in MJD patients is one of the most affected regions (Rosenberg, 1992; Sudarsky and Coutinho, 1995; Durr *et al.*, 1996; Rub *et al.*, 2006; Alves *et al.*, 2008). The L7 promoter that drives expression of the C-terminal mutant ataxin-3 transgene is associated with expression in Purkinje cells. Even though MJD neuropathology has been associated with preferential degeneration of the deep cerebellar nuclei there are growing evidences that Purkinje cells are a type of cells that are affected in MJD patients (Munoz *et al.*, 2002; Scherzed *et al.*, 2012), which confers physiological relevance to the model.

Severe cerebellar atrophy and ataxic phenotype were already present at the time of initiation of CBZ treatment at three weeks of age. Nevertheless, CBZ led to rescue of the pathology, mediating a significant decrease in aggregated and soluble mutant ataxin-3 content within a short period of treatment, and attenuation of the phenotype upon longer term administration. This marked reduction in soluble and aggregated mutant ataxin-3 in the cerebellum is concordant with the results obtained regarding the clearance of antitrypsin Z in the liver of a transgenic model of hepatic fibrosis upon CBZ treatment (Hidvegi *et al.*, 2010). The capacity to enhance degradation of both insoluble and soluble ataxin-3 may represent an important characteristic of CBZ as a potential therapeutic drug.

The motor behavior assessment when using the rotarod was not conclusive, however the activity box tests revealed a modest but consistent tendency in the improvement of transgenic mice performance. The observed small but non-significant tendency for improvement in the rotarod performance could be explained by a) the short period of treatment, which may not have been sufficient to maximize the therapeutic effects in this severe model of disease, and b) the variability and reduced number of animals, which was aggravated by the use of animals of both

genders. Nevertheless, a clear tendency for an improved activity profile was observed in CBZ treated mice when compared to the controls at the 10 and 12 weeks time-points. Treated mice travelled longer distances, spent less time resting and more time moving fast. These results are in accordance with the hypoactivity reported for severe or late-stage disease models (Goti *et al.*, 2004; Bichelmeier *et al.*, 2007; Boy *et al.*, 2010), and are expected to arise from improvement of neuropathology. This could indicate that activation of autophagy pathway may delay the progression of the disease, even in an advanced stage of the pathology, evidencing that impairment of the autophagy pathway, of protein clearance and of neuronal dysfunction may not be irreversible. These results are in accordance with studies in SCA1, MJD and Huntington's disease where it is shown that even in a late stage of the disease, the cells which preferentially degenerate in the disorder retain at least some ability to repair the damage caused by mutant ataxin-1 (Zu *et al.*, 2004).

Altogether, these data provide strong evidence that stimulation of autophagy by CBZ is able to prevent neuropathological and behavioral MJD-associated abnormalities when administered in an early stage and to partially rescue the pathological phenotype when administered in a late stage of the disease. Therefore, the results presented in this thesis suggest that the autophagy pathway plays an important role in the cellular degradation of mutant ataxin-3 and in the prevention of consequent pathology. Importantly, this work suggests that CBZ treatment could be explored as possible therapeutic strategy to MJD.

CHAPTER 5 – CONCLUSIONS AND PERSPECTIVES

This work is a contribution to understand the potential of CBZ, a mood-stabilizing drug, in the mitigation of neurodegenerative disorders such as Machado-Joseph disease. Our results suggest that CBZ mediates activation of autophagy, degradation of mutant ataxin-3 and alleviation of MJD-associated neurophatology and motor behaviour, and should be further investigated for therapy of Machado-Joseph disease.

CBZ is an autophagy-enhancing drug, which signals through the phosphoinositol pathway thereby up-regulating autophagy (Sarkar and Rubinsztein, 2006; Hidvegi *et al.*, 2010). Previous work from our group indicated that activation of autophagy through an mTOR independent pathway might alleviate MJD (Nascimento-Ferreira *et al* 2011). Therefore we sought to investigate whether CBZ could be used as new non-invasive therapy for this severe disease for which no efficient treatment is available. Using an *in vitro* neuroblastoma model, and two in vivo models of MJD, we evaluated disease-modifying properties of CBZ. We provide *in vitro* and *in vivo* evidence that CBZ promotes activation of autophagy, reduction of mutant ataxin-3 levels, and of MJD-associated neurophatology and motor behaviour.

Overall, this study suggests that activation of autophagy by treatment with CBZ may be a promising therapeutic approach to MJD. Furthermore, combination of CBZ with other strategies, as previously reported in HD with combination of lithium and rapamycin (Sarkar *et al.*, 2008) may also be beneficial. More studies with longer times of treatment, and more animals will need to be done, to assess the real impact of this drug in the clearance of mutant protein and alleviation of MJD, and in order to develop an effective therapeutic for this incapacitating disease.

CHAPTER 6 – REFERENCES

Albrecht, M., M. Golatta, U. Wullner and T. Lengauer (2004). "Structural and functional analysis of ataxin-2 and ataxin-3." Eur J Biochem **271**(15): 3155-3170.

Alves, S., E. Regulier, I. Nascimento-Ferreira, R. Hassig, N. Dufour, A. Koeppen, A. L. Carvalho, S. Simoes, M. C. de Lima, E. Brouillet, V. C. Gould, N. Deglon and L. P. de Almeida (2008). "Striatal and nigral pathology in a lentiviral rat model of Machado-Joseph disease." Hum Mol Genet **17**(14): 2071-2083.

Ambrosio, A. F., P. Soares-Da-Silva, C. M. Carvalho and A. P. Carvalho (2002). "Mechanisms of action of carbamazepine and its derivatives, oxcarbazepine, BIA 2-093, and BIA 2-024." Neurochem Res **27**(1-2): 121-130.

Anglade, P., S. Vyas, F. Javoy-Agid, M. T. Herrero, P. P. Michel, J. Marquez, A. Mouatt-Prigent, M. Ruberg, E. C. Hirsch and Y. Agid (1997). "Apoptosis and autophagy in nigral neurons of patients with Parkinson's disease." Histol Histopathol **12**(1): 25-31.

Antony, P. M., S. Mantele, P. Mollenkopf, J. Boy, R. H. Kehlenbach, O. Riess and T. Schmidt (2009). "Identification and functional dissection of localization signals within ataxin-3." Neurobiol Dis **36**(2): 280-292.

Atlashkin, V., V. Kreykenbohm, E. L. Eskelinen, D. Wenzel, A. Fayyazi and G. Fischer von Mollard (2003). "Deletion of the SNARE vti1b in mice results in the loss of a single SNARE partner, syntaxin 8." Mol Cell Biol **23**(15): 5198-5207.

Axe, E. L., S. A. Walker, M. Manifava, P. Chandra, H. L. Roderick, A. Habermann, G. Griffiths and N. T. Ktistakis (2008). "Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum." J Cell Biol **182**(4): 685-701.

Baba, M., M. Osumi and Y. Ohsumi (1995). "Analysis of the membrane structures involved in autophagy in yeast by freeze-replica method." Cell Struct Funct **20**(6): 465-471.

Balgi, A. D., B. D. Fonseca, E. Donohue, T. C. Tsang, P. Lajoie, C. G. Proud, I. R. Nabi and M. Roberge (2009). "Screen for chemical modulators of autophagy reveals novel therapeutic inhibitors of mTORC1 signaling." PLoS One **4**(9): e7124.

Bence, N. F., R. M. Sampat and R. R. Kopito (2001). "Impairment of the ubiquitin-proteasome system by protein aggregation." Science **292**(5521): 1552-1555.

Bennett, E. J., N. F. Bence, R. Jayakumar and R. R. Kopito (2005). "Global impairment of the ubiquitin-proteasome system by nuclear or cytoplasmic protein aggregates precedes inclusion body formation." Mol Cell **17**(3): 351-365.

Berger, Z., B. Ravikumar, F. M. Menzies, L. G. Oroz, B. R. Underwood, M. N. Pangalos, I. Schmitt, U. Wullner, B. O. Evert, C. J. O'Kane and D. C. Rubinsztein (2006). "Rapamycin alleviates toxicity of different aggregate-prone proteins." Hum Mol Genet **15**(3): 433-442.

Berke, S. J., Y. Chai, G. L. Marrs, H. Wen and H. L. Paulson (2005). "Defining the role of ubiquitin-interacting motifs in the polyglutamine disease protein, ataxin-3." J Biol Chem **280**(36): 32026-32034.

Berridge, M. J. (1987). "Inositol trisphosphate and diacylglycerol: two interacting second messengers." Annu Rev Biochem **56**: 159-193.

Berridge, M. J., M. D. Bootman and H. L. Roderick (2003). "Calcium signalling: dynamics, homeostasis and remodelling." Nat Rev Mol Cell Biol **4**(7): 517-529.

Bertilsson, L. (1978). "Clinical pharmacokinetics of carbamazepine." Clin Pharmacokinet **3**(2): 128-143.

Bettencourt, C. and M. Lima (2011). "Machado-Joseph Disease: from first descriptions to new perspectives." Orphanet J Rare Dis **6**: 35.

Bettencourt, C., C. Santos, R. Montiel, C. Costa Mdo, P. Cruz-Morales, L. R. Santos, N. Simoes, T. Kay, J. Vasconcelos, P. Maciel and M. Lima (2010). "Increased transcript diversity: novel splicing variants of Machado-Joseph disease gene (ATXN3)." Neurogenetics **11**(2): 193-202.

Bichelmeier, U., T. Schmidt, J. Hubener, J. Boy, L. Ruttiger, K. Habig, S. Poths, M. Bonin, M. Knipper, W. J. Schmidt, J. Wilbertz, H. Wolburg, F. Laccone and O. Riess (2007). "Nuclear localization of ataxin-3 is required for the manifestation of symptoms in SCA3: in vivo evidence." J Neurosci **27**(28): 7418-7428.

Bjorkoy, G., T. Lamark, A. Brech, H. Outzen, M. Perander, A. Overvatn, H. Stenmark and T. Johansen (2005). "p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death." J Cell Biol **171**(4): 603-614.

Boy, J., T. Schmidt, U. Schumann, U. Grasshoff, S. Unser, C. Holzmann, I. Schmitt, T. Karl, F. Laccone, H. Wolburg, S. Ibrahim and O. Riess (2010). "A transgenic mouse model of spinocerebellar

ataxia type 3 resembling late disease onset and gender-specific instability of CAG repeats." Neurobiol Dis **37**(2): 284-293.

Burnett, B., F. Li and R. N. Pittman (2003). "The polyglutamine neurodegenerative protein ataxin-3 binds polyubiquitylated proteins and has ubiquitin protease activity." Hum Mol Genet **12**(23): 3195-3205.

Burnett, B. G. and R. N. Pittman (2005). "The polyglutamine neurodegenerative protein ataxin 3 regulates aggresome formation." Proc Natl Acad Sci U S A **102**(12): 4330-4335.

Cancel, G., N. Abbas, G. Stevanin, A. Durr, H. Chneiweiss, C. Neri, C. Duyckaerts, C. Penet, H. M. Cann, Y. Agid and et al. (1995). "Marked phenotypic heterogeneity associated with expansion of a CAG repeat sequence at the spinocerebellar ataxia 3/Machado-Joseph disease locus." Am J Hum Genet **57**(4): 809-816.

Carlson, K. M., J. M. Andresen and H. T. Orr (2009). "Emerging pathogenic pathways in the spinocerebellar ataxias." Curr Opin Genet Dev **19**(3): 247-253.

Chai, Y., S. S. Berke, R. E. Cohen and H. L. Paulson (2004). "Poly-ubiquitin binding by the polyglutamine disease protein ataxin-3 links its normal function to protein surveillance pathways." J Biol Chem **279**(5): 3605-3611.

Chai, Y., S. L. Koppenhafer, N. M. Bonini and H. L. Paulson (1999). "Analysis of the role of heat shock protein (Hsp) molecular chaperones in polyglutamine disease." J Neurosci **19**(23): 10338-10347.

Chai, Y., S. L. Koppenhafer, S. J. Shoesmith, M. K. Perez and H. L. Paulson (1999). "Evidence for proteasome involvement in polyglutamine disease: localization to nuclear inclusions in SCA3/MJD and suppression of polyglutamine aggregation in vitro." Hum Mol Genet **8**(4): 673-682.

Chai, Y., J. Shao, V. M. Miller, A. Williams and H. L. Paulson (2002). "Live-cell imaging reveals divergent intracellular dynamics of polyglutamine disease proteins and supports a sequestration model of pathogenesis." Proc Natl Acad Sci U S A **99**(14): 9310-9315.

Chan, E. Y. and S. A. Tooze (2009). "Evolution of Atg1 function and regulation." Autophagy **5**(6): 758-765.

Chang, Y. Y. and T. P. Neufeld (2009). "An Atg1/Atg13 complex with multiple roles in TOR-mediated autophagy regulation." Mol Biol Cell **20**(7): 2004-2014.

Ciechanover, A. (2001). "Ubiquitin-mediated degradation of cellular proteins: why destruction is essential for construction, and how it got from the test tube to the patient's bed." Isr Med Assoc J **3**(5): 319-327.

Ciechanover, A. (2005). "Proteolysis: from the lysosome to ubiquitin and the proteasome." Nat Rev Mol Cell Biol **6**(1): 79-87.

Codogno, P. and A. J. Meijer (2005). "Autophagy and signaling: their role in cell survival and cell death." Cell Death Differ **12 Suppl 2**: 1509-1518.

Costa, M. C., J. Gomes-da-Silva, C. J. Miranda, J. Sequeiros, M. M. Santos and P. Maciel (2004). "Genomic structure, promoter activity, and developmental expression of the mouse homologue of the Machado-Joseph disease (MJD) gene." Genomics **84**(2): 361-373.

Coutinho, P. and C. Andrade (1978). "Autosomal dominant system degeneration in Portuguese families of the Azores Islands. A new genetic disorder involving cerebellar, pyramidal, extrapyramidal and spinal cord motor functions." Neurology **28**(7): 703-709.

Coutinho, P., A. Guimaraes and F. Scaravilli (1982). "The pathology of Machado-Joseph disease. Report of a possible homozygous case." Acta Neuropathol **58**(1): 48-54.

Criollo, A., M. C. Maiuri, E. Tasdemir, I. Vitale, A. A. Fiebig, D. Andrews, J. Molgo, J. Diaz, S. Lavandero, F. Harper, G. Pierron, D. di Stefano, R. Rizzuto, G. Szabadkai and G. Kroemer (2007). "Regulation of autophagy by the inositol trisphosphate receptor." Cell Death Differ **14**(5): 1029-1039.

Cruzado, J. M. (2008). "Nonimmunosuppressive effects of mammalian target of rapamycin inhibitors." Transplant Rev (Orlando) **22**(1): 73-81.

Cuervo, A. M. (2004). "Autophagy: in sickness and in health." Trends Cell Biol **14**(2): 70-77.

Cummings, C. J. and H. Y. Zoghbi (2000). "Trinucleotide repeats: mechanisms and pathophysiology." Annu Rev Genomics Hum Genet **1**: 281-328.

de Almeida, L. P., C. A. Ross, D. Zala, P. Aebischer and N. Deglon (2002). "Lentiviral-mediated delivery of mutant huntingtin in the striatum of rats induces a selective neuropathology modulated by polyglutamine repeat size, huntingtin expression levels, and protein length." J Neurosci **22**(9): 3473-3483.

de Almeida, L. P., D. Zala, P. Aebischer and N. Deglon (2001). "Neuroprotective effect of a CNTF-expressing lentiviral vector in the quinolinic acid rat model of Huntington's disease." Neurobiol Dis **8**(3): 433-446.

De Duve, C. and R. Wattiaux (1966). "Functions of lysosomes." Annu Rev Physiol **28**: 435-492.

do Carmo Costa, M., F. Bajanca, A. J. Rodrigues, R. J. Tome, G. Corthals, S. Macedo-Ribeiro, H. L. Paulson, E. Logarinho and P. Maciel (2010). "Ataxin-3 plays a role in mouse myogenic differentiation through regulation of integrin subunit levels." PLoS One **5**(7): e11728.

Doss-Pepe, E. W., E. S. Stenroos, W. G. Johnson and K. Madura (2003). "Ataxin-3 interactions with rad23 and valosin-containing protein and its associations with ubiquitin chains and the proteasome are consistent with a role in ubiquitin-mediated proteolysis." Mol Cell Biol **23**(18): 6469-6483.

Duenas, A. M., R. Goold and P. Giunti (2006). "Molecular pathogenesis of spinocerebellar ataxias." Brain **129**(Pt 6): 1357-1370.

Dunn, W. A., Jr. (1990). "Studies on the mechanisms of autophagy: maturation of the autophagic vacuole." J Cell Biol **110**(6): 1935-1945.

Durr, A., G. Stevanin, G. Cancel, C. Duyckaerts, N. Abbas, O. Didierjean, H. Chneiweiss, A. Benomar, O. Lyon-Caen, J. Julien, M. Serdaru, C. Penet, Y. Agid and A. Brice (1996). "Spinocerebellar ataxia 3 and Machado-Joseph disease: clinical, molecular, and neuropathological features." Ann Neurol **39**(4): 490-499.

Erlich, S., E. Shohami and R. Pinkas-Kramarski (2006). "Neurodegeneration induces upregulation of Beclin 1." Autophagy **2**(1): 49-51.

Evert, B. O., U. Wullner, J. B. Schulz, M. Weller, P. Groscurth, Y. Trottier, A. Brice and T. Klockgether (1999). "High level expression of expanded full-length ataxin-3 in vitro causes cell death and formation of intranuclear inclusions in neuronal cells." Hum Mol Genet **8**(7): 1169-1176.

Feder, J. H., J. M. Rossi, J. Solomon, N. Solomon and S. Lindquist (1992). "The consequences of expressing hsp70 in Drosophila cells at normal temperatures." Genes Dev **6**(8): 1402-1413.

Feldman, M. E., B. Apsel, A. Uotila, R. Loewith, Z. A. Knight, D. Ruggero and K. M. Shokat (2009). "Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2." PLoS Biol **7**(2): e38.

Ferrigno, P. and P. A. Silver (2000). "Polyglutamine expansions: proteolysis, chaperones, and the dangers of promiscuity." Neuron **26**(1): 9-12.

Fimia, G. M., A. Stoykova, A. Romagnoli, L. Giunta, S. Di Bartolomeo, R. Nardacci, M. Corazzari, C. Fuoco, A. Ucar, P. Schwartz, P. Gruss, M. Piacentini, K. Chowdhury and F. Cecconi (2007). "Ambra1 regulates autophagy and development of the nervous system." Nature **447**(7148): 1121-1125.

Fu, J., C. J. Shao, F. R. Chen, H. K. Ng and Z. P. Chen (2010). "Autophagy induced by valproic acid is associated with oxidative stress in glioma cell lines." Neuro Oncol **12**(4): 328-340.

Fu, Y. H., D. P. Kuhl, A. Pizzuti, M. Pieretti, J. S. Sutcliffe, S. Richards, A. J. Verkerk, J. J. Holden, R. G. Fenwick, Jr., S. T. Warren and et al. (1991). "Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox." Cell **67**(6): 1047-1058.

Fujita, N., T. Itoh, H. Omori, M. Fukuda, T. Noda and T. Yoshimori (2008). "The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy." Mol Biol Cell **19**(5): 2092-2100.

Furuta, N., N. Fujita, T. Noda, T. Yoshimori and A. Amano (2010). "Combinational soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins VAMP8 and Vti1b mediate fusion of antimicrobial and canonical autophagosomes with lysosomes." Mol Biol Cell **21**(6): 1001-1010.

Ganley, I. G., H. Lam du, J. Wang, X. Ding, S. Chen and X. Jiang (2009). "ULK1.ATG13.FIP200 complex mediates mTOR signaling and is essential for autophagy." J Biol Chem **284**(18): 12297-12305.

Gatchel, J. R. and H. Y. Zoghbi (2005). "Diseases of unstable repeat expansion: mechanisms and common principles." Nat Rev Genet **6**(10): 743-755.

Geng, J., U. Nair, K. Yasumura-Yorimitsu and D. J. Klionsky (2010). "Post-Golgi Sec proteins are required for autophagy in Saccharomyces cerevisiae." Mol Biol Cell **21**(13): 2257-2269.

Glickman, M. H. and A. Ciechanover (2002). "The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction." Physiol Rev **82**(2): 373-428.

Gomez-Sintes, R. and J. J. Lucas (2010). "NFAT/Fas signaling mediates the neuronal apoptosis and motor side effects of GSK-3 inhibition in a mouse model of lithium therapy." J Clin Invest **120**(7): 2432-2445.

Goti, D., S. M. Katzen, J. Mez, N. Kurtis, J. Kiluk, L. Ben-Haiem, N. A. Jenkins, N. G. Copeland, A. Kakizuka, A. H. Sharp, C. A. Ross, P. R. Mouton and V. Colomer (2004). "A mutant ataxin-3 putative-cleavage fragment in brains of Machado-Joseph disease patients and transgenic mice is cytotoxic above a critical concentration." J Neurosci **24**(45): 10266-10279.

Guertin, D. A. and D. M. Sabatini (2009). "The pharmacology of mTOR inhibition." Sci Signal **2**(67): pe24.

Gutierrez, M. G., D. B. Munafo, W. Beron and M. I. Colombo (2004). "Rab7 is required for the normal progression of the autophagic pathway in mammalian cells." J Cell Sci **117**(Pt 13): 2687-2697.

Hailey, D. W., A. S. Rambold, P. Satpute-Krishnan, K. Mitra, R. Sougrat, P. K. Kim and J. Lippincott-Schwartz (2010). "Mitochondria supply membranes for autophagosome biogenesis during starvation." Cell **141**(4): 656-667.

Hara, T. and N. Mizushima (2009). "Role of ULK-FIP200 complex in mammalian autophagy: FIP200, a counterpart of yeast Atg17?" Autophagy **5**(1): 85-87.

Hara, T., K. Nakamura, M. Matsui, A. Yamamoto, Y. Nakahara, R. Suzuki-Migishima, M. Yokoyama, K. Mishima, I. Saito, H. Okano and N. Mizushima (2006). "Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice." Nature **441**(7095): 885-889.

Harding, T. M., A. Hefner-Gravink, M. Thumm and D. J. Klionsky (1996). "Genetic and phenotypic overlap between autophagy and the cytoplasm to vacuole protein targeting pathway." J Biol Chem **271**(30): 17621-17624.

Harris, G. M., K. Dodelzon, L. Gong, P. Gonzalez-Alegre and H. L. Paulson (2010). "Splice isoforms of the polyglutamine disease protein ataxin-3 exhibit similar enzymatic yet different aggregation properties." PLoS One **5**(10): e13695.

Havel, L. S., S. Li and X. J. Li (2009). "Nuclear accumulation of polyglutamine disease proteins and neuropathology." Mol Brain **2**: 21.

Hayashi-Nishino, M., N. Fujita, T. Noda, A. Yamaguchi, T. Yoshimori and A. Yamamoto (2009). "A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation." Nat Cell Biol **11**(12): 1433-1437.

He, C., C. R. Bartholomew, W. Zhou and D. J. Klionsky (2009). "Assaying autophagic activity in transgenic GFP-Lc3 and GFP-Gabarap zebrafish embryos." Autophagy **5**(4): 520-526.

Hemelaar, J., V. S. Lelyveld, B. M. Kessler and H. L. Ploegh (2003). "A single protease, Apg4B, is specific for the autophagy-related ubiquitin-like proteins GATE-16, MAP1-LC3, GABARAP, and Apg8L." J Biol Chem **278**(51): 51841-51850.

Hidvegi, T., M. Ewing, P. Hale, C. Dippold, C. Beckett, C. Kemp, N. Maurice, A. Mukherjee, C. Goldbach, S. Watkins, G. Michalopoulos and D. H. Perlmutter (2010). "An autophagy-enhancing drug promotes degradation of mutant alpha1-antitrypsin Z and reduces hepatic fibrosis." Science **329**(5988): 229-232.

Hirsimaki, Y., P. Hirsimaki and K. Lounatmaa (1982). "Vinblastine-induced autophagic vacuoles in mouse liver and Ehrlich ascites tumor cells as assessed by freeze-fracture electron microscopy." Eur J Cell Biol **27**(2): 298-301.

Hosokawa, N., T. Hara, T. Kaizuka, C. Kishi, A. Takamura, Y. Miura, S. Iemura, T. Natsume, K. Takehana, N. Yamada, J. L. Guan, N. Oshiro and N. Mizushima (2009). "Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy." Mol Biol Cell **20**(7): 1981-1991.

Ichikawa, Y., J. Goto, M. Hattori, A. Toyoda, K. Ishii, S. Y. Jeong, H. Hashida, N. Masuda, K. Ogata, F. Kasai, M. Hirai, P. Maciel, G. A. Rouleau, Y. Sakaki and I. Kanazawa (2001). "The genomic structure and expression of MJD, the Machado-Joseph disease gene." J Hum Genet **46**(7): 413-422.

Imarisio, S., J. Carmichael, V. Korolchuk, C. W. Chen, S. Saiki, C. Rose, G. Krishna, J. E. Davies, E. Ttofi, B. R. Underwood and D. C. Rubinsztein (2008). "Huntington's disease: from pathology and genetics to potential therapies." Biochem J **412**(2): 191-209.

Itakura, E., C. Kishi, K. Inoue and N. Mizushima (2008). "Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG." Mol Biol Cell **19**(12): 5360-5372.

Iwata, A., J. C. Christianson, M. Bucci, L. M. Ellerby, N. Nukina, L. S. Forno and R. R. Kopito (2005). "Increased susceptibility of cytoplasmic over nuclear polyglutamine aggregates to autophagic degradation." Proc Natl Acad Sci U S A **102**(37): 13135-13140.

Jahreiss, L., F. M. Menzies and D. C. Rubinsztein (2008). "The itinerary of autophagosomes: from peripheral formation to kiss-and-run fusion with lysosomes." Traffic **9**(4): 574-587.

Jardim, L. B., I. Silveira, M. L. Pereira, A. Ferro, I. Alonso, M. do Ceu Moreira, P. Mendonca, F. Ferreirinha, J. Sequeiros and R. Giugliani (2001). "A survey of spinocerebellar ataxia in South Brazil - 66 new cases with Machado-Joseph disease, SCA7, SCA8, or unidentified disease-causing mutations." J Neurol **248**(10): 870-876.

Jeong, H., F. Then, T. J. Melia, Jr., J. R. Mazzulli, L. Cui, J. N. Savas, C. Voisine, P. Paganetti, N. Tanese, A. C. Hart, A. Yamamoto and D. Krainc (2009). "Acetylation targets mutant huntingtin to autophagosomes for degradation." Cell **137**(1): 60-72.

Jiang, H., B. S. Tang, B. Xu, G. H. Zhao, L. Shen, J. G. Tang, Q. H. Li and K. Xia (2005). "Frequency analysis of autosomal dominant spinocerebellar ataxias in mainland Chinese patients and clinical and molecular characterization of spinocerebellar ataxia type 6." Chin Med J (Engl) **118**(10): 837-843.

Jung, C. H., C. B. Jun, S. H. Ro, Y. M. Kim, N. M. Otto, J. Cao, M. Kundu and D. H. Kim (2009). "ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery." Mol Biol Cell **20**(7): 1992-2003.

Kabeya, Y., N. Mizushima, T. Ueno, A. Yamamoto, T. Kirisako, T. Noda, E. Kominami, Y. Ohsumi and T. Yoshimori (2000). "LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing." EMBO J **19**(21): 5720-5728.

Kanda, T., E. Isozaki, S. Kato, H. Tanabe and M. Oda (1989). "Type III Machado-Joseph disease in a Japanese family: a clinicopathological study with special reference to the peripheral nervous system." Clin Neuropathol **8**(3): 134-141.

Kawaguchi, Y., T. Okamoto, M. Taniwaki, M. Aizawa, M. Inoue, S. Katayama, H. Kawakami, S. Nakamura, M. Nishimura, I. Akiguchi and et al. (1994). "CAG expansions in a novel gene for Machado-Joseph disease at chromosome 14q32.1." Nat Genet **8**(3): 221-228.

Khan, L. A., T. Yamanaka and N. Nukina (2008). "Genetic impairment of autophagy intensifies expanded polyglutamine toxicity in Caenorhabditis elegans." Biochem Biophys Res Commun **368**(3): 729-735.

Kiffin, R., C. Christian, E. Knecht and A. M. Cuervo (2004). "Activation of chaperone-mediated autophagy during oxidative stress." Mol Biol Cell **15**(11): 4829-4840.

Kihara, A., Y. Kabeya, Y. Ohsumi and T. Yoshimori (2001). "Beclin-phosphatidylinositol 3-kinase complex functions at the trans-Golgi network." EMBO Rep **2**(4): 330-335.

Kim, J. and D. J. Klionsky (2000). "Autophagy, cytoplasm-to-vacuole targeting pathway, and pexophagy in yeast and mammalian cells." Annu Rev Biochem **69**: 303-342.

Kimura, S., T. Noda and T. Yoshimori (2008). "Dynein-dependent movement of autophagosomes mediates efficient encounters with lysosomes." Cell Struct Funct **33**(1): 109-122.

Kirik, D., L. E. Annett, C. Burger, N. Muzyczka, R. J. Mandel and A. Bjorklund (2003). "Nigrostriatal alpha-synucleinopathy induced by viral vector-mediated overexpression of human alpha-synuclein: a new primate model of Parkinson's disease." Proc Natl Acad Sci U S A **100**(5): 2884-2889.

Kirkin, V., T. Lamark, Y. S. Sou, G. Bjorkoy, J. L. Nunn, J. A. Bruun, E. Shvets, D. G. McEwan, T. H. Clausen, P. Wild, I. Bilusic, J. P. Theurillat, A. Overvatn, T. Ishii, Z. Elazar, M. Komatsu, I. Dikic and T. Johansen (2009). "A role for NBR1 in autophagosomal degradation of ubiquitinated substrates." Mol Cell **33**(4): 505-516.

Kirkin, V., D. G. McEwan, I. Novak and I. Dikic (2009). "A role for ubiquitin in selective autophagy." Mol Cell **34**(3): 259-269.

Klionsky, D. J., H. Abeliovich, P. Agostinis, D. K. Agrawal, G. Aliev, D. S. Askew, M. Baba, E. H. Baehrecke, B. A. Bahr, A. Ballabio, B. A. Bamber, D. C. Bassham, E. Bergamini, X. Bi, M. Biard-Piechaczyk, J. S. Blum, D. E. Bredesen, J. L. Brodsky, J. H. Brumell, U. T. Brunk, W. Bursch, N. Camougrand, E. Cebollero, F. Cecconi, Y. Chen, L. S. Chin, A. Choi, C. T. Chu, J. Chung, P. G. Clarke, R. S. Clark, S. G. Clarke, C. Clave, J. L. Cleveland, P. Codogno, M. I. Colombo, A. Coto-Montes, J. M. Cregg, A. M. Cuervo, J. Debnath, F. Demarchi, P. B. Dennis, P. A. Dennis, V. Deretic, R. J. Devenish, F. Di Sano, J. F. Dice, M. Difiglia, S. Dinesh-Kumar, C. W. Distelhorst, M. Djavaheri-Mergny, F. C. Dorsey, W. Droge, M. Dron, W. A. Dunn, Jr., M. Duszenko, N. T. Eissa, Z. Elazar, A. Esclatine, E. L. Eskelinen, L. Fesus, K. D. Finley, J. M. Fuentes, J. Fueyo, K. Fujisaki, B. Galliot, F. B. Gao, D. A. Gewirtz, S. B. Gibson, A. Gohla, A. L. Goldberg, R. Gonzalez, C. Gonzalez-Estevez, S. Gorski, R. A. Gottlieb, D. Haussinger, Y. W. He, K. Heidenreich, J. A. Hill, M. Hoyer-Hansen, X. Hu, W. P. Huang, A. Iwasaki, M. Jaattela, W. T. Jackson, X. Jiang, S. Jin, T. Johansen, J. U. Jung, M. Kadowaki, C. Kang, A. Kelekar, D. H. Kessel, J. A. Kiel, H. P. Kim, A. Kimchi, T. J. Kinsella, K. Kiselyov, K. Kitamoto, E. Knecht, M. Komatsu, E. Kominami, S. Kondo, A. L. Kovacs, G. Kroemer, C. Y. Kuan, R. Kumar, M. Kundu, J. Landry, M. Laporte, W. Le, H. Y. Lei, M. J. Lenardo, B. Levine, A. Lieberman, K. L. Lim, F. C. Lin, W. Liou, L. F. Liu, G. Lopez-Berestein, C. Lopez-Otin, B. Lu, K. F. Macleod, W. Malorni, W. Martinet, K. Matsuoka, J. Mautner, A. J. Meijer, A. Melendez, P. Michels, G. Miotto, W. P. Mistiaen, N. Mizushima, B. Mograbi, I. Monastyrska, M. N. Moore, P. I. Moreira, Y. Moriyasu, T. Motyl, C. Munz, L. O. Murphy, N. I. Naqvi, T. P. Neufeld, I. Nishino, R. A.

Nixon, T. Noda, B. Nurnberg, M. Ogawa, N. L. Oleinick, L. J. Olsen, B. Ozpolat, S. Paglin, G. E. Palmer, I. Papassideri, M. Parkes, D. H. Perlmutter, G. Perry, M. Piacentini, R. Pinkas-Kramarski, M. Prescott, T. Proikas-Cezanne, N. Raben, A. Rami, F. Reggiori, B. Rohrer, D. C. Rubinsztein, K. M. Ryan, J. Sadoshima, H. Sakagami, Y. Sakai, M. Sandri, C. Sasakawa, M. Sass, C. Schneider, P. O. Seglen, O. Seleverstov, J. Settleman, J. J. Shacka, I. M. Shapiro, A. Sibirny, E. C. Silva-Zacarin, H. U. Simon, C. Simone, A. Simonsen, M. A. Smith, K. Spanel-Borowski, V. Srinivas, M. Steeves, H. Stenmark, P. E. Stromhaug, C. S. Subauste, S. Sugimoto, D. Sulzer, T. Suzuki, M. S. Swanson, I. Tabas, F. Takeshita, N. J. Talbot, Z. Talloczy, K. Tanaka, I. Tanida, G. S. Taylor, J. P. Taylor, A. Terman, G. Tettamanti, C. B. Thompson, M. Thumm, A. M. Tolkovsky, S. A. Tooze, R. Truant, L. V. Tumanovska, Y. Uchiyama, T. Ueno, N. L. Uzcategui, I. van der Klei, E. C. Vaquero, T. Vellai, M. W. Vogel, H. G. Wang, P. Webster, J. W. Wiley, Z. Xi, G. Xiao, J. Yahalom, J. M. Yang, G. Yap, X. M. Yin, T. Yoshimori, L. Yu, Z. Yue, M. Yuzaki, O. Zabirnyk, X. Zheng, X. Zhu and R. L. Deter (2008). "Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes." Autophagy **4**(2): 151-175.

Klionsky, D. J., J. M. Cregg, W. A. Dunn, Jr., S. D. Emr, Y. Sakai, I. V. Sandoval, A. Sibirny, S. Subramani, M. Thumm, M. Veenhuis and Y. Ohsumi (2003). "A unified nomenclature for yeast autophagy-related genes." Dev Cell **5**(4): 539-545.

Klionsky, D. J. and S. D. Emr (2000). "Autophagy as a regulated pathway of cellular degradation." Science **290**(5497): 1717-1721.

Komatsu, M., S. Waguri, T. Chiba, S. Murata, J. Iwata, I. Tanida, T. Ueno, M. Koike, Y. Uchiyama, E. Kominami and K. Tanaka (2006). "Loss of autophagy in the central nervous system causes neurodegeneration in mice." Nature **441**(7095): 880-884.

Kraft, C., F. Reggiori and M. Peter (2009). "Selective types of autophagy in yeast." Biochim Biophys Acta **1793**(9): 1404-1412.

Krebs, R. A. and M. E. Feder (1997). "Deleterious consequences of Hsp70 overexpression in Drosophila melanogaster larvae." Cell Stress Chaperones **2**(1): 60-71.

Kuhlbrodt, K., J. Mouysset and T. Hoppe (2005). "Orchestra for assembly and fate of polyubiquitin chains." Essays Biochem **41**: 1-14.

la Cour, T., R. Gupta, K. Rapacki, K. Skriver, F. M. Poulsen and S. Brunak (2003). "NESbase version 1.0: a database of nuclear export signals." Nucleic Acids Res **31**(1): 393-396.

La Spada, A. R., E. M. Wilson, D. B. Lubahn, A. E. Harding and K. H. Fischbeck (1991). "Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy." Nature **352**(6330): 77-79.

Lamark, T., V. Kirkin, I. Dikic and T. Johansen (2009). "NBR1 and p62 as cargo receptors for selective autophagy of ubiquitinated targets." Cell Cycle **8**(13): 1986-1990.

Laplante, M. and D. M. Sabatini (2009). "mTOR signaling at a glance." J Cell Sci **122**(Pt 20): 3589-3594.

Lauwers, E., Z. Debyser, J. Van Dorpe, B. De Strooper, B. Nuttin and V. Baekelandt (2003). "Neuropathology and neurodegeneration in rodent brain induced by lentiviral vector-mediated overexpression of alpha-synuclein." Brain Pathol **13**(3): 364-372.

Li, F., T. Macfarlan, R. N. Pittman and D. Chakravarti (2002). "Ataxin-3 is a histone-binding protein with two independent transcriptional corepressor activities." J Biol Chem **277**(47): 45004-45012.

Li, X., H. Li and X. J. Li (2008). "Intracellular degradation of misfolded proteins in polyglutamine neurodegenerative diseases." Brain Res Rev **59**(1): 245-252.

Liang, C., J. S. Lee, K. S. Inn, M. U. Gack, Q. Li, E. A. Roberts, I. Vergne, V. Deretic, P. Feng, C. Akazawa and J. U. Jung (2008). "Beclin1-binding UVRAG targets the class C Vps complex to coordinate autophagosome maturation and endocytic trafficking." Nat Cell Biol **10**(7): 776-787.

Lima, L. and P. Coutinho (1980). "Clinical criteria for diagnosis of Machado-Joseph disease: report of a non-Azorena Portuguese family." Neurology **30**(3): 319-322.

Lima, M., F. Mayer, P. Coutinho and A. Abade (1997). "Prevalence, geographic distribution, and genealogical investigation of Machado-Joseph disease in the Azores (Portugal)." Hum Biol **69**(3): 383-391.

Lo Bianco, C., J. L. Ridet, B. L. Schneider, N. Deglon and P. Aebischer (2002). "alpha -Synucleinopathy and selective dopaminergic neuron loss in a rat lentiviral-based model of Parkinson's disease." Proc Natl Acad Sci U S A **99**(16): 10813-10818.

Loewith, R., E. Jacinto, S. Wullschleger, A. Lorberg, J. L. Crespo, D. Bonenfant, W. Oppliger, P. Jenoe and M. N. Hall (2002). "Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control." Mol Cell **10**(3): 457-468.

Maciel, P., M. C. Costa, A. Ferro, M. Rousseau, C. S. Santos, C. Gaspar, J. Barros, G. A. Rouleau, P. Coutinho and J. Sequeiros (2001). "Improvement in the molecular diagnosis of Machado-Joseph disease." Arch Neurol **58**(11): 1821-1827.

Maciel, P., C. Gaspar, A. L. DeStefano, I. Silveira, P. Coutinho, J. Radvany, D. M. Dawson, L. Sudarsky, J. Guimaraes, J. E. Loureiro and et al. (1995). "Correlation between CAG repeat length and clinical features in Machado-Joseph disease." Am J Hum Genet **57**(1): 54-61.

Maeda, T. and F. Eisenberg, Jr. (1980). "Purification, structure, and catalytic properties of L-myo-inositol-1-phosphate synthase from rat testis." J Biol Chem **255**(18): 8458-8464.

Majerus, P. W. (1992). "Inositol phosphate biochemistry." Annu Rev Biochem **61**: 225-250.

Martinez-Vicente, M., Z. Talloczy, E. Wong, G. Tang, H. Koga, S. Kaushik, R. de Vries, E. Arias, S. Harris, D. Sulzer and A. M. Cuervo (2010). "Cargo recognition failure is responsible for inefficient autophagy in Huntington's disease." Nat Neurosci **13**(5): 567-576.

Maruyama, H., S. Nakamura, Z. Matsuyama, T. Sakai, M. Doyu, G. Sobue, M. Seto, M. Tsujihata, T. Oh-i, T. Nishio and et al. (1995). "Molecular features of the CAG repeats and clinical manifestation of Machado-Joseph disease." Hum Mol Genet **4**(5): 807-812.

Masino, L., V. Musi, R. P. Menon, P. Fusi, G. Kelly, T. A. Frenkiel, Y. Trottier and A. Pastore (2003). "Domain architecture of the polyglutamine protein ataxin-3: a globular domain followed by a flexible tail." FEBS Lett **549**(1-3): 21-25.

Masino, L., G. Nicastro, L. Calder, M. Vendruscolo and A. Pastore (2011). "Functional interactions as a survival strategy against abnormal aggregation." FASEB J **25**(1): 45-54.

Masino, L., G. Nicastro, R. P. Menon, F. Dal Piaz, L. Calder and A. Pastore (2004). "Characterization of the structure and the amyloidogenic properties of the Josephin domain of the polyglutamine-containing protein ataxin-3." J Mol Biol **344**(4): 1021-1035.

Matsunaga, K., E. Morita, T. Saitoh, S. Akira, N. T. Ktistakis, T. Izumi, T. Noda and T. Yoshimori (2010). "Autophagy requires endoplasmic reticulum targeting of the PI3-kinase complex via Atg14L." J Cell Biol **190**(4): 511-521.

McCampbell, A., J. P. Taylor, A. A. Taye, J. Robitschek, M. Li, J. Walcott, D. Merry, Y. Chai, H. Paulson, G. Sobue and K. H. Fischbeck (2000). "CREB-binding protein sequestration by expanded polyglutamine." Hum Mol Genet **9**(14): 2197-2202.

Melendez, A., Z. Talloczy, M. Seaman, E. L. Eskelinen, D. H. Hall and B. Levine (2003). "Autophagy genes are essential for dauer development and life-span extension in C. elegans." Science **301**(5638): 1387-1391.

Menzies, F. M., J. Huebener, M. Renna, M. Bonin, O. Riess and D. C. Rubinsztein (2010). "Autophagy induction reduces mutant ataxin-3 levels and toxicity in a mouse model of spinocerebellar ataxia type 3." Brain **133**(Pt 1): 93-104.

Menzies, F. M., K. Moreau, C. Puri, M. Renna and D. C. Rubinsztein (2012). "Measurement of autophagic activity in mammalian cells." Curr Protoc Cell Biol **Chapter 15**: Unit 15 16.

Menzies, F. M., B. Ravikumar and D. C. Rubinsztein (2006). "Protective roles for induction of autophagy in multiple proteinopathies." Autophagy **2**(3): 224-225.

Meusser, B., C. Hirsch, E. Jarosch and T. Sommer (2005). "ERAD: the long road to destruction." Nat Cell Biol **7**(8): 766-772.

Mizushima, N., T. Noda, T. Yoshimori, Y. Tanaka, T. Ishii, M. D. George, D. J. Klionsky, M. Ohsumi and Y. Ohsumi (1998). "A protein conjugation system essential for autophagy." Nature **395**(6700): 395-398.

Mizushima, N., H. Sugita, T. Yoshimori and Y. Ohsumi (1998). "A new protein conjugation system in human. The counterpart of the yeast Apg12p conjugation system essential for autophagy." J Biol Chem **273**(51): 33889-33892.

Mizushima, N., A. Yamamoto, M. Matsui, T. Yoshimori and Y. Ohsumi (2004). "In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker." Mol Biol Cell **15**(3): 1101-1111.

Mizushima, N. and T. Yoshimori (2007). "How to interpret LC3 immunoblotting." Autophagy **3**(6): 542-545.

Mizushima, N., T. Yoshimori and Y. Ohsumi (2002). "Mouse Apg10 as an Apg12-conjugating enzyme: analysis by the conjugation-mediated yeast two-hybrid method." FEBS Lett **532**(3): 450-454.

Montgomery, S. M., D. L. Morris, N. P. Thompson, J. Subhani, R. E. Pounder and A. J. Wakefield (1998). "Prevalence of inflammatory bowel disease in British 26 year olds: national longitudinal birth cohort." BMJ **316**(7137): 1058-1059.

Montie, H. L., M. S. Cho, L. Holder, Y. Liu, A. S. Tsvetkov, S. Finkbeiner and D. E. Merry (2009). "Cytoplasmic retention of polyglutamine-expanded androgen receptor ameliorates disease via

autophagy in a mouse model of spinal and bulbar muscular atrophy." Hum Mol Genet **18**(11): 1937-1950.

Muchowski, P. J., G. Schaffar, A. Sittler, E. E. Wanker, M. K. Hayer-Hartl and F. U. Hartl (2000). "Hsp70 and hsp40 chaperones can inhibit self-assembly of polyglutamine proteins into amyloid-like fibrils." Proc Natl Acad Sci U S A **97**(14): 7841-7846.

Munoz, E., M. J. Rey, M. Mila, A. Cardozo, T. Ribalta, E. Tolosa and I. Ferrer (2002). "Intranuclear inclusions, neuronal loss and CAG mosaicism in two patients with Machado-Joseph disease." J Neurol Sci **200**(1-2): 19-25.

Nakano, K. K., D. M. Dawson and A. Spence (1972). "Machado disease. A hereditary ataxia in Portuguese emigrants to Massachusetts." Neurology **22**(1): 49-55.

Nakatogawa, H., Y. Ichimura and Y. Ohsumi (2007). "Atg8, a ubiquitin-like protein required for autophagosome formation, mediates membrane tethering and hemifusion." Cell **130**(1): 165-178.

Nascimento-Ferreira, I., T. Santos-Ferreira, L. Sousa-Ferreira, G. Auregan, I. Onofre, S. Alves, N. Dufour, V. F. Colomer Gould, A. Koeppen, N. Deglon and L. Pereira de Almeida (2011). "Overexpression of the autophagic beclin-1 protein clears mutant ataxin-3 and alleviates Machado-Joseph disease." Brain **134**(Pt 5): 1400-1415.

Nicastro, G., R. P. Menon, L. Masino, P. P. Knowles, N. Q. McDonald and A. Pastore (2005). "The solution structure of the Josephin domain of ataxin-3: structural determinants for molecular recognition." Proc Natl Acad Sci U S A **102**(30): 10493-10498.

Nicastro, G., S. V. Todi, E. Karaca, A. M. Bonvin, H. L. Paulson and A. Pastore (2010). "Understanding the role of the Josephin domain in the PolyUb binding and cleavage properties of ataxin-3." PLoS One **5**(8): e12430.

Nixon, R. A., J. Wegiel, A. Kumar, W. H. Yu, C. Peterhoff, A. Cataldo and A. M. Cuervo (2005). "Extensive involvement of autophagy in Alzheimer disease: an immuno-electron microscopy study." J Neuropathol Exp Neurol **64**(2): 113-122.

Noda, T., N. Fujita and T. Yoshimori (2008). "The Ubi brothers reunited." Autophagy **4**(4): 540-541.

Oh-oka, K., H. Nakatogawa and Y. Ohsumi (2008). "Physiological pH and acidic phospholipids contribute to substrate specificity in lipidation of Atg8." J Biol Chem **283**(32): 21847-21852.

Orr, H. T. and H. Y. Zoghbi (2007). "Trinucleotide repeat disorders." Annu Rev Neurosci **30**: 575-621.

Oue, M., K. Mitsumura, T. Torashima, C. Koyama, H. Yamaguchi, N. Furuya and H. Hirai (2009). "Characterization of mutant mice that express polyglutamine in cerebellar Purkinje cells." Brain Res **1255**: 9-17.

Padiath, Q. S., A. K. Srivastava, S. Roy, S. Jain and S. K. Brahmachari (2005). "Identification of a novel 45 repeat unstable allele associated with a disease phenotype at the MJD1/SCA3 locus." Am J Med Genet B Neuropsychiatr Genet **133B**(1): 124-126.

Pankiv, S., T. H. Clausen, T. Lamark, A. Brech, J. A. Bruun, H. Outzen, A. Overvatn, G. Bjorkoy and T. Johansen (2007). "p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy." J Biol Chem **282**(33): 24131-24145.

Pattingre, S., A. Tassa, X. Qu, R. Garuti, X. H. Liang, N. Mizushima, M. Packer, M. D. Schneider and B. Levine (2005). "Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy." Cell **122**(6): 927-939.

Paulson, H. L. (2007). "Dominantly inherited ataxias: lessons learned from Machado-Joseph disease/spinocerebellar ataxia type 3." Semin Neurol **27**(2): 133-142.

Paulson, H. L., S. S. Das, P. B. Crino, M. K. Perez, S. C. Patel, D. Gotsdiner, K. H. Fischbeck and R. N. Pittman (1997). "Machado-Joseph disease gene product is a cytoplasmic protein widely expressed in brain." Ann Neurol **41**(4): 453-462.

Paulson, H. L., M. K. Perez, Y. Trottier, J. Q. Trojanowski, S. H. Subramony, S. S. Das, P. Vig, J. L. Mandel, K. H. Fischbeck and R. N. Pittman (1997). "Intranuclear inclusions of expanded polyglutamine protein in spinocerebellar ataxia type 3." Neuron **19**(2): 333-344.

Perry, C. N., S. Kyoi, N. Hariharan, H. Takagi, J. Sadoshima and R. A. Gottlieb (2009). "Novel methods for measuring cardiac autophagy in vivo." Methods Enzymol **453**: 325-342.

Petiot, A., E. Ogier-Denis, E. F. Blommaart, A. J. Meijer and P. Codogno (2000). "Distinct classes of phosphatidylinositol 3'-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells." J Biol Chem **275**(2): 992-998.

Pickford, F., E. Masliah, M. Britschgi, K. Lucin, R. Narasimhan, P. A. Jaeger, S. Small, B. Spencer, E. Rockenstein, B. Levine and T. Wyss-Coray (2008). "The autophagy-related protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid beta accumulation in mice." J Clin Invest **118**(6): 2190-2199.

Ranum, L. P., J. K. Lundgren, L. J. Schut, M. J. Ahrens, S. Perlman, J. Aita, T. D. Bird, C. Gomez and H. T. Orr (1995). "Spinocerebellar ataxia type 1 and Machado-Joseph disease: incidence of CAG

expansions among adult-onset ataxia patients from 311 families with dominant, recessive, or sporadic ataxia." Am J Hum Genet **57**(3): 603-608.

Ravikumar, B., A. Acevedo-Arozena, S. Imarisio, Z. Berger, C. Vacher, C. J. O'Kane, S. D. Brown and D. C. Rubinsztein (2005). "Dynein mutations impair autophagic clearance of aggregate-prone proteins." Nat Genet **37**(7): 771-776.

Ravikumar, B., R. Duden and D. C. Rubinsztein (2002). "Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy." Hum Mol Genet **11**(9): 1107-1117.

Ravikumar, B., K. Moreau, L. Jahreiss, C. Puri and D. C. Rubinsztein (2010). "Plasma membrane contributes to the formation of pre-autophagosomal structures." Nat Cell Biol **12**(8): 747-757.

Ravikumar, B., A. Stewart, H. Kita, K. Kato, R. Duden and D. C. Rubinsztein (2003). "Raised intracellular glucose concentrations reduce aggregation and cell death caused by mutant huntingtin exon 1 by decreasing mTOR phosphorylation and inducing autophagy." Hum Mol Genet **12**(9): 985-994.

Ravikumar, B., C. Vacher, Z. Berger, J. E. Davies, S. Luo, L. G. Oroz, F. Scaravilli, D. F. Easton, R. Duden, C. J. O'Kane and D. C. Rubinsztein (2004). "Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease." Nat Genet **36**(6): 585-595.

Ravikumar. B., S. Sarkar, J. E. Davies, M. Futter, M- Garcia-Arencibia, Z. W. Green-Thompson, M. Jimenez-Sanchez, V. I. Korolchuk, M. Lichtenberg, S. Luo, D. C. O. Massey, F. M. Menzies, K. Moreau, U. Narayanan, M. Renna, F. H. Siddiqi, Benjamin R. Underwood, Ashley R. Winslow, and D. C. Rubinsztein (2010). "Regulation of Mammalian Autophagy in Physiology and Pathophysiology" Physiol Rev 90: 1383–1435.

Reggiori, F., C. W. Wang, U. Nair, T. Shintani, H. Abeliovich and D. J. Klionsky (2004). "Early stages of the secretory pathway, but not endosomes, are required for Cvt vesicle and autophagosome assembly in Saccharomyces cerevisiae." Mol Biol Cell **15**(5): 2189-2204.

Riess, O., U. Rub, A. Pastore, P. Bauer and L. Schols (2008). "SCA3: neurological features, pathogenesis and animal models." Cerebellum **7**(2): 125-137.

Rodrigues, A. J., M. do Carmo Costa, T. L. Silva, D. Ferreira, F. Bajanca, E. Logarinho and P. Maciel (2010). "Absence of ataxin-3 leads to cytoskeletal disorganization and increased cell death." Biochim Biophys Acta **1803**(10): 1154-1163.

Rodrigues, A. J., A. Neves-Carvalho, A. Ferro, A. Rokka, G. Corthals, E. Logarinho and P. Maciel (2009). "ATX-3, CDC-48 and UBXN-5: a new trimolecular complex in Caenorhabditis elegans." Biochem Biophys Res Commun **386**(4): 575-581.

Rodrigues, A. J., A. Neves-Carvalho, A. Teixeira-Castro, A. Rokka, G. Corthals, E. Logarinho and P. Maciel (2011). "Absence of ataxin-3 leads to enhanced stress response in C. elegans." PLoS One **6**(4): e18512.

Roizin, L., S. Stellar, N. Willson, J. Whittier and J. C. Liu (1974). "Electron microscope and enzyme studies in cerebral biopsies of Huntington's chorea." Trans Am Neurol Assoc **99**: 240-243.

Romanul, F. C., H. L. Fowler, J. Radvany, R. G. Feldman and M. Feingold (1977). "Azorean disease of the nervous system." N Engl J Med **296**(26): 1505-1508.

Rose, C., F. M. Menzies, M. Renna, A. Acevedo-Arozena, S. Corrochano, O. Sadiq, S. D. Brown and D. C. Rubinsztein (2010). "Rilmenidine attenuates toxicity of polyglutamine expansions in a mouse model of Huntington's disease." Hum Mol Genet **19**(11): 2144-2153.

Rosenberg, R. N. (1992). "Machado-Joseph disease: an autosomal dominant motor system degeneration." Mov Disord **7**(3): 193-203.

Rosenberg, R. N., W. L. Nyhan, C. Bay and P. Shore (1976). "Autosomal dominant striatonigral degeneration. A clinical, pathologic, and biochemical study of a new genetic disorder." Neurology **26**(8): 703-714.

Rub, U., R. A. de Vos, E. R. Brunt, T. Sebesteny, L. Schols, G. Auburger, J. Bohl, E. Ghebremedhin, K. Gierga, K. Seidel, W. den Dunnen, H. Heinsen, H. Paulson and T. Deller (2006). "Spinocerebellar ataxia type 3 (SCA3): thalamic neurodegeneration occurs independently from thalamic ataxin-3 immunopositive neuronal intranuclear inclusions." Brain Pathol **16**(3): 218-227.

Rubinsztein, D. C. (2006). "The roles of intracellular protein-degradation pathways in neurodegeneration." Nature **443**(7113): 780-786.

Rubinsztein, D. C., A. M. Cuervo, B. Ravikumar, S. Sarkar, V. Korolchuk, S. Kaushik and D. J. Klionsky (2009). "In search of an "autophagomometer"." Autophagy **5**(5): 585-589.

Rusten, T. E., K. Lindmo, G. Juhasz, M. Sass, P. O. Seglen, A. Brech and H. Stenmark (2004). "Programmed autophagy in the Drosophila fat body is induced by ecdysone through regulation of the PI3K pathway." Dev Cell **7**(2): 179-192.

Rusten, T. E., T. Vaccari, K. Lindmo, L. M. Rodahl, I. P. Nezis, C. Sem-Jacobsen, F. Wendler, J. P. Vincent, A. Brech, D. Bilder and H. Stenmark (2007). "ESCRTs and Fab1 regulate distinct steps of autophagy." Curr Biol **17**(20): 1817-1825.

Sakai, T., M. Ohta and H. Ishino (1983). "Joseph disease in a non-Portuguese family." Neurology **33**(1): 74-80.

Sarbassov, D. D., S. M. Ali and D. M. Sabatini (2005). "Growing roles for the mTOR pathway." Curr Opin Cell Biol **17**(6): 596-603.

Sarkar, S., R. A. Floto, Z. Berger, S. Imarisio, A. Cordenier, M. Pasco, L. J. Cook and D. C. Rubinsztein (2005). "Lithium induces autophagy by inhibiting inositol monophosphatase." J Cell Biol **170**(7): 1101-1111.

Sarkar, S., G. Krishna, S. Imarisio, S. Saiki, C. J. O'Kane and D. C. Rubinsztein (2008). "A rational mechanism for combination treatment of Huntington's disease using lithium and rapamycin." Hum Mol Genet **17**(2): 170-178.

Sarkar, S., E. O. Perlstein, S. Imarisio, S. Pineau, A. Cordenier, R. L. Maglathlin, J. A. Webster, T. A. Lewis, C. J. O'Kane, S. L. Schreiber and D. C. Rubinsztein (2007). "Small molecules enhance autophagy and reduce toxicity in Huntington's disease models." Nat Chem Biol **3**(6): 331-338.

Sarkar, S. and D. C. Rubinsztein (2006). "Inositol and IP3 levels regulate autophagy: biology and therapeutic speculations." Autophagy **2**(2): 132-134.

Schaeffer, V., I. Lavenir, S. Ozcelik, M. Tolnay, D. T. Winkler and M. Goedert (2012). "Stimulation of autophagy reduces neurodegeneration in a mouse model of human tauopathy." Brain **135**(Pt 7): 2169-2177.

Schaffar, G., P. Breuer, R. Boteva, C. Behrends, N. Tzvetkov, N. Strippel, H. Sakahira, K. Siegers, M. Hayer-Hartl and F. U. Hartl (2004). "Cellular toxicity of polyglutamine expansion proteins: mechanism of transcription factor deactivation." Mol Cell **15**(1): 95-105.

Scherzed, W., E. R. Brunt, H. Heinsen, R. A. de Vos, K. Seidel, K. Burk, L. Schols, G. Auburger, D. Del Turco, T. Deller, H. W. Korf, W. F. den Dunnen and U. Rub (2012). "Pathoanatomy of Cerebellar Degeneration in Spinocerebellar Ataxia Type 2 (SCA2) and Type 3 (SCA3)." Cerebellum **11**(3): 749-760.

Schmidt, T., G. B. Landwehrmeyer, I. Schmitt, Y. Trottier, G. Auburger, F. Laccone, T. Klockgether, M. Volpel, J. T. Epplen, L. Schols and O. Riess (1998). "An isoform of ataxin-3 accumulates in the nucleus of neuronal cells in affected brain regions of SCA3 patients." Brain Pathol **8**(4): 669-679.

Schmidt, T., K. S. Lindenberg, A. Krebs, L. Schols, F. Laccone, J. Herms, M. Rechsteiner, O. Riess and G. B. Landwehrmeyer (2002). "Protein surveillance machinery in brains with spinocerebellar ataxia type 3: redistribution and differential recruitment of 26S proteasome subunits and chaperones to neuronal intranuclear inclusions." Ann Neurol **51**(3): 302-310.

Schols, L., G. Amoiridis, T. Buttner, H. Przuntek, J. T. Epplen and O. Riess (1997). "Autosomal dominant cerebellar ataxia: phenotypic differences in genetically defined subtypes?" Ann Neurol **42**(6): 924-932.

Schols, L., P. Bauer, T. Schmidt, T. Schulte and O. Riess (2004). "Autosomal dominant cerebellar ataxias: clinical features, genetics, and pathogenesis." Lancet Neurol **3**(5): 291-304.

Schols, L., J. Haan, O. Riess, G. Amoiridis and H. Przuntek (1998). "Sleep disturbance in spinocerebellar ataxias: is the SCA3 mutation a cause of restless legs syndrome?" Neurology **51**(6): 1603-1607.

Schols, L., A. M. Vieira-Saecker, S. Schols, H. Przuntek, J. T. Epplen and O. Riess (1995). "Trinucleotide expansion within the MJD1 gene presents clinically as spinocerebellar ataxia and occurs most frequently in German SCA patients." Hum Mol Genet **4**(6): 1001-1005.

Scott, R. C., O. Schuldiner and T. P. Neufeld (2004). "Role and regulation of starvation-induced autophagy in the Drosophila fat body." Dev Cell **7**(2): 167-178.

Seidel, K., S. Siswanto, E. R. Brunt, W. den Dunnen, H. W. Korf and U. Rub (2012). "Brain pathology of spinocerebellar ataxias." Acta Neuropathol **124**(1): 1-21.

Sengupta, S., T. R. Peterson and D. M. Sabatini (2010). "Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress." Mol Cell **40**(2): 310-322.

Sequeiros, J. and P. Coutinho (1993). "Epidemiology and clinical aspects of Machado-Joseph disease." Adv Neurol **61**: 139-153.

Shao, J. and M. I. Diamond (2007). "Polyglutamine diseases: emerging concepts in pathogenesis and therapy." Hum Mol Genet **16 Spec No. 2**: R115-123.

Simoes, A. T., N. Goncalves, A. Koeppen, N. Deglon, S. Kugler, C. B. Duarte and L. Pereira de Almeida (2012). "Calpastatin-mediated inhibition of calpains in the mouse brain prevents mutant ataxin 3 proteolysis, nuclear localization and aggregation, relieving Machado-Joseph disease." Brain **135**(Pt 8): 2428-2439.

Singh, R. and A. M. Cuervo (2011). "Autophagy in the cellular energetic balance." Cell Metab **13**(5): 495-504.

Spencer, B., R. Potkar, M. Trejo, E. Rockenstein, C. Patrick, R. Gindi, A. Adame, T. Wyss-Coray and E. Masliah (2009). "Beclin 1 gene transfer activates autophagy and ameliorates the neurodegenerative pathology in alpha-synuclein models of Parkinson's and Lewy body diseases." J Neurosci **29**(43): 13578-13588.

Stevanin, G., P. S. Sousa, G. Cancel, A. Durr, O. Dubourg, G. A. Nicholson, J. Weissenbach, E. Jardim, Y. Agid, E. Cassa and et al. (1994). "The gene for Machado-Joseph disease maps to the same 3-cM interval as the spinal cerebellar ataxia 3 gene on chromosome 14q." Neurobiol Dis **1**(1-2): 79-82.

Sridhar, S., Y. Botbol, F. Macian and A. M. Cuervo (2012). " Autophagy and disease: always two sides to a problem". J Pathol 226: 255–273.

Sudarsky, L. and P. Coutinho (1995). "Machado-Joseph disease." Clin Neurosci **3**(1): 17-22.

Sun, Q., W. Fan, K. Chen, X. Ding, S. Chen and Q. Zhong (2008). "Identification of Barkor as a mammalian autophagy-specific factor for Beclin 1 and class III phosphatidylinositol 3-kinase." Proc Natl Acad Sci U S A **105**(49): 19211-19216.

Tait, D., M. Riccio, A. Sittler, E. Scherzinger, S. Santi, A. Ognibene, N. M. Maraldi, H. Lehrach and E. E. Wanker (1998). "Ataxin-3 is transported into the nucleus and associates with the nuclear matrix." Hum Mol Genet **7**(6): 991-997.

Takiyama, Y., M. Nishizawa, H. Tanaka, S. Kawashima, H. Sakamoto, Y. Karube, H. Shimazaki, M. Soutome, K. Endo, S. Ohta and et al. (1993). "The gene for Machado-Joseph disease maps to human chromosome 14q." Nat Genet **4**(3): 300-304.

Tan, J. M., E. S. Wong, D. S. Kirkpatrick, O. Pletnikova, H. S. Ko, S. P. Tay, M. W. Ho, J. Troncoso, S. P. Gygi, M. K. Lee, V. L. Dawson, T. M. Dawson and K. L. Lim (2008). "Lysine 63-linked ubiquitination promotes the formation and autophagic clearance of protein inclusions associated with neurodegenerative diseases." Hum Mol Genet **17**(3): 431-439.

Tanida, I., E. Tanida-Miyake, T. Ueno and E. Kominami (2001). "The human homolog of Saccharomyces cerevisiae Apg7p is a Protein-activating enzyme for multiple substrates including human Apg12p, GATE-16, GABARAP, and MAP-LC3." J Biol Chem **276**(3): 1701-1706.

Taroni, F. and S. DiDonato (2004). "Pathways to motor incoordination: the inherited ataxias." Nat Rev Neurosci **5**(8): 641-655.

Thompson, L. M., C. T. Aiken, L. S. Kaltenbach, N. Agrawal, K. Illes, A. Khoshnan, M. Martinez-Vincente, M. Arrasate, J. G. O'Rourke, H. Khashwji, T. Lukacsovich, Y. Z. Zhu, A. L. Lau, A. Massey, M. R. Hayden, S. O. Zeitlin, S. Finkbeiner, K. N. Green, F. M. LaFerla, G. Bates, L. Huang, P. H. Patterson, D. C. Lo, A. M. Cuervo, J. L. Marsh and J. S. Steffan (2009). "IKK phosphorylates Huntingtin and targets it for degradation by the proteasome and lysosome." J Cell Biol **187**(7): 1083-1099.

Thoreen, C. C., S. A. Kang, J. W. Chang, Q. Liu, J. Zhang, Y. Gao, L. J. Reichling, T. Sim, D. M. Sabatini and N. S. Gray (2009). "An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1." J Biol Chem **284**(12): 8023-8032.

Tian, Y., V. Bustos, M. Flajolet and P. Greengard (2011). "A small-molecule enhancer of autophagy decreases levels of Abeta and APP-CTF via Atg5-dependent autophagy pathway." FASEB J **25**(6): 1934-1942.

Tooze, S. A. and T. Yoshimori (2010). "The origin of the autophagosomal membrane." Nat Cell Biol **12**(9): 831-835.

Torashima, T., C. Koyama, A. Iizuka, K. Mitsumura, K. Takayama, S. Yanagi, M. Oue, H. Yamaguchi and H. Hirai (2008). "Lentivector-mediated rescue from cerebellar ataxia in a mouse model of spinocerebellar ataxia." EMBO Rep **9**(4): 393-399.

Trottier, Y., G. Cancel, I. An-Gourfinkel, Y. Lutz, C. Weber, A. Brice, E. Hirsch and J. L. Mandel (1998). "Heterogeneous intracellular localization and expression of ataxin-3." Neurobiol Dis **5**(5): 335-347.

Tsuji, S. (1997). "Molecular genetics of triplet repeats: unstable expansion of triplet repeats as a new mechanism for neurodegenerative diseases." Intern Med **36**(1): 3-8.

Twist, E. C., L. K. Casaubon, M. H. Ruttledge, V. S. Rao, P. M. Macleod, J. Radvany, Z. Zhao, R. N. Rosenberg, L. A. Farrer and G. A. Rouleau (1995). "Machado Joseph disease maps to the same region of chromosome 14 as the spinocerebellar ataxia type 3 locus." J Med Genet **32**(1): 25-31.

Tzvetkov, N. and P. Breuer (2007). "Josephin domain-containing proteins from a variety of species are active de-ubiquitination enzymes." Biol Chem **388**(9): 973-978.

Vale, J., P. Bugalho, I. Silveira, J. Sequeiros, J. Guimaraes and P. Coutinho (2010). "Autosomal dominant cerebellar ataxia: frequency analysis and clinical characterization of 45 families from Portugal." Eur J Neurol **17**(1): 124-128.

van de Warrenburg, B. P., R. J. Sinke, C. C. Verschuuren-Bemelmans, H. Scheffer, E. R. Brunt, P. F. Ippel, J. A. Maat-Kievit, D. Dooijes, N. C. Notermans, D. Lindhout, N. V. Knoers and H. P. Kremer (2002). "Spinocerebellar ataxias in the Netherlands: prevalence and age at onset variance analysis." Neurology **58**(5): 702-708.

van der Vaart, A., J. Griffith and F. Reggiori (2010). "Exit from the Golgi is required for the expansion of the autophagosomal phagophore in yeast Saccharomyces cerevisiae." Mol Biol Cell **21**(13): 2270-2284.

Venkatraman, P., R. Wetzel, M. Tanaka, N. Nukina and A. L. Goldberg (2004). "Eukaryotic proteasomes cannot digest polyglutamine sequences and release them during degradation of polyglutamine-containing proteins." Mol Cell **14**(1): 95-104.

Volinia, S., R. Dhand, B. Vanhaesebroeck, L. K. MacDougall, R. Stein, M. J. Zvelebil, J. Domin, C. Panaretou and M. D. Waterfield (1995). "A human phosphatidylinositol 3-kinase complex related to the yeast Vps34p-Vps15p protein sorting system." EMBO J **14**(14): 3339-3348.

Wang, C. W. and D. J. Klionsky (2003). "The molecular mechanism of autophagy." Mol Med **9**(3-4): 65-76.

Wang, G., K. Ide, N. Nukina, J. Goto, Y. Ichikawa, K. Uchida, T. Sakamoto and I. Kanazawa (1997). "Machado-Joseph disease gene product identified in lymphocytes and brain." Biochem Biophys Res Commun **233**(2): 476-479.

Wang, H., N. Jia, E. Fei, Z. Wang, C. Liu, T. Zhang, J. Fan, M. Wu, L. Chen, N. Nukina, J. Zhou and G. Wang (2007). "p45, an ATPase subunit of the 19S proteasome, targets the polyglutamine disease protein ataxin-3 to the proteasome." J Neurochem **101**(6): 1651-1661.

Wang, Q., L. Li and Y. Ye (2006). "Regulation of retrotranslocation by p97-associated deubiquitinating enzyme ataxin-3." J Cell Biol **174**(7): 963-971.

Warrick, J. M., H. Y. Chan, G. L. Gray-Board, Y. Chai, H. L. Paulson and N. M. Bonini (1999). "Suppression of polyglutamine-mediated neurodegeneration in Drosophila by the molecular chaperone HSP70." Nat Genet **23**(4): 425-428.

Webb, J. L., B. Ravikumar, J. Atkins, J. N. Skepper and D. C. Rubinsztein (2003). "Alpha-Synuclein is degraded by both autophagy and the proteasome." J Biol Chem **278**(27): 25009-25013.

Weidberg, H., T. Shpilka, E. Shvets, A. Abada, F. Shimron and Z. Elazar (2011). "LC3 and GATE-16 N termini mediate membrane fusion processes required for autophagosome biogenesis." Dev Cell **20**(4): 444-454.

Wilburn, B., D. D. Rudnicki, J. Zhao, T. M. Weitz, Y. Cheng, X. Gu, E. Greiner, C. S. Park, N. Wang, B. L. Sopher, A. R. La Spada, A. Osmand, R. L. Margolis, Y. E. Sun and X. W. Yang (2011). "An antisense CAG repeat transcript at JPH3 locus mediates expanded polyglutamine protein toxicity in Huntington's disease-like 2 mice." Neuron **70**(3): 427-440.

Williams, A., S. Sarkar, P. Cuddon, E. K. Ttofi, S. Saiki, F. H. Siddiqi, L. Jahreiss, A. Fleming, D. Pask, P. Goldsmith, C. J. O'Kane, R. A. Floto and D. C. Rubinsztein (2008). "Novel targets for Huntington's disease in an mTOR-independent autophagy pathway." Nat Chem Biol **4**(5): 295-305.

Williams, R. S., L. Cheng, A. W. Mudge and A. J. Harwood (2002). "A common mechanism of action for three mood-stabilizing drugs." Nature **417**(6886): 292-295.

Wong, E. and A. M. Cuervo (2010). "Autophagy gone awry in neurodegenerative diseases." Nat Neurosci **13**(7): 805-811.

Wong, E. and A. M. Cuervo (2010). "Integration of clearance mechanisms: the proteasome and autophagy." Cold Spring Harb Perspect Biol **2**(12): a006734.

Woods, B. T. and H. H. Schaumburg (1972). "Nigro-spino-dentatal degeneration with nuclear ophthalmoplegia. A unique and partially treatable clinico-pathological entity." J Neurol Sci **17**(2): 149-166.

Wullschleger, S., R. Loewith and M. N. Hall (2006). "TOR signaling in growth and metabolism." Cell **124**(3): 471-484.

Xiong, N., M. Jia, C. Chen, J. Xiong, Z. Zhang, J. Huang, L. Hou, H. Yang, X. Cao, Z. Liang, S. Sun, Z. Lin and T. Wang (2011). "Potential autophagy enhancers attenuate rotenone-induced toxicity in SH-SY5Y." Neuroscience **199**: 292-302.

Yamada, M., T. Sato, S. Tsuji and H. Takahashi (2008). "CAG repeat disorder models and human neuropathology: similarities and differences." Acta Neuropathol **115**(1): 71-86.

Yamamoto, A., Y. Tagawa, T. Yoshimori, Y. Moriyama, R. Masaki and Y. Tashiro (1998). "Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells." Cell Struct Funct **23**(1): 33-42.

Yang, W., J. R. Dunlap, R. B. Andrews and R. Wetzel (2002). "Aggregated polyglutamine peptides delivered to nuclei are toxic to mammalian cells." Hum Mol Genet **11**(23): 2905-2917.

Yang, Z. and D. J. Klionsky (2009). "An overview of the molecular mechanism of autophagy." Curr Top Microbiol Immunol **335**: 1-32.

Yla-Anttila, P., H. Vihinen, E. Jokitalo and E. L. Eskelinen (2009). "3D tomography reveals connections between the phagophore and endoplasmic reticulum." Autophagy **5**(8): 1180-1185.

Yoshimoto, K., H. Hanaoka, S. Sato, T. Kato, S. Tabata, T. Noda and Y. Ohsumi (2004). "Processing of ATG8s, ubiquitin-like proteins, and their deconjugation by ATG4s are essential for plant autophagy." Plant Cell **16**(11): 2967-2983.

Zhang, H., M. Bosch-Marce, L. A. Shimoda, Y. S. Tan, J. H. Baek, J. B. Wesley, F. J. Gonzalez and G. L. Semenza (2008). "Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia." J Biol Chem **283**(16): 10892-10903.

Zhang, L., J. Yu, H. Pan, P. Hu, Y. Hao, W. Cai, H. Zhu, A. D. Yu, X. Xie, D. Ma and J. Yuan (2007). "Small molecule regulators of autophagy identified by an image-based high-throughput screen." Proc Natl Acad Sci U S A **104**(48): 19023-19028.

Zhang, X., L. Li, S. Chen, D. Yang, Y. Wang, Z. Wang and W. Le (2011). "Rapamycin treatment augments motor neuron degeneration in SOD1(G93A) mouse model of amyotrophic lateral sclerosis." Autophagy **7**(4): 412-425.

Zhong, X. and R. N. Pittman (2006). "Ataxin-3 binds VCP/p97 and regulates retrotranslocation of ERAD substrates." Hum Mol Genet **15**(16): 2409-2420.

Zoghbi, H. Y. (2000). "Spinocerebellar ataxias." Neurobiol Dis **7**(5): 523-527.

Zoghbi, H. Y. and H. T. Orr (2000). "Glutamine repeats and neurodegeneration." Annu Rev Neurosci **23**: 217-247.

Zois, C. E., A. Giatromanolaki, E. Sivridis, M. Papaiakovou, H. Kainulainen and M. I. Koukourakis (2011). ""Autophagic flux" in normal mouse tissues: focus on endogenous LC3A processing." Autophagy **7**(11): 1371-1378.

Zu, T., L. A. Duvick, M. D. Kaytor, M. S. Berlinger, H. Y. Zoghbi, H. B. Clark and H. T. Orr (2004). "Recovery from polyglutamine-induced neurodegeneration in conditional SCA1 transgenic mice." J Neurosci **24**(40): 8853-8861.