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ROLE OF ALPHA-SYNUCLEIN IN NEURODEGENERATION

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Para a Susana

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Interplay between α-synuclein and ataxin-3

Abbreviations

18F-DOPA	6-[18F]fluoro L-DOPA
6-OHDA	6-hydroxydopamine
AD	Alzheimer's disease
ANOVA	Analysis of variance
α-syn	α-synuclein
Atx3	Ataxin-3
BSA	Bovine serum albumin
CAG	Cytosine-adenine-guanine
CBD	Corticobasal degeneration
DAT	Dopamine transporter
DCF	Dichlorodihydrofluorescein
DLB	Dementia with Lewy bodies
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DOX	Doxyclicine
DRPLA	Dentatorubral-pallidoluysian atrophy
DUB	Deubiquitinating enzyme
ECF	Enhanced chemifluorescence
ECL	Enhanced chemiluminescence
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
GSH	Glutathione
HD	Huntington's disease
HSP70	Heat-shock protein 70
K	Lysine
LRRK2	Leucine-rich repeat kinase 2
MJD	Machado-Joseph disease
MSA	Multiple system atrophy
NAC	Non-amyloid beta-component
PAGE	Polyacrilamide gel electrophoresis

PBS	Phosphate buffered solution
PET	Positron emission tomography
PFA	Paraformaldehyde
PINK1	PTEN-induced putative kinase 1
PD	Parkinson’s Disease
polyQ	Polyglutamine
PSP	Progressive Supranuclear Palsy
REM	Rapid eye movement
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute’s medium
SCA	Spinocerebellar ataxia
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SPECT	Single-photon emission computed tomography
Ser	Serine
SNpc	Substantia nigra pars compacta
SNCA	α-synuclein
TH	Tyrosine hydroxylase
Ub	Ubiquitin
UCHL1	Ubiquitin C-terminal hydrolase 1
UIM	Ubiquitin-interacting motif
UPS	Ubiquitin-proteasome system
VCP/p97	Valosin-containing protein/protein 97

Resumo

A doença de Parkinson e a doença de Machado-Joseph são duas doenças neurodegenerativas essencialmente motoras, caracterizadas histologicamente por agregados proteicos. A doença de Machado-Joseph é uma doença hereditária, associada a uma mutação no gene ATXN3 que codifica a proteína ataxina-3. A doença de Parkinson é uma doença com inúmeros factores etiológicos propostos, incluindo mutações em 14 *loci* diferentes. Um destes *loci* codifica a proteína α-synucleína. Ainda que separadas geneticamente, a doença de Parkinson e a doença de Machado-Joseph, tal como outras doenças neurodegenerativas, partilham mecanismos neuropatológicos. Curiosamente, a sintomatologia caracteristicamente atribuída à doença de Parkinson, tremor de repouso, rigidez e bradicinesia, pode ser o modo de apresentação de numerosas doenças neurológicas, incluindo a de doentes com Machado-Joseph.

Nesta tese, revemos sucintamente os mecanismos que contribuem para a regulação da homeostase proteica dentro da célula, dando especial ênfase às proteínas associadas com estas duas doenças. Adicionalmente, discutimos a desregulação destas vias e dos mecanismos patológicos que levam à doença de Parkinson e à doença de Machado-Joseph, enquanto revemos a sua apresentação clínica e possível correlação patológica.

Finalmente, tendo em conta as evidências que propõem a interacção entre proteínas associadas a diferentes doenças neurodegenerativas, exploramos a associação entre a ataxina-3 e a α-synucleína, com o objectivo de compreender se a sua interacção pode contribuir para a patogénese destas duas doenças. Os nossos resultados evidenciam um papel tanto da ataxina-3 mutante como da sobre-expressão da α-sinucleína no aumento dos níveis de stress oxidativo celular. Adicionalmente, a ataxina-3 mutante parece aumentar os níveis intracelulares de α-sinucleína. Por outro lado, os resultados sugerem um papel protector para a ataxina-3 wild-type. O aumento dos níveis da

α-sinucleína induzidos pela ataxina-3 expandida e subsequente aumento do stress oxidativo celular, evidenciam um possível papel da α-sinucleína na patogénese da doença de Machado-Joseph.

Abstract

Conformational diseases are a broad group of disorders histologically characterized by proteinaceous aggregates that include Parkinson's disease and Machado-Joseph disease, two motor neurodegenerative diseases.

Machado-Joseph disease is a fatal, inherited neurodegerative disease, linked to a mutation in ATXN3 gene encoding for ataxin-3. Parkinson's disease, on its turn, has been extensively associated with numerous etiological factors, including mutations in 14 loci. One of these *loci* encodes α-synuclein. Although genetically separated, common neuropathological mechanisms are proposed to underlie the pathogenesis of both Parkinson's disease and Machado-Joseph disease. Curiously, the traditional clinical presentation of Parkinson's disease patients, resting tremor, rigidity and bradykinesia, can be the unique features displayed by Machado-Joseph patients.

In this thesis, we make a short review of the mechanisms contributing for proteostasis within the cell and discuss how impairment of these pathways may lead to disease pathogenesis. Moreover, taking into special consideration the proteins associated to both Machado-Joseph disease and Parkinson's disease, we expose the clinical presentation of these diseases and associated neuropathology.

Furthermore, increasing evidence support interactions between proteins associated to different neurodegenerative disorders. Hence, we studied the association between ataxin-3 and α-synuclein, aiming to understand whether this interaction could contribute for disease pathogenesis. Our results show that both the expanded ataxin-3 and overexpression of α-synuclein are associated with increased levels of oxidative stress within the cell. Moreover, the expanded ataxin-3 seems to increase α-synuclein levels. On the other hand, our data suggest a protective role for wild-type ataxin-3. The increased levels of α-synuclein induced by expanded ataxin-3, and consequent increased oxidative stress, evidence a possible role for α-synuclein in Machado-Joseph disease pathogenesis.

CHAPTER I - Proteostasis in Parkinson's disease and Machado-Joseph disease

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1. Introduction

Several diseases affecting the central nervous system are histologically linked by the common feature of protein inclusions - illustrious examples include Alzheimer's disease (AD), Huntington's disease (HD) and Parkinson's disease (PD)[1,2,3] (Table 1).

Such conformational diseases arise from pathologic changes in protein size or shape, which ultimately lead to protein aggregation and deposition in different locations within the cell, with consequent cellular dysfunction. These diseases typically present histologically with the hallmarks of intra- or extracellular aggregates[4,5,6] (Figure 1). The exact correlation between protein aggregation and neurodegeneration, however, is still not completely understood[7,4,8]. Even though the same basic mechanism, protein aggregation, is proposed to be the pathogenic basis of this group of diseases, the clinical features characteristic of each disease indicate that there might be disease specific mechanisms.

Thoroughly agreed is the crucial need of proteostasis for basic cellular functioning. The existence of mechanisms that deal with protein and organelle that need to be cleared up in eukaryotic cells, such as the ubiquitin-proteasome system (UPS) and the autophagy pathways, have a major impact on cell survival and organ function[9,10]. These catabolic systems play a fundamental role in neuronal development, including synaptogenesis and synaptic plasticity, as well as in neuronal homeostasis

and disease[11]. Moreover, abnormalities in both the UPS and autophagy have been associated with several diseases[9,12,13,14,15]. Interestingly, even though the changes in a particular protein are present since early in life, as pointed out by inherited diseases such as HD, its intracellular accumulation may have to reach a defined threshold for clinical manifestation, which are only detected later in life. Although a functional decay of the surveillance mechanisms with age could explain the late onset of these diseases, further insults, such as oxidative stress and mitochondrial dysfunction, appear to be indispensable contributors to disease progression[16].

Table 1. Protein aggregation and Neurodegenerative disease.

Toxic protein	Protein deposit	Gene mutated	Disease
α-synuclein	Lewy bodies Lewy neurites	SNCA	PD, DLB
	Glial cystoplasmic inclusions		MSA
Tau	Neuronal and glial inclusions	MAPT	AD, CBD, PSP
Polyglutamine repeat expansions	Nuclear and cytoplamic inclusions	HD DRPLA ATXN1 ATXN2 ATXN3	HD DPRLA SCA1 SCA2 SCA3 (MJD)
ß-amyloid	Senile plaques	APP PS1 PS2	AD

Various neurodegenerative disorders are characterized by the common hallmark of protein deposition. Notice that the same protein may be associated with distinct diseases and that one disease may as well have more than one protein contributing to its neuropathology. Parkinson's disease (PD), Dementia with Lewy bodies (DLB), Multiple System Atrophy (MSA), Huntington's disease (HD), Dentatorubral-pallidoluysian atrophy (DPRLA), Spinocerebellar ataxia type 1 (SCA1) and type 2 (SCA2), Machado-Joseph disease (MJD), Alzheimer's disease (AD) (adapted from Forman et al. 2004[6]).

Neurodegenerative diseases are a vast and heterogeneous group. The traditional view of these diseases, with isolated mechanisms and clinical manifestations, is now being questioned. Many proteins initially correlated to a clinical entity have now been shown to interact with proteins

associated with another disease[17,18,19]. Interestingly, PD is questionable as a single clinical entity[20, 21]. Its clinical heterogeneity, age of onset and progression rate, in addition to the arduous differential diagnosis from the parkinsonian syndromes, is an example of the immensity of cellular pathways, stimulus and protein-protein interactions that may give rise to a certain disease phenotype. Moreover, many proteins have been indicated as having a role in PD's pathogenesis and parkinsonian syndromes[22,23,24,25,26,27,28].

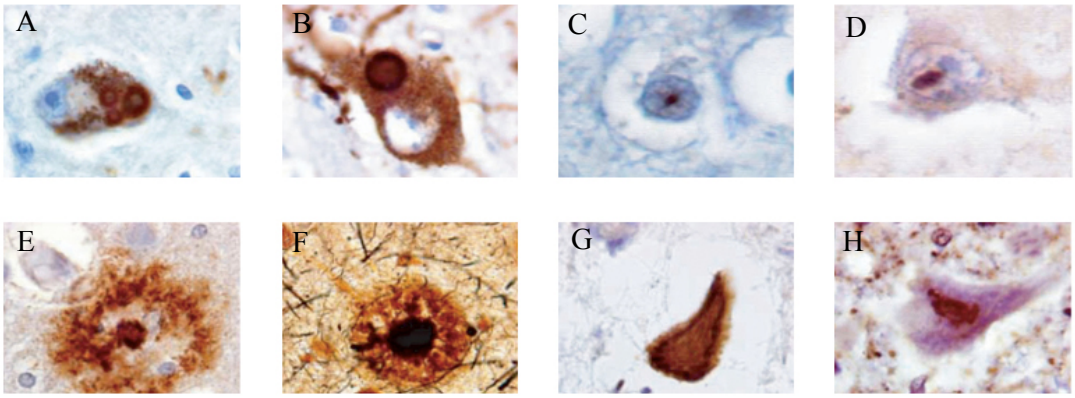

Figure 1. Characteristic histopathological lesions of neurodegenerative diseases. Protein deposition differs among the different diseases. In HD the inclusions are typically intranuclear (labeled for ubiquitin **(C)** and huntingtin **(D)**), while in AD the neuritic plaques are extracellular deposits (labeled for Aß protein **(E)** and silver stained **(F)**). In PD (labeled for α-synuclein **(A)** and phosphorilated α-synuclein **(B)**) and ALS (labeled for neurofilament **(G)** and ubiquitin **(H)**) the deposits are cytoplasmic (Adapted from Ross and Poirier 2004[1]).

In this review, we focus on the pathogenesis of PD and parkinsonian syndromes, taking into consideration the role and interactions of α-synuclein - a protein implicated not only in PD, but also in other synucleinopathies. Moreover, we describe the pathogenesis of Machado-Joseph disease (MJD), an hereditary neurodegenerative disorder caused by a mutation in ataxin-3, since some patients with this disease exhibit a parkinsonian phenotype[29,30,31].

2. Pathways that clear aggregate-prone proteins

Misfolded proteins are harmful to the cell. When misfolded, the normal function of a protein is altered, acquiring different biochemical properties, namely a greater tendency to aggregate through changes in solubility. Thus, protein degradation is crucial for cellular well-functioning, and when deregulated, can lead to a wide range of diseases, the so called conformational diseases[4,7,32].

The UPS and autophagy pathways are the two main routes of protein and organelle clearance in eukaryotic cells, allowing a dynamic state between synthesis and degradation. Moreover, UPS and autophagy have been associated and are thought to have a synergistic role in neuroprotection[33]. Here, we make a short review of these pathways.

2.1. The Ubiquitin- Proteasome System

Proteasomes are multiprotein complexes that typically degrade short-lived nuclear and cytosolic proteins, as well as abnormal proteins that need to be eliminated from cells[34]. Misfolded proteins in the endoplasmic reticulum may also be retrotranslocated back to the cytosol to be degraded by the proteasome[35].

The UPS seems to have an important role in regulating various cellular processes, including cell growth and proliferation, apoptosis, protein quality control, DNA repair and transcription, as well as the immune and inflammatory response[36].

The majority of the proteins are targeted for proteasomal degradation after modification with ubiquitin, which tags the protein as an UPS substrate. This conjugation typically involves three types of enzymes, namely E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase), in three successive steps (Figure 2).

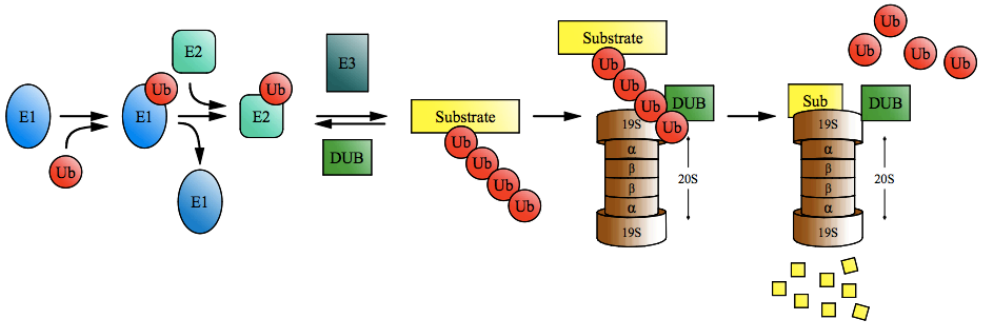

Figure 2. The Ubiquitin-Proteasome system. Ubiquitin (Ub) is activated and conjugate to the target substrate through the sequential actions of E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin-ligase enzyme). The deubiquitinating enzymes (DUB) oppose E3 activity (adapted from Eldridge and O'Brien 2010[36]).

First, an E1 uses ATP to activate ubiquitin by creating a thioester-linked conjugate between itself and ubiquitin. Then, one of the several E2s receives ubiquitin from E1 and forms a similar thioester intermediate. Finally, E3 binds both E2 and the substrate, and catalyzes the last step by transferring the ubiquitin to the substrate with a covalent attachment. Importantly, E3 enzymes are classified in different classes, each with numerous enzymes, according to the mechanism that catalyzes ubiquitin transfer. Therefore, a singe E1 enzyme first activates ubiquitin and transfers it to one of the multiple existing E2 enzymes. Most E2 enzymes, in turn, interact with several different E3 enzymes, which generally are found to interact with various substrates with similar recognition motifs. Moreover, even though a crucial step in targeting of a protein to the proteasome consists in specific binding of the protein to the appropriate E3, some substrates can be targeted by more than one E3. As a consequence, a complex and well-regulated chain of events leads to the targeting of different proteins to the proteasome by the ubiquitin system[37].

The sequential addition of activated ubiquitin moieties through isopeptide bonds on the previously conjugated residue of the ubiquitin molecule, which serves as a substrate, creates a polyubiquitin

chain. Ubiquitin has several residues that can be ubiquitinated including the methionine in position 1 (M1) and the lysines in positions 11(K11), 48 (K48) and 63 (K63)[38]. However, only chains of four or more ubiquitin molecules linked by K48 residues are uniformly agreed to form a recognition signal that allows substrates to be shuttled to the proteasome. In contrast, monoubiquitinated proteins or polyubiquitinated at K63, K11 or M1 residues may not be transported to the proteasome, assumedly being associated with DNA repair, cell cycle regulation and/or inflammation[38]. Recent reports suggest that the residue through each the polyubiquitin chain is linked may not have a specific and exclusive role within the cell. For instance, cyclin B1 can be targeted from proteasomal degradation through a polyubiquitinated chain linked by residues K48, K63 and K11, rather than K48-linked chains alone[39].

The proteasome (also known as 26S proteasome) is a 2.5 MDa, large multicatalytic protease complex, highly conserved among all eukaryotes and composed of two subcomplexes: a 20S core particle (CP), with the proteasomal catalytic activity, and a 19S regulatory particle (RP) (Figure 3).

The 20S CP gives the barrel-shape to the proteasome and is made up of four rings, each with seven subunits. Only the two inner rings of the 20S CP have proteolytic activity[40]. The 19S RP recognizes the ubiquitinated proteins and other putative substrates of the proteasome, and is attached to the outer rings of the 20S CP, to form the 26S proteasome[4,41]. Classically, a polyubiquitin chain of at least four-ubiquitin residues linked at K48 efficiently binds the proteasome, through recognition of its structure[42].

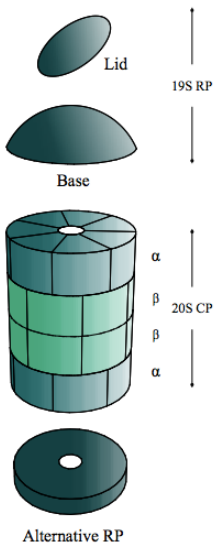

Figure 3. Structure of the proteasome. The proteasome is composed of one or two regulatory particles (RP) attached to the core particle (CP). The CP is composed of four ring, two α-rings and two ß-rings, and contains the proteolytic activity. The 19S RP is composed of two eight-subunit complexes, a lid and a base. Numerous chaperone proteins, ubiquitinating and deubiquitinating enzymes can interact with the RP (Adapted from Glickman and Chiechanover 2002[43]).

Each substrate of the proteasome enters and is degraded within the proteasome in a process called proteolysis. During proteolysis, the cleavage of the peptide bonds by proteases releases free amino acids and small peptides, as well as the energy invested previously in the synthesis of the bond. In most cases, small peptides between 3 and 23 amino acids are the result of the proteolytic cleavage and the pattern of peptides derived from a determined protein is stable over time. Additionally, these short-term peptide products do not accumulate in the cell as they are rapidly cleaved by proteases or aminopeptidases[43].

Interestingly, not all proteasomal substrates are completely cleaved to monomers and small peptides. Proteasomal degradation can result in a truncated form of a certain substrate, as it might, as well, merely transform the conformation of unfolded proteins and accelerate their refolding[44]. Therefore, proteasome activity is required not only to degrade abnormal tagged proteins, but also to alter the cellular activities or behavior of a certain protein, by changing its size or conformation. As a consequence, aggregation of this otherwise misfolded proteins is disabled.

Proteasomal degradation, as well as the folding of misfolded proteins is crucial for cellular integrity, thus its regulation must be delicately adjusted. Firstly, control of the proteasomal activity is mainly mediated by regulatory proteins - activators and inhibitors of proteasomal-mediated proteolysis. Hence, cells may recruit or inhibit the proteasome according to their needs by altering levels of these proteins. Additionally, deubiquitinating enzymes (DUBs), which counteract the activity of the three ubiquitin pathway enzymes (E1, E2 and E3), rescue substrate proteins from proteasomal degradation. Cleavage of the proteasomal degradation recycles ubiquitin - whose transcription is done at relatively low levels - and controls protein function or localization within the cell[43].

2.1.1. Ataxin-3: a Deubiquitinating enzyme (DUB)

There are now approximately 100 human DUBs identified[45]. These proteases belong to five distinct subclasses: Ub C-terminal hydrolases (UCHs), Ub-specific proteases (USPs), Machado-Joseph disease protein domain proteases (MJDs), ovarian tumor proteases (OTUs) and JAMM motif (zinc metalloproteases)[45]. While the importance of DUBs is well documented, the understanding of DUB substrate specificity and contribution to regulation of the UPS is comparatively poorly understood[45].

Ataxin-3 is a DUB protein, which was first described in 1994 by Kawaguchi and collaborators, but still remains a puzzling protein. Ataxin-3 is encoded by the MJD-associated gene on the long arm of chromosome 14 (14q24.3-32.1), ATXN3[46,47]. The translated protein has a molecular weight of approximately 42 kDa in healthy individuals[48].

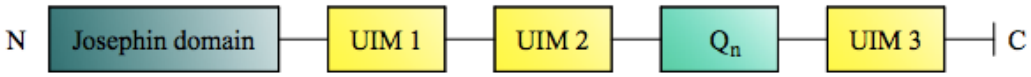

Figure 4. Structure of ataxin-3 (adapted from Matos et al. 2011[57]).

Ataxin-3 is composed of an N-terminal domain, the Josephin domain, and a flexible C-terminal domain, containing a changeable tract of polyglutamines (polyQ)[49]. The Josephin domain grants Ataxin-3 its ubiquitin protease activity[49]. The C-terminal domain comprises either two or three ubiquitin-interacting motifs (UIM) and the polyQ tract (Figure 4). Interestingly, the ubiquitin-binding sites in the Josephin domain have low affinity for ubiquitin but were found indispensable for binding and correct positioning the polyubiquitin chains to posterior proteolytic cleavage[50]. Thus, a proposed mechanism shows cooperation between the UIMs and the ubiquitin-binding sites of the Josephin domain, as UIM recruit and bind the polyubiquitin chains and then ubiquitin-binding sites adjust the chains' position, allowing its cleavage[50].

The polyQ tract has a variable length reflecting the number of CAG repeats in the ATXN3 allele. Similarly to other polyQ diseases, the phenotype of Machado Joseph's disease is expressed once the ATXN3 gene includes over 54 CAG repeats, usually 61-84 repeats[48,51].

Many human ataxin-3 isoforms have been described, derived from alternative splicing of the ATXN3 gene[52]. From the 20 expected isoforms[53], the most common isoform expressed in the human brain presents an additional UIM in the C-terminal region[54]. However, the biological relevance of such isoforms is still unknown[54].

Ataxin-3 is ubiquitously expressed[55,52] and throughout the brain, different regions apparently present distinct expression levels[52]. Furthermore, expression of ataxin-3 has been detected in both the nucleus and cytoplasm of neuronal cells, though nuclear shuttling of ataxin-3 seems to be tightly controlled. Concordantly, it seems that nuclear localization of ataxin-3 displays a particular role, potentially associated with heat stress response[56].

The precise physiological role of most DUBs remains unknown. Taking into account the variety of ubiquitination signals, arising from the great variety in ubiquitin arrangements, one may assume that a certain DUB recognizes only some types of ubiquitination signals[57]. Moreover, ubiquitination regulates a multiplicity of cellular mechanisms additionally to protein degradation, including DNA

repair, chromatin remodeling, cell cycle, subcellular localization and pathway signaling[58]. Thus, different DUBs could be responsible for distinct tasks concerning cellular homeostasis within the cell.

In the specific case of ataxin-3, it is able to bind polyubiquitin chain through its UIMs, interacting with both K48 and K63-linked ubiquitin chains[51]. Increased amounts and accumulation of ubiquitinated proteins were found to result from inhibition of its catalytic activity or lower levels of this enzyme[59,60]. Moreover, concordant levels of accumulated proteins were achieved in knock-out mice for ataxin-3[61]. This supports the idea that ataxin-3 targets proteins for proteosomal degradation. Moreover, ataxin-3 typically binds to ubiquitin chains with a minimum of four ubiquitin monomers, including ubiquitin chains linked by residue K48, which constitute the signal for proteasome targeting[61,62].

Although much is still left to understand about ataxin-3 function, the protein may exert its role by modifying the ubiquitin framework linked to a certain substrate, consequently altering its role within the cell[62]. Moreover, some data suggested that ataxin-3 cleaves mostly K63-linked ubiquitin chains, or chains of mixed K48 and K63 linkage, thus eliciting ataxin-3 activity as regulator of polyubiquitin chain framework[62]. Isolation of the Josephin domain of ataxin-3 showed that its association with K48-linked ubiquitin chains is stronger and cleavage more efficient than with K63-linked chains, which contrasts with the affinity of the full-length protein[50,62]. Ataxin-3 is thus more likely to edit the polyubiquitin chain attached to a substrate, rather than disassemble ubiquitin chain in order to release free ubiquitin monomers[50,62]. Similarly to ataxin-3, other DUBs typically relate to a particular substrate, to which they have maximal affinity and, thus, catalytic activity. Additionally, most DUBs' activity is carefully regulated as they may greatly influence cell homeostasis[58]. Moreover, ataxin-3 proteolytic activity *in vitro* was shown to be quite slow, suggesting that optimal conditions, such as the addition of the appropriate substrate may be required

for optimal proteolysis [58,63]. Moreover, stimulators of ataxin-3 activity have been reported, namely VCP/p97[63].

These data shows that ataxin-3 has a role in deubiquitination and that most likely it is involved in the UPS. Other cellular functions attributed to ataxin-3 include transcription regulation[64,65,66], cytoskeleton organization[67,68] and myogenesis[69].

The pathogenic effect of polyQ expansion on ataxin-3 (defined in section 4) appears to affect ataxin-3 normal function, particularly the more studied ataxin-3 deubiquitinating activity, and possibly other still unknown function(s) of the proteins[58].

2.2. Autophagy

Autophagy (from the greek words “auto” and “phagein”, self and eat, respectively) is the mechanism by which intracellular components, including proteins and organelles, are degraded in lysosomes[70].

Many intracellular mechanisms require a proper function of the autophagic machinery, including defense against pathogenic organisms, cellular differentiation, growth control and mechanisms related to cell death and cell survival in response to stress stimulus. Moreover, autophagy plays an important role in proteostasis or protein control systems[70].

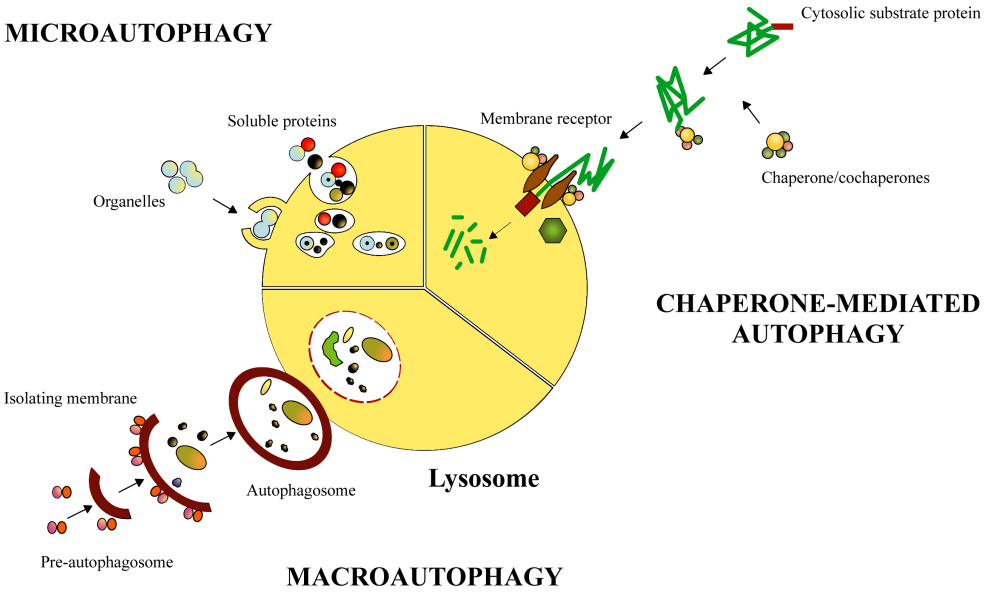

Figure 5. The different types of autophagy. Macroautophagy results in degradation of intracellular components through formation of an isolation membrane and subsequent autophagosome, that later fuses with lysosomes. In microautophagy, the lysosomal membrane deforms to wrap regions of the cytosol. Chaperone-mediated autophagy targets proteins to lysosomes by associating the substrate to chaperones that are further translocated into lysosomal lumen (adapted from Bandhyopadhyay and Cuervo 2007[284]).

Three different types of autophagy have been proposed, depending on the mechanism used to deliver the substrate to the lysosome: macroautophagy, microautophagy and chaperone-mediated autophagy[70,71] (Figure 5). Each of these different mechanisms is activated in different conditions, due to its individual characteristics.

Macroautophagy occurs through activation of two pathways, a lipid-to-protein conjugation pathway and a protein-to-protein conjugation pathway, resulting in the formation of an isolation membrane. The isolation membrane elongates and wraps around the substrates, and then finally closes creating a double membrane vesicle, called autophagosome, which isolates a region of the cytosol that contains organelles, soluble proteins, protein aggregates and the target substrate[72]. The autophagosome later fuses with lysosomes, exposing the substrates to lysosomal acidic proteases for degradation. Macroautophagy can be activated under basal conditions[73] or be inducible by stress, to achieve cellular homeostasis and energy balance. Moreover, activation of macroautophagy

occurs in response to nutritional stress (in order to degrade proteins into amino acids, which can then be used as an energy source) and in response to both endogenous and exogenous stressors, such as viral infections[74]. Impairment of macroautophagy has been suggested to be involved in the pathogenesis of various diseases, as cancer, metabolic and neurodegenerative diseases[75].

In microautophagy, lysosome deforms itself and wraps the part of the cytosol containing the target substrate. This process is thought to be constitutively active, allowing the continuous turnover of cellular constituents in basal conditions. However, its understanding in mammals is still very limited[76].

Chaperone-mediated autophagy differs from the other two types of autophagy as there is selective delivery of the target substrate to the lysosomes to be degraded. In order to be delivered, the substrate must contain a KFERQ signal motif recognized by cytosolic chaperone, heat shock cognate protein of 70 kDa (hsc70), which targets the substrate to the lysosomal surface. Then, the interaction between the hsc70/substrate complex and the lysosome-associated membrane protein type 2 (LAMP-2A) enables the passage of the substrate through the lysosomal membrane[77,78]. Although chaperone-mediated autophagy was found in most types of mammalian cells, its activity varies among cell types and cellular conditions. Moreover, maximal activation of chaperone-mediated autophagy has been described under stress conditions, including mild oxidative stress or toxic exposure that cause damage in particular proteins[71]. Thus, chaperone-mediated autophagy allows the specific removal of modified proteins, without affecting adjacent functional proteins. Different neurodegenerative diseases have been associated to alterations in chaperone-mediated autophagy[71].

The three different types of autophagy seem thus to be activated in response to different stimulus. However, evidences support that all the three mechanisms are interconnected, this being crucial in conditions associated with failure of one of these systems[71].

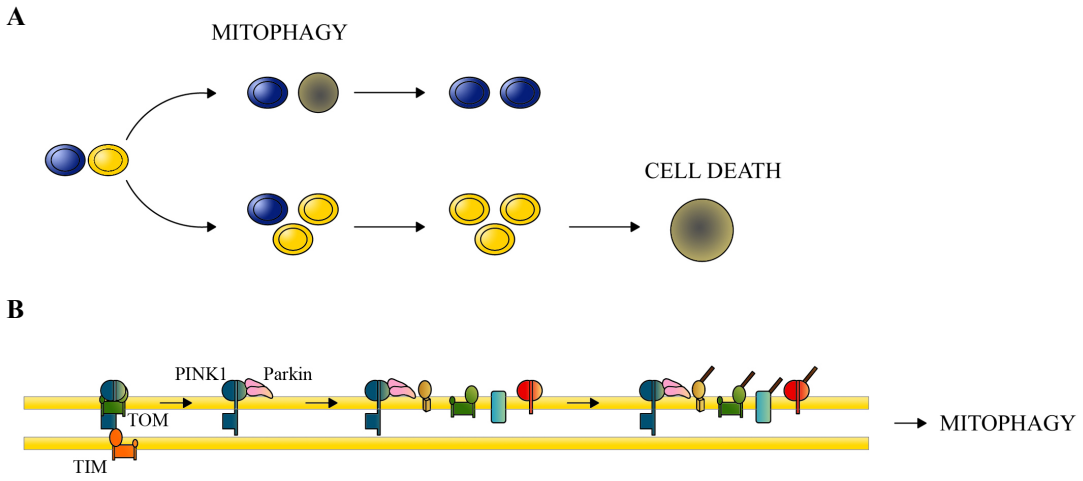

Figure 6. Mechanisms of Mitophagy. (A) The selective degradation of depolarized mitochondria prevents cell death induced by the accumulation of defective mitochondria **(B)** PINK1 accumulates in the outer mitochodria and recruits Parkin. Parkin triggers the recruitment of numerous mediators, culminating in the mitophagic process (adapted from Jin and Youle 2012[138]).

Mitophagy is the selective degradation of mitochondria by lysosome-dependent processes (Figure 6). This process differs from the three typical processes of autophagy as dysfunctional mitochondria are selectively engulfed by autophagossomes after loss of mitochondrial membrane potential[79,80]. Moreover, mitophagy seems to control not only mitochondria quality, but also mitochondrial number, helping to eliminate this organelle during the development of specialized cells or tissues[81].

The mechanism proposed for mitophagy includes two proteins whose mutations are linked to PD, parkin and PINK1. First, PINK1 accumulates in the outer mitochondrial membrane of defective mitochondria differentiating them from healthy mitochondria. Then, PINK1 selectively recruits Parkin to mitochondria[79,80]. Following translocation to the mitochondrial surface, Parkin ubiquitinates numerous proteins in the outer mitochondrial membrane, which in turn recruit other proteins and cytosolic factors needed for the initiation of mitophagy[80,82]. Some examples are VCP, a protein linked to endoplasmic-reticulum associated protein degradation (ERAD)[83], proteasomal subunits[84] and histone deacetylase 6 (HDAC6)[82].

3. Parkinson's disease

In 1817, James Parkinson published the first description of a group of six patients presenting similar clinical features, namely rigidity, tremor and festination, in "An Essay on the Shaking Palsy"[85]. Nearly two centuries have passed from his first description; the syndrome that was named after him as Parkinson's disease (PD) is now known as an age-dependent neurodegenerative disorder, traditionally characterized by the triad of resting tremor, rigidity and bradykinesia[20,86].

PD is the second most frequent neurodegenerative disorder and the major affecting movement, with 1% of the population over the age of 65 diagnosed with this disorder. The mean age of diagnosis is 70 years old, with early-onset disease (before age of 55) having a prevalence of 4%[28,87,88]. While the majority of PD cases are sporadic and rely in a combination of environmental causes and genetic susceptibility, 15-20% of PD patients report a familial history of the disease[28,89,90]. Increasing age has been identified as a major risk factor for the both the familial and sporadic forms of PD[89].

3.1. Clinical Presentation

The onset of PD is typically insidious and progressive. Symptoms are initially most commonly found unilaterally with subsequent spread for the rest of the body. Supported by imaging studies of 6-[18F]fluoro L-DOPA (F-DOPA) uptake, the rate of decline of striatal dopamine innervation is thought to be faster in the starting years, with slower progression in later stages of the disease [91] (Figure 7).

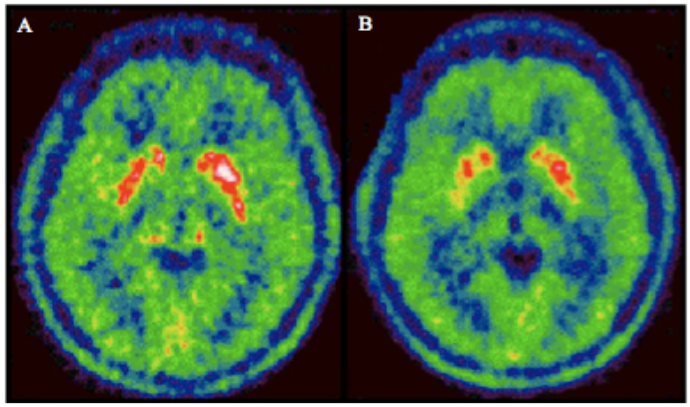

Figure 7. F-DOPA uptake in a PD patient. A: at diagnosis; **B:** 2 years after follow-up (Adapted from Brooks and Pavese 2009).

A therapeutical window could therefore arise from this data. Unfortunately, few PD diagnosis are done in these early stages, with evidence showing that motor symptoms manifest only after a significant cell loss has occurred[91]. In addition, PD patients, typically in their 70s, often mistake their symptoms as caused by physiological aging or rheumatic disease, with consequent later seek for medical assistance.

The most frequent first noticed symptom is tremor of the hand. Rigidity contributes to the flexed posture characteristic of PD patients. Postural instability, gait dysfunction with festination and freezing (motor and speech), hypomimia and impaired postural reflexes are also frequently recognized[92]. Increasing evidence is supporting the concept of premotor PD with the onset of olfactory dysfunction, sleep abnormalities, cardiac sympathetic denervation, constipation, depression and pain presenting before the typical motor symptoms, which could have an impact on treatment options and strategies, if the diagnosis would be set at an earlier stage of the disease [17,93,94] (Table 2). Moreover, current treatments for PD (dopaminergic replacement and functional neurosurgery) act primarily to motor symptoms control, largely targeting and reverting tremor, rigidity and bradykinesia. Nevertheless, other disabilities, such as gait dysfunction, swallowing and speech difficulties, autonomic dysfunction and cognitive decline, also found in PD patients, rise as they are less influenced by therapeutic strategies[94,93].

Table 2. The clinical features of PD.

Motor symptoms	Bradykinesia Rigidity Resting tremor Gait and balance Freezing gait Falls Speech difficulties Swallowing difficulties
Neuropsychiatric symptoms	Dementia Hallucinations Depression Attention deficit
Visual symptoms	Visuospacial and color discrimination Diplopia Blurred vision
Sleep disturbances	Daytime sleepiness Fatigue Insomnia REM sleep behavior disorder Restless legs and periodic limb movements
Autonomic dysfunction	Urinary symptoms Urgency Nocturia Frequency Gastrointestinal symptoms Constipation Fecal continence Dribbling of saliva Unsatisfactory voiding of bowel Cardiac sympathetic denervation Sweating Orthostatic hypotension Sexual dysfunction
Other	Olfactory dysfunction

Adapted from Maetzler et al. 2009[286] and Chaudhury et al. 2006[287].

Strikingly, dementia is a critical major long-term cause of disability, affecting around 30-40% of PD patients[21,95,96] which indicate an approximate 4 to 6-fold increased risk of dementia compared to age-matched general population[97]. Moreover, a study showed that the prevalence of dementia after 5 years of PD diagnosis was 28%, increasing to 48% at 15 years and 83% at 20 years after diagnosis[96]. Another study showed that 10 years after diagnosis, approximately 75% of PD patients develop dementia[97].

However, neither of these symptoms are pathognomonic for PD and though the course and symptom association suggest the specific diagnosis, differential diagnosis of parkinsonian syndromes is challenging and should always be kept in mind.

Table 3. Clinical features in the differential diagnosis between PD and Parkinson-plus syndromes.

	PD	DLB	MSA	PSP	CBD
Dementia		+			
Apraxia					+
Akinesia	+	+	+	+	+
Rigidity	+	+	+	+	+
Tremor	+	+			
Gait disorder		+	+	+	+
Falls		+		+	+
Dysarthria		+	+	+	+
Dysphagia		+		+	
Gaze palsy				+	
Autonomic failure		+			

Adapted from Tolosa et al. 2006[100].

PD diagnosis still depends on clinical expertise, with no currently reliable test available. Approximately 80% of patients with parkinsonian features are clinically diagnosed with a "probable PD" after a longitudinal evaluation[98,99,100]. In clinical practice the combination of cardinal motor features, with absence of atypical symptoms - early hallucinations and dysautonomia, ophtalmoparesis or ataxia (Table 3) - associated with a good response to L-DOPA, strongly suggests the diagnosis. Moreover, the diagnostic challenge of PD increases in the early course of the disease when signs and symptoms overlap with those of other syndromes[99]. Many of the prominent features of PD may also occur due to physiological aging or from other co-morbidities, as well as

medications. A definite diagnosis requires post-mortem analysis of patient's brain, findings of dopaminergic neuronal loss and depigmentation of the *substantia nigra* confirm the diagnosis, with frequent identification of Lewy bodies and Lewy neurites spreading from the brainstem[28,101,102].

3.2. Neuropathological mechanisms

Scientific evidence linked the cardinal motor symptoms of the disease to the loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) in the brainstem (Figure 8). Post-mortem analysis of PD patients brain showed macroscopical loss of neurons in SNpc that matched the later finding of decreased levels of dopamine in the striatum[103]. Injected L-DOPA was first shown to improve akinesia in PD patients in 1961 and later in the same decade oral L-DOPA was available[104].

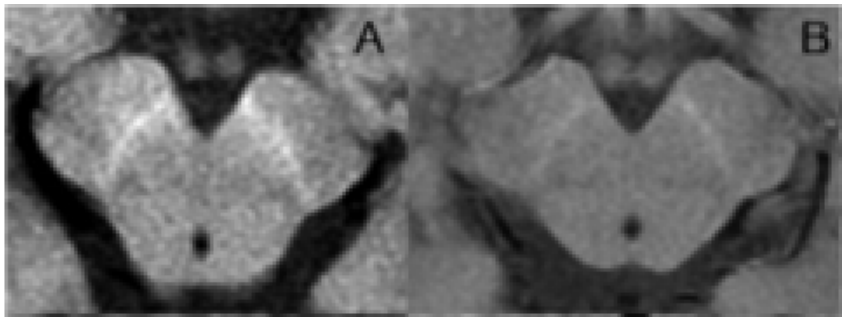

Figure 8. MRI studies of the *substantia nigra*. Representative neuromelanin of the SNpc obtained by T1-weighted MRI comparing a control **(A)** with acute ischemic stroke to a PD patient **(B)** (adapted from Kashihara et al. 2011[288]).

Once a step was made towards the understanding of PD pathogenesis, numerous defects have risen as contributors to disease pathogenesis, including organelle dysfunction, increased oxidative stress, decreased ATP formation, inflammation and DNA mutations, all leading to cell damage and, ultimately, death of dopaminergic and non-dopaminergic neurons in PD patients brains[105]. Besides the neuronal loss, PD is histologically characterized by the presence of Lewy bodies and Lewy neurites in the more susceptible neurons. These are intracellular protein inclusions present in the

neuronal cell body (Lewy bodies) or neuronal processes (Lewy neurites) of damaged neurons, composed primarily of α-synuclein[101,102].

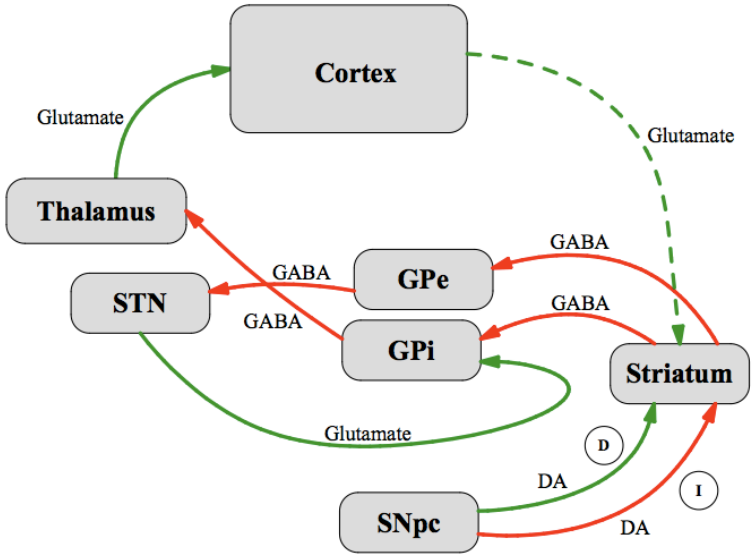

Figure 9. The direct and indirect pathways of movement. The direct pathway of movement initiates in the SNpc and comprises the sequential activation of the Striatum, *Globus Pallidus internus* (GPi), Thalamus and Cortex. This pathway can also be activated through corticostriatal feedback. In the indirect pathway, which may also be stimulated through corticostriatal feedback, neurons in the Striatum activated by the nigrostriatal pathway, project to the *Globus Pallidus externus* (GPe) and thence to the Subtalamic Nucleus (STN), GPi, Thalamus and Cortex. Red indicates and inhibitory projection while green shows stimulation (adapted from Quik and Wonnacott 2011[289]).

Early explanations of PD pathogenesis relied on the loss of dopaminergic transmission in the striatum, due to SNpc neuronal loss, to explain classic PD motor symptoms due to impairment of the non-pyramidal nigrostriatal pathway (Figure 9). Dopamine metabolism itself is considered to be one of the reasons that leads to damage of ventrolateral SNpc cells. Auto-oxidation of dopamine in the presence of iron produces toxic dopamine-quinone species, superoxide radicals and hydrogen peroxide. On the other hand, the enzyme monoamine oxidase (MAO) can deaminated dopamine into 3,4-dihydroxyphenylacetic acid (DOPAC) and hydrogen peroxide[106]. The reactive oxygen species arising as by-products of dopamine metabolism, if not properly handled by anti-oxidant

defenses within the cell, can further oxidize lipids (causing lipid peroxidation) and other biomolecules, and impair mitochondrial function[107,108]. Hydrogen peroxide arising from dopamine metabolism can be further catalyzed by the iron-associated Fenton reaction into reactive oxygen species (ROS)[109]. Moreover, the pattern of cell loss includes neuromelanin-containing neurons, thought to be more susceptible to these insults, as neuromelanin itself or its precursors might be cytotoxic[110,111].

However, α-synuclein-positive Lewy bodies are not confined to these neurons, showing that neuropathology is more extensive and independent of neuromelanin[111]. Based on clinical heterogeneity, the impact of Lewy bodies and Lewy neurites as determinants of clinical progression is questioned[20].

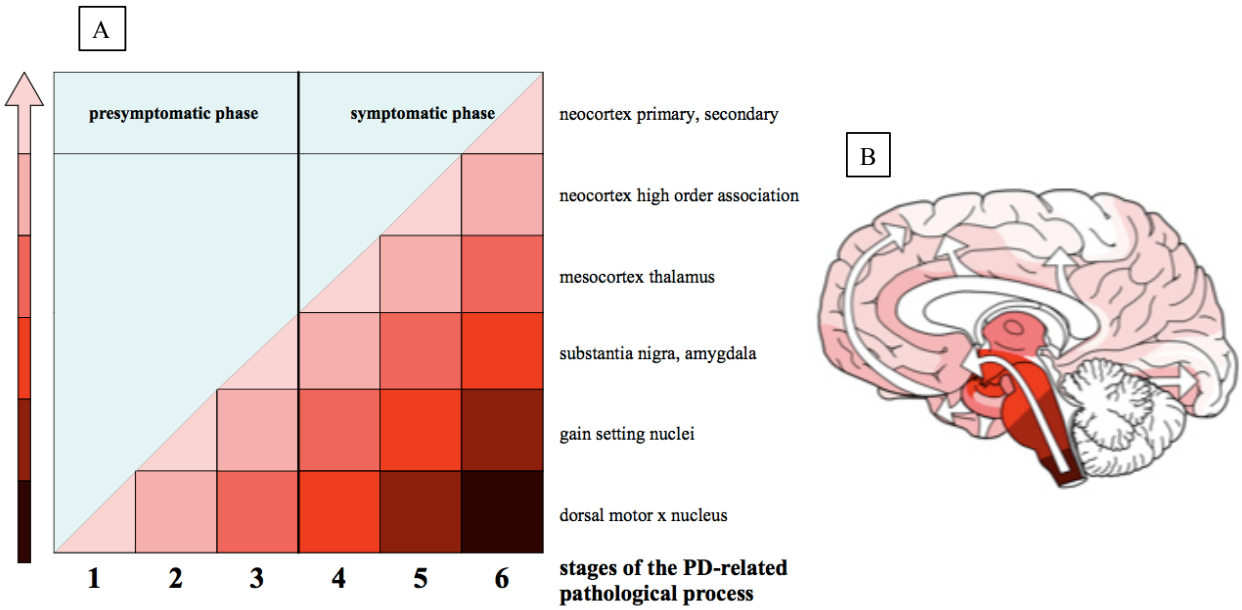

Figure 10. Braak's Staging for PD. Braak and colleagues suggest an ascending pathological process starting in the brainstem. **(A)** There is sequential compromising of brain areas (from region 1 until region 6) and the typical PD motor symptoms arise after neuropathological damage in region 3 (including the SN); **(B)** Diagram showing the ascending pathological process. (adapted from Braak et al. 2004[117]).

Braak and colleagues conceived a staging system for sporadic PD based on the neuropathological finding of Lewy neurites and Lewy bodies (Figure 10). Accordingly, they proposed that PD is a synucleinopathy that affects the central nervous systems and that it begins in non-dopaminergic neurons in the lower brainstem or in the olfactory bulb. From the brainstem (region 1, including dorsal motor nucleus of the vagus nerve) the progression is done towards the *locus coeruleus* (region 2), the *mesencephalum* (region 3, including *substantia nigra*), the mesocortex and thalamus (region 4) and the neocortex (region 5 and region 6). Hence, by the time PD diagnosis is made, lower brainstem involvement accompanies mesencephalic disease, as parkinsonism-associated clinical signs that enable the "PD diagnosis" by current criteria only appear with SNpc neuronal loss in stage 3 or after. Moreover, this system supports the existence of a pre-motor PD, as the synucleinopathy predictably progresses stepwise caudo-rostrally from the brainstem to the neocortex[112,113,114].

Reports of pyramidal neuronal loss in the pre-motor cortex in post-mortem analyses of patients who died after a relatively short course of the disease (less than 5 years), along with the typical loss in the SNpc[115], counterpose Braak's staging, as they suggest that the cerebral cortex may be affected early in disease course. Nonetheless, with the short progression period of the patients described, features of many other syndromes overlap with those of PD and thus, one might argue the accuracy of the diagnosis.

Other neurons involved in the neurodegeneration include monoaminergic neurons in the *locus coeruleus* and raphe nuclei and cholinergic neurons in the nucleus basalis of Meynert and the pedunculopontine tegmental nucleus[116,117].

Current treatment options, however, also do not have impact in disease course, highlighting the imperative better understanding of disease etiology and pathological mechanisms. Unraveling PD pathogenesis will enable the development of therapies that halt pathological mechanisms, and thus, disease progression. In addition, putative disease biomarkers may arise from the clarification of

disease mechanisms. Subsequently, an early diagnosis can be matched with disease modifying drugs that will mitigate the development of both motor and non-motor symptoms.

Both sporadic and inherited forms of the disease are thought to develop from the same neuropathological mechanisms. Nonetheless, little is known about PD etiopathogenesis. Environmental and genetic factors, including genetic predisposition have been described as major contributors to PD pathogenesis[118]. Age plays an unquestionable role; however, with the lack of understanding how exactly it contributes to disease pathogenesis, explanations rely on decreased ability of physiological processes to deal with damaging exposures. Environmental exposures were proposed after evidence linked postencephalitic parkinsonism to a viral infection[119] and the development of parkinsonism after toxin exposure, as 1-methyl-4-phenyl-1,2,3,6-tetrahydroxy-pyridine (MPTP)[120] and rotenone[121]. Other environmental exposures related to PD pathogenesis include industrial exposure, rural environment, plant toxins, pesticides, paraquat, carbon monoxide and carbon disulfide[121]. MPTP was shown to decrease mitochondrial respiratory chain complex I activity in the *substantia nigra*[121], while the pesticide rotenone, another complex I inhibitor, showed the ability to induce pathological, biochemical and clinical features of PD in rodent models[122]. Nonetheless, little is known about PD etiopathogenesis. Environmental and genetic factors, including genetic predisposition have been described as major contributors to PD pathogenesis[118].

Evidences on the role played by the mitochondria in PD started with the identification of PD-causing toxins, rotenone and MPTP, able to directly damage the mitochondria and cause selective death of dopaminergic neurons[121,124]. Later, complex 1 deficiency and deletions in mitochondrial DNA were also identified in PD patient's *substantia nigra*. The toxin 6-hydroxydopamine (6-OH-DA), which induce a parkinsonian phenotype in models of PD, also acts through increasing mitochondrial reactive oxygen species and impairing the mitochondrial respiratory chain[121].

Oxidative stress, on its turn, has been implicated in PD pathogenesis by multiple studies showing oxidative damage to proteins, lipids and DNA, as well as decreased glutathione (GSH) levels[124,125].

Moreover, deficiencies in the major antioxidant enzymes - catalase, superoxide dismutase and glutathione peroxidase - in brain have been identified and support oxidative stress as a contributor to PD pathogenesis[124].

Postmortem studies demonstrated that nigral cell degeneration could be the result of oxidative stress and oxidizing toxins[124], and SNpc shows basal high levels of oxidative stress in the normal brain, which is increased in PD patients[126,127]. In addition, L-DOPA treatment may increase oxidative load, through dopamine metabolism[127]. Markers of oxidative damage in the periphery have been found in PD, suggesting that oxidative stress may not be restricted to the brain[128]. Additionally, despite the major emphasis in dopaminergic neuronal dysfunction in PD, the activation of glial cells was shown to have an important role in cell death[129,129,130,131]. Some studies suggest that glial cells are involved in PD pathogenesis as oxidative stress may originate in these cells[129].

Additional studies in diverse models of PD, including cellular models, neurotoxin and genetic models in animals, corroborated the role played by mitochondrial impairment[132,133,134]. Deficits in mitochondrial respiratory chain, increased levels of oxidative stress and impaired calcium handling capacity, have been described in multiple PD models[121,124,135].

Hence, not only increased damage to mitochondria is as an explanation for the neuronal death, as there might be a cellular decreased ability to deal with dysfunctional mitochondria. Additionally, gene mutations associated with familial forms of PD have been shown to either directly or indirectly affect mitochondria; this include PTEN-induced kinase 1 (PINK1), parkin, DJ-1, α-synuclein and leucine-rich repeat kinase 2 (LRRK2) (section 3.2), which contribute for maintaining mitochondrial integrity and organelle quality control systems[136,137,138]. Thus, malfunction of the quality control systems for mitochondria may lead to the accumulation of defective mitochondria and cell death[138].

Alterations in protein ubiquitination and degradation by the UPS may also be primary contributors to PD pathogenesis[139,140]. Moreover, various genes encoding proteins that participate or are

associated with UPS, were found to be mutated in PD, including parkin and UCH-L1[23,141]. The relationship between UPS dysfunction and these mutations and familial PD cases is not completely established.

In addition to the familial cases of the disease, sporadic PD has been linked to UPS dysfunction. A propensity to develop proteolytic stress with age, due to decreased proteasomal function, has been linked to protein aggregation and disruption of normal cell homeostasis, with impaired intracellular functions[142]. Additionally, inhibition of proteasomal function has been shown to cause mitochondrial dysfunction and oxidative stress[132]; both mechanisms, as discussed previously, have been proposed to explain neuronal death in PD. Thus, whether proteasomal defects are primary in sporadic PD pathogenesis or derived from mitochondrial dysfunction and oxidative stress is not known[132].

Apart from the UPS, the autophagy-lysosome pathway, another protein clearance mechanism in the cell, has been also associated to PD. Genes causing familial PD - parkin, PARK1 - have been associated to the this pathway[138]. Since autophagy is involved in cellular homeostasis, contributing for organelle turnover and decreasing protein aggregation, reduced activity of this pathway is easily suggested to play a role in disease associated with protein deposition. Moreover, both macroautophagy and chaperone-mediated autophagy were found to decrease with age[143], possibly contributing for the late onset of PD.

A-synuclein, the major constituent of Lewy-bodies and Lewy neurites, is degraded by macroautophagy and chaperone-mediated autophagy[15,71]. The most common α-synuclein mutants, A53T and A30P, inhibit chaperone-mediated autophagy, as they compete and show a higher affinity towards LAMP2A[71]. Previous evidences show that these mutants may induce α-synuclein aggregation by inhibiting chaperone-mediated autophagy[71].

Additionally, 193M missense mutation in UCH-L1 also exhibits increased affinity towards LAMP2A and Hsc70, thus showing a negative effect on the chaperone-mediated autophagy

pathway. Overexpression of this mutant leads to increased levels of α-synuclein, suggesting that UCH-L1 may exert an important role in in PD[144].

Nevertheless, excessive activation of autophagy has been associated with neuronal loss[145]. It is important to clarify whether excessive activation of this pathway does in fact happen in PD. Previous studies have shown abnormal presence of autophagic vacuoles in PD patients brains, which contrast to the rare detection in the brains of general population; however, instead of correlating with increased activation of macroautophagy, this finding could be explained by decreased vacuole clearance ability[146]. Nonetheless, as previously mentioned, overexpression of α-synuclein mutants have been shown to activate macroautophagy[71]. Thus, further studies are needed to understand the true role of autophagy in PD.

Other pathologic mechanisms proposed to contribute to PD pathogenesis include inflammation, excitotoxicity, ER-to-Golgi traffic dysfunction and nitric oxide toxicity[15,73]. Hence, a variety of mechanisms have been proposed to play a role in PD pathogenesis. Nevertheless, it remains to be clarified whether there are initiating factors and mechanisms that propagate the existing neuronal damage or whether all these mechanisms are equal contributors to disease initiation and propagation.

3.3. Parkinson's disease associated mutations

The efforts to understand PD pathogenesis have been creating a growing list of mutations. These account for fewer than 10% of PD cases. Although a high percentage of patients present a family history of parkinsonism[28,90], many of these seem not to be hereditary, but denote an increased familial susceptibility or the environmental impact, due to common exposures. Moreover, PD is not fully penetrant, with individuals within the same family affected by monogenic disease presenting with variable manifestations[90].

Table 4. PD-associated loci.

	Gene	Locus	Inheritance
PARK1/4	SNCA	4q21	AD
PARK2	PRKN	6q25.2-q27	AR
PARK3	-	2p13	AD
PARK5	UCHL1	4q14	AD
PARK6	PINK1	1p36	AR
PARK7	DJ1	1p36	AR
PARK8	LRRK2	12q12	AD
PARK9	ATP13A2	1p36	AR
PARK10	-	1p32	AD
PARK11	GIGYF2	2q37.1	AD
PARK12	-	Xq21-q25	X-linked
PARK13	Omi/HtrA2	2p12	AD
PARK14	PLA2G6	22q13.1	AR
PARK15	FBX07	22q12-q13	AR
PARK16	-	1q32	-

Adapted from Sundal et al. 2012[27].

From the 16 loci (PARK1-PARK16) identified through the years as having a putative role in PD, 11 were found to have in fact mutations that could be associated to PD pathogenesis (Table 4) - PARK1 and PARK4 (α-synuclein)[22], PARK2 (parkin)[23], PARK5 (UCHL1), PARK6 (PINK1)[24], PARK7 (DJ-1)[25], PARK8 (LRRK2)[26], PARK9 (ATP13A2), PARK11 (GIGGYF2), PARK13 (HTRA2), PARK14 (PLA2G6) and PARK15 (FBXO7).[27] PARK5, PARK11, PARK13 and PARK16 are still to be confirmed as PD-associated loci[27].

However, mutations in some loci, PARK9 and PARK14, give rise to atypical clinical presentation with diagnosis within parkinsonism-plus syndromes, while new loci not included in PARK loci, as

those encoding eukaryotic translation initiation factor 4 (EIF4G1) and vacuolar protein sorting 35 (VPS35), appear to be associated with typical clinical presentation[27].

Dominantly inherited mutations include α-synuclein, SNCA gene and LRRK2, PARK8 gene[28]. The identification of mutations in the SNCA gene were found to be associated with other diseases than PD, including Lewy body disorders[147,148,149]. A-synuclein will be discussed further in this thesis.

PARK8 mutations are a more frequent cause of autosomal dominant PD[27,28,150]. Typically, mutated LRRK2 leads to late-onset, asymmetrical, L-DOPA responsive PD. Clinical presentation is variable, including age of onset and phenotype severity[150]. Many LRRK2 mutations linked to PD were identified, being 6099G>A (Gly2019Ser) the most frequent mutation among Caucasians, causing 0.5-2.0% of sporadic and 5% of hereditary PD. Moreover, disease penetrance is age-dependent; 17% of patients with this mutation at age 50 will develop the disease, while this number increases to 85% at age 70[150]. LRRK2 is a large protein with 2527 amino acids, with numerous roles attributed to it, including dopaminergic signaling[151]. It is expressed in most brain regions, however, was never found to be aggregated in any specific brain lesion, including Lewy bodies or Lewy neurites. Moreover, typical Lewy body disease was found in most cases of LRRK2-associated parkinsonism. Some cases, on the contrary, were positive for tau aggregation without the presence of Lewy bodies[152].

Recent data described a new PD-associated autosomal dominant mutation on chromosome 3, with typical late onset and a good response to L-DOPA therapy[153,154]. Elongation initiation factor 4G1 (eIFG4G1), the protein encoded in this locus, helps cell survival in response to environmental stress. Moreover, the clinical presentation of patients with EIFG4G1 mutations seems to match the clinical presentation of patients with α-synuclein accumulation and Lewy body formation[153,154].

Recessive inherited mutations include parkin, PARK2 gene; DJ-1, PARK7 gene and PINK1, PARK7 gene. These rare mutations lead to loss of function of the encoding proteins, with typical early onset (before age 40-50), slow progressing and L-DOPA responsive PD[27,28].

Parkin is an E3 enzyme with 465 amino acid protein, encoded by PARK2 gene. Parkin localizes mostly to cytosol but it has been also identified in the mitochondrial outer membrane and in synaptic vesicles[23,155,156,157]. Various deletions and point mutations in the gene have been found in 50% of patients with autosomal recessive PD (AR-PD), one of the most common familial forms of PD and the most frequent cause of early-onset PD (under 40-50 years)[23,158]. Parkin gene missense mutations seem to influence the cellular localization, catalytic activity, solubility and stability of parkin, while PARK2 deletions, which extend through several exons, suggest a great loss of parkin function[159]. Altogether, these alterations in PARK2, even if through different mechanisms, seem to result in decreased levels of parkin function.

PINK1 mutations account for 1-2% of early-onset PD cases. These mutations seem to alter stability, localization and function of PINK1, a mitochondrial protein kinase, thought to have a role in mitochondrial protection. Both PARK6 and PARK8 encode protein kinases, PINK1 and LRRK2 respectively, that may influence the phosphorylation state of proteins that accumulate in end-stage PD. Interestingly, α-synuclein and parkin and, were identified as putative substrates for LRRK2[160] and PINK1[161], respectively. Indeed, phosphorylated α-synuclein is a common finding in Lewy Bodies (~90%)[162]. Additionaly, PINK1 and parkin have been shown to cooperate synergistically to promote neuronal survival in the *substantia nigra*, allowing the identification and further degradation of dysfunctional mitochondria by autophagy[80].

DJ1 is an oncogene, which deletions and missense mutations were identified as causing <1% of early-onset parkinsonism[25]. DJ1 protein expression is induced by oxidative stress. Susceptibility to oxidative stress and proteasome inhibition, both implicated in sporadic PD, were found in drosophila model with low levels of DJ1 expression[163,164].

Mutations in glucocerebrosidase, responsible for Gaucher's disease, have been also linked with 5-fold increased risk of developing early-onset PD. Moreover, typical phenotype of patients with mutations in glucocerebrosidase gene, matches typical PD phenotype[165].

Lastly, genetic variability in microtubule-associated protein tau (MAPT) gene, which encodes protein tau and is commonly linked with chromosome 17 tauopathies, may contribute to PD susceptibility. Moreover, even though the predominant feature of tauopathies is dementia - as seen in AD -, parkinsonism is also often described[166]. Moreover, tau and α-synuclein may have a common contribution to PD susceptibility, as α-synuclein has been identified in traditional tauopathies[167] and tau deposition, which on its turn may be observed in both sporadic and familial forms of PD[168].

As described, PD is a non-completely penetrant disease, with identified mutations and environmental exposures that increase the probability of disease development. However, many intriguing details about disease pathogenesis are still to be unraveled. The factors that define the rate of motor function decline, the predisposing factors leading to cognitive impairment, the role played by dopamine and the anatomical location, SNpc, are among the questions still in debate. Further questions also concern the mechanisms found to explain PD pathogenesis - whether a mechanism is preponderant in PD or whether the interaction of disabled cellular mechanisms is what truly causes PD, is not fully understood either. Finally, the variability of clinical phenotypes sets the argue on whether PD is an heterogeneous disease or if, on the other hand, many different diseases that share common symptoms, are being erroneous diagnosed as PD.

Indeed, neurodegenerative disorders often show mixed features of one another - as easily seen in PD, a movement disorder, in which patients often present dementia in later stages of the disease. Moreover, proteins earlier associated with a particular disease seem to have defined roles in different pathologies. A good example of this is α-synuclein[147,148,149].

In order to understand disease pathogenesis is not only important to understand protein function and interactions, but also to realize whether these results could be achieved with physiological levels of protein expression. Additionally, it would be important to understand whether increasing age - known to influence the activity of UPS and autophagy-lysosome pathway - also influences protein

expression and/or creates the cellular conditions that further increase its aggregation. Notably, models of disease usually lack to reproduce this major contributor to PD pathogenesis, with young animal model used to recreate disease conditions and conclude putative underlying mechanisms.

3.4. Parkinsonian syndromes and protein aggregation

Parkinsonism refers to a neurological syndrome characterized by bradykinesia, muscle rigidity and tremor at rest. Parkisonian syndromes can be divided in 4 categories based on whether they are idiopathic (primary parkinsonism, as PD), acquired (secondary parkinsonism, as drug-induced or vascular parkinsonism), hereditary (heredodegenerative parkinsonism, as parkinsonism in type 4 Machado Joseph disease) or associated to multiple system degeneration (parkinsonism-plus syndromes)[169].

The first step into the diagnosis is ruling out any secondary cause of parkinsonism, most importantly drug-induced and vascular parkinsonism (pseudo-parkinsonism)[99]. A detailed medical history, including medications, and a careful clinical examination are extremely important for this distinction. Wilson's disease and other toxic causes are rare but reversible and treatable causes of parkinsonism, which should be ruled out[169].

Atypical parkinsonian syndromes are identified by the premature manifestation of additional clinical features, as the name Parkinson-plus indicate. These sometimes subtle clinical features usually are referred to as "red flags" and must direct the diagnosis into the group of atypical parkinsonian syndromes. Early postural instability with frequent falls should bring atypical parkinsonism to mind, as it is common to all subtypes, though most prominent in progressive supranuclear palsy[170]. Cycling ability ("bycicle sign") could for example be questioned, as the majority of atypical patients who have this activity need to stop it early after disease onset, while PD patients usually maintain this activity for longer periods[171].

Table 5. PD differential diagnosis.

Degenerative parkinsonian syndomes	Multiple system atrophy Progressive supranuclear palsy Dementia with Lewy bodies Corticobasal degeneration Parkinsonism linked to AD Machado-Joseph disease Huntington's disease Prion disorders	Neuroacanthocytosis Wilson's disease Whipple's disease X-linked dystonia-parkinsonism Neuronal brain iron accumulating syndromes Parkinsonism-dementia-amyotrophic lateral sclerosis complex
Infections	Prion disease Encephalitis lethargica	HIV-related parkinsonism
Toxins	MPTP carbon monoxide carbon disulfide manganese	paraquat hexane rotenone toluene
Structural lesions	hydrocephalus Falx cerebrii /motor strip meningioma Cavernoma	Arteriovenous malformation Infective cysts Tumors of basal ganglia
Cerebrovascular disorders	Vascular pseudoparkinsonism	

Adapted from Chaudhuri and Ondo 2010[169].

Moreover, in the early course of the disease findings of autonomic symptoms (postural hypotension and incontinence), ocular signs and hallucinations or psychosis, in addition to the characteristic parkinsonian symptoms, favor the diagnosis within the Parkinson-plus syndromes[99]. In the later stages of PD these signs and symptoms are also prevalent. In addition, symmetry of symptoms in early stages of disease also questions PD as the correct diagnosis[169]. The importance of a correct diagnosis relies also on the fact that these syndromes typically have a poorer response to dopaminergic therapy (L-DOPA or dopamine agonists) and should not be treated by deep brain stimulation[99]. Moreover, dopaminergic therapy may worsen atypical features such as orthostatic hypotension or confusion[95]. The prognosis can also be estimated based on the correct parkinsonism subtype.

In addition to secondary parkinsonism and Parkinson-plus syndromes, parkinsonian features can be among the clinical presentation of heredodegenerative and some cases have been reported where

parkinsonism is the unique feature at clinical onset[169]. Parkinsonism can also be the only symptomatology identified in patients with corticobasal degeneration (CBD) or essential tremor, particularly in early stages[100]. Multiple system atrophy patients (MSA) and progressive supranuclear palsy (PSP), a parkinson-plus syndrome, often have an initial good response to L-DOPA[100], as do some patients with Machado Joseph disease (MJD)[29,30,31], with later loss of therapeutic efficacy.

It may be impossible to distinguish idiopathic PD from other parkinsonian syndromes based on clinical presentation. Equivocal diagnosis of idiopathic PD (approximately 15%) can therefore be easily done, and 80% misdiagnosed patients actually have multiple system atrophy or progressive supranuclear palsy, the two most common Parkinson-plus syndromes, both with a worst prognosis and decreased life expectancy[148]. In clinical practice, the most relevant diseases presenting with parkinsonism apart from PD are MSA, PSP, DLB and CBD. Oculomotor signs contribute to the differential between these various subtypes - supranuclear gaze palsy is the main characteristic of PSP; saccadic latency in the horizontal plane (oculomotor apraxia) presents early in CBD patients, while suppression of vestibular-ocular reflex is frequent in MSA patients[172].

Genetic findings can also be used for the differential diagnosis. In fact, as previously described, a patient that initially presented parkinsonism with a positive response to dopaminergic therapy, may end up presenting progressive features of cerebellar ataxia making genetic screening an indispensable test for the correct diagnosis[173]. On the other hand, Parkinson-plus syndromes, rather than being genetically separated, are defined as either synucleinopathies or tauopathies[174]. However, although wild-type α-synuclein overexpression has been shown to cause PD[175], it does not predict the movement disorder, neither are α-synuclein positive aggregates markers of neuronal dysfunction[176].

Motor findings among the different causes of parkinsonism are sometimes independent of the loss of *substantia nigra* and deafferentation of the striatum. Importantly, despite the traditional

correlation with proteinaceous aggregates, and post-mortem analysis displaying Lewy bodies and Lewy neurites as disease hallmarks; the only consistent feature of PD is the progressive loss of dopaminergic neurons[28]. Nonetheless, aggregation of misfolded proteins contributes to the neurodegenerative process in PD and Parkinson-plus syndromes. These proteinopathies involve mainly two proteins: tau and α-synuclein 174. Interestingly, tau has been proposed to contribute to PD pathogenesis[18]. Tau (MAPT gene) has several isoforms according to the number of repeats in the micro-tubule binding domain: 4 repeats (4R), associated with PSP, CBD and FTDP17, and 3 repeats (3R), prevalent in Pick's disease[177]. A-synuclein is also associated with LBD and MSA, in addition to PD[149,174].

4. Machado-Joseph disease

Machado-Joseph disease, also known as spinocerebellar ataxia type 3 (MJD/SCA3), is an inherited autosomal dominant neurodegenerative disorder, belonging to the expanded CAG/polyQ ataxias[178]. It was first described by Nakano and collaborators in 1972 in Machado family of Portuguese immigrants[179].

More than 20 years after the initial description, the gene associated with MJD was localized to chromosome 14 - 14q24.3-32.1[48] and later on a CAG expansion was identified in the same locus[46], encoding ataxin-3, a DUB protein described in section 2.1.1. The normal number of CAG repeats in the MJD1 gene ranges from 14-34; 14 repeats were identified not only as the shortest number of repeats, but also the most common[180]. In MJD patients, CAG expansion ranges from 61-84 repeats, with larger expansion associated with paternal transmission[180], as in HD.

MJD is the most common spinocerebellar disease, with prevalence estimated from 0.3 to 2.0 per 100,000[181], with higher prevalence found in particular areas around the world, including the Azores Islands; indeed, the highest prevalence is found in Flores island (1/239)[182]. The relative frequency

of MJD among other SCAs varies between populations: it is very high in Brazil (92% of all cases of SCA), Portugal (58-74%), Japan (63%), China (48-49%) and Germany (42%)[182].

4.1. Clinical manifestations

MJD is a multisystem neurodegenerative disorder that predominantly involves motor pathways - cerebellar, pyramidal, extrapyramidal and oculomotor. Gait impairment tends to be the initial symptom observed among MJD patients[178].

The mean age at onset is around the fourth decade of life - cases were report at age 4[183] and 70 years old - and mean survival time of 21 years[182].

From the multitude and heterogeneity of symptoms presented by MJD patients, the diagnosis is initially suspected by the development of progressive cerebellar ataxia, which may be further accompanied by pyramidal signs as spasticity, extrapyramidal signs as dystonia or limb incoordination and motor neuron signs as fasciculations, usually facial and lingual[178,184]. Dysarthria and dysphagia are also often observed. The oculomotor system affection is translated into the gaze-evoked nystagmus, diplopia, altered saccadic movements, supranuclear vertical gaze palsy and, less often, external progressive ophtalmoplegia. Moreover, MJD patients may exhibit impaired vestibulo-ocular reflex[185].

Dysautonomic features of MJD include genitourinary and sudomotor alterations, as nicturia, urinary incontinence or hyperhidrosis[186]. Proprioceptive and tactile sensation can also be compromised[187]. Non-motor features of the disease include sleep disturbances (daytime sleepiness and insomnia) [188,189], behavioral changes, with some cases of dementia reported[190]. Additionally, verbal and visual memory deficits, with visuospatial and constructional dysfunction and verbal fluency deficits are also characteristic of MJD[191]. The more specific signs of MJD are external progressive

ophtalmoplegia, dystonia, lingual and facial fasciculations, as well as eyelid retracting and reduced blinking, creating the distinctive "bulging eyes appearance"[184].

The high clinical heterogeneity led to efforts to characterize the disease within more homogeneous phenotypic groups. The initial classification consisted of 3 types[178], to which was afterwards added a fourth[192] and more recently a fifth type[193]. Type 1 ("type Joseph" - 13% of patients) describes early onset cases of MJD, usually at age 20, with rapid progression of the disease and are characterized by predominant pyramidal and extrapyramidal signs - rigidity, spasticity, bradykinesia, dystonia, as well as the characteristic cerebellar ataxia, that is relatively less pronounced. Patients with type 2 ("type Thomas"), the most common type (57%), have usually an intermediate age of onset (20-50 years old) and a slow disease progression; in addition to the ataxia, patients usually present external progressive ophtalmoplegia and may, or not, have pyramidal signs. Type 3 ("type Machado" - 30%) patients more often have a late onset of the disease (40-75 years old), motor neuropathy and muscle atrophy in addition to the cerebellar ataxia.The rarest presentations are types 4 and 5. Type 4 (further described in section 4.2.) is typically associated with parkinsonism, while type 5 is associated with spastic paraplegia with no cerebellar ataxia[182]. Thus, the distinction between the different clinical types is based on extrapiramidal and motor neuron signs. However, some patients exhibit symptoms described in different clinical types and might even change among the different types as disease progresses[29,31,173,192]. In addition, there is little correlation between the CAG repeat length and the clinical presentation, although patients with type 1 usually have a greater number of CAG repeats[31,180].

4.2. Parkinsonian phenotype

Type 4 MJD has very few reports. Parkinsonian phenotype in MJD is rare and traditionally the description of parkinsonian features was associated with other more common symptomatology including cerebellar ataxia, oculomotor involvement and fasciculations or other signs of peripheral neuropathy[173]. Moreover, the clinical presentation of

parkinsonism and peripheral neuropathy should recall MJD, and the physician must rule out this disease through genetic screening[192]. From the few existing reports, some describe a presentation of the disease with at least two features of the characteristic parkinsonism triad of tremor, bradykinesia and rigidity, a positive L-DOPA therapy response and absence of atypical symptoms[29,30,31] - an indistinguishable phenotype from PD (Table 6). Progression of the disease later exposed the more typical findings of MJD, namely cerebellar signs, 8 to 10 years after parkinsonian syndrome onset[30,31].

Table 6. Clinical findings in four MJD patients

Clinical Findings	Patient 1	Patient 2	Patient 3	Patient 4
Age of onset (years)	31	30	57	50
Bradykinesia	+	+	+	-
Facial masking	+	+	+	-
Rigidity	+	+	-	-
Postural instability	-	+	+	-
Shuffling	-	+	+	-
Dystonia	-	-	-	+
Asymmetric onset	+	+	+	+
Hypokinetic dysarthria	-	+	+	-
Dopamine responsiveness	+	+	+	unknown
Resting tremor	-	+	+	-
Intension tremor	-	-	-	-
Ataxic gait	-	-	-	-
Saccadic slowing	-	+	+	+
Peripheral neuronopathy/ absent deep tendon reflexes	+	-	-	-
CAG repeats	73	67	68	75

Adapted from Gwinn-Hardy et al. 2001[29].

4.3. Neuropathological Mechanisms

The heterogeneity of clinical features of MJD relies on the multitude of system affected by the neurodegenerative process associated to the disease. Degeneration of the cerebellum, midbrain,

pons and pontine nuclei, medulla oblongata, as well as all the cranial nerves emerging from these structures (III-XII) have been described[194,195,196,197] (Figure 10). Depigmentation of *substantia nigra* was also reported[194], possibly accounting for the parkinsonian features MJD patients may develop. Additionally, a variety of nuclei and other structures, including the thalamus and the spinal cord, were found to be compromised in MJD patients[198,199,200]. Curiously, enlargement of the fourth ventricle is the most consistent feature found in magnetic resonance imaging (MRI)[201], and not the expect cerebellar and brainstem degeneration. The atrophy of these two structures is progressive and dependent on CAG repeat length[202]. Structural damage of particular anatomic regions results in impairment of several important pathways, with consequent motor, somatosensory, visual, auditory and autonomic dysfunction[196]. Blood perfusion to frontal, parietal, temporal lobes, basal ganglia and cerebellum were documented with SPECT (*single-photon emission computed tomography*) studies of MJD patient's brains[201], as well as a decrease in F-DOPA uptake in the cerebral cortex and striatum by PET (*positron emission tomography*) studies[203]. The metabolic activity in the cerebellum, brainstem, cerebral cortex, thalamus and putamen, was shown to be decreased[200]. The evolution of neurodegeneration within MJD brain is not well established, although it is known not to be synchronous among the different regions affected.

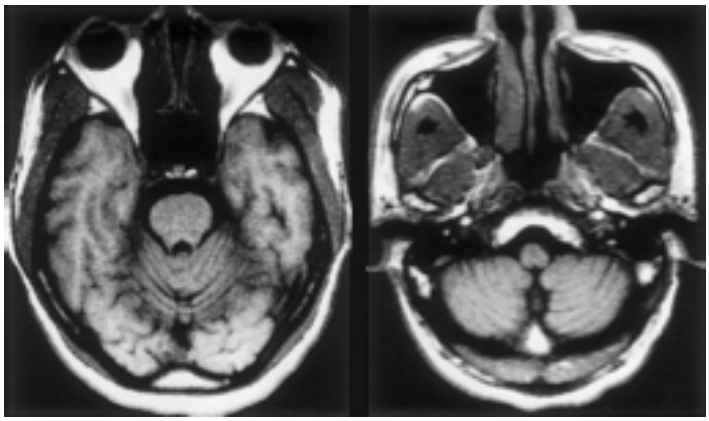

Figure 11. MRI studies of a MJD patient. The cerebellar signs (ataxia) presented by the patient correlate with the cerebellar atrophy shown (adapted from Buhman et al. 2003[30]).

4.4. The expanded ataxin-3

The broad group of conformational diseases includes polyQ diseases, in which CAG expansion in the encoding gene, creates an anomalous protein with expanded glutamine residues and altered function, including increased predisposition to aggregate. Indeed, polyQ diseases are characterized by intracellular and intranuclear neuronal inclusions, composed by these abnormal proteins[52,55]. In polyQ expansion disorders, disease severity increases with glutamine repeat number and age of onset decreases with increase of repeat number[182]. It is thus accepted that the CAG expansion in the coding gene leads to the histologic hallmarks and specific clinical phenotype of the different polyQ diseases, through mechanisms that need further clarification[182]. Expanded polyQ domain in ATX3 gene seems to confer different physiological properties to the mutant protein, when compared to the wild-type Ataxin-3, including higher propensity to misfold, and hence increased propensity to aggregate[204]. Apart from the C-terminal domain containing the polyQ tract, other regions, including the Josephin domain (at the N-terminal) determine specific protein properties, playing an important role in alterations of ataxin-3 structure, which may determine its conformational state and thus influence its tendency to aggregate[204,205].

Many explanations have been created in order to try to explain how the expanded glutamine tract give rise to disease's phenotype. All the theories rely on cellular changes induced by the expanded ataxin-3 and three hypotheses have been raised to explain the underlying pathways. One attributes the toxic effects to protein aggregation. The others, to the toxicity of expanded ataxin-3, either through interactions and roles within the cell distinct from the wild-type protein or through the toxicity of its fragments after proteolytic cleavage[57]. As described before in this thesis, a common feature to all polyQ diseases is the formation of intracellular aggregates. In MJD, the characteristic hallmark is aggregation in the form of nuclear inclusions[52,55], even though axonal inclusions have also been described[206]. Ataxin-3 aggregates contain ß-rich fibrillar structures of amyloid nature, as

observed in Alzheimer's disease[207] and as in other amyloid-forming proteins. The process of converting the soluble protein into amyloid aggregates creates intermediary metabolites; current knowledge hypothesizes that the smaller aggregates or oligomers are indeed the most cytotoxic species[208]. Moreover, these aggregates were found to sequester different proteins involved in a variety of cellular processes as transcription regulation, proteasome constituents and molecular chaperones, axonal transport and various ubiquitin-binding proteins[55,206]. Thus, the toxicity of expanded ataxin-3 could be exerted through the impairment of this crucial cellular functions. However, as for other conformational diseases, the true pathogeny of cellular aggregates, whether they are toxic or protective, is still to be understood. The finding of nuclear inclusions in unaffected brain areas of MJD patients[52,55,209] supported the suggested idea that protein inclusions could result from protective mechanisms of the cell to deal with expanded ataxin-3 toxicity[204], similar to what was proposed for Huntington's disease, another polyQ disease[210].

Whether toxic or protective, the presence of nuclear aggregates emphasizes the role played by this cellular compartment. The shuttling of wild-type ataxin-3 from the cytoplasm to the nucleus is tightly controlled[56] and exerts an important role in transcription regulation[64,65,66]; thus modifications induced by expanded polyQ tract that disrupt normal nuclear role of ataxin-3 may be detrimental to the cell[57]. A less effective degradation of expanded ataxin-3 in the nucleus when compared to the cytoplasm, could also justify the characteristic inclusion site[211]. Furthermore, the toxic fragment hypothesis supports that proteolytic cleavage of expanded ataxin-3 results in shorter fragments, described to promote aggregation and cell death[212]. Moreover, high levels of these fragments were reported in affected regions of MJD patient's and animal model's brains[211,212,213]; also, C-terminal fragments of expanded ataxin-3 were found to lead to aggregation and cell death in animal models of MJD[55,211]. Taking into account that the Josephin domain and other regions in expanded ataxin-3 are of significant importance to protein conformation[205] and contains the catalytic site of ataxin-3[50], it is therefore conceivable that proteolytic cleavage of expanded ataxin-3 is needed for the changes

in protein conformation and activity, leading to altered protein interactions and disturbance of protein normal function or increased propensity to aggregate[213]. In addition, various enzymes were found to be able to cleave ataxin-3 and lack of these cleavage sites was shown to reduce C-terminal fragment formation, and reduce protein aggregation and cellular toxicity in a drosophila model of MJD[213]. The third proposed explanatory mechanism, the gain-of-function hypothesis of expanded ataxin-3, proposes that the CAG expansion enables different protein interactions, including a stronger interaction with its substrates and, due to its higher propensity to aggregate, consequent sequestration of its substrates, with accumulation of ubiquitinated proteins in the cell[51,214]. Expanded ataxin-3 was shown to interact with typical substrates of wild-type ataxin-3[51,62]. Moreover, the extent of interaction with proteins such as p97/VCP[83] or the extent of deubiquitination of parkin [215], was found to be increased, suggesting that the expanded ataxin-3 may alter normal function of these protein within the cell[83,215]. Impairment of ERAD by compromising p97/VCP[83] and other neuroprotective pathways, such as mitophagy, which involve parkin's activity[80], are possible mechanisms through which expansion of ataxin-3 leads to MJD phenotype[57]. Apart from contributing to greater protein aggregation and increase toxicity within the cell, the pathogenesis associated with the expanded ataxin-3 may also arise through disruption of ataxin-3 normal functions[62,206,211]. Nevertheless, ataxin-3 activity has been proven not to be crucial for survival, with no morphologic abnormalities and no presentation of motor features[61]. Both ataxin-3 and expanded ataxin-3 were shown to have overall comparable deubiquitinating activities[51], although some differences have been described[62,215]. The accumulation of ubiquitinated substrates, as seen in disease associated nuclear inclusions, with aberrant or overwhelmed protein clearing mechanisms, are a probable link between CAG expansion and altered deubiquitination of the expanded ataxin-3[63]. Other roles of ataxin-3 have been also shown to be compromised. These include the role of ataxin-3 as a transcription regulator[64,65,66] , which was found to be abnormal, with increased expression[64,209] and transcriptional deregulation[216] of several genes. The finding of

axonal inclusions in degenerated areas of MJD patient's brain[206] and the role in cytoskeleton organization proposed for ataxin-3[67,68] also add disruption of axonal transportation as a potential pathway leading to neurodegeneration[206]. Finally, similarly to wild-type ataxin-3, stress may induced nuclear transport of the expanded ataxin-3[56]. Nuclear shuttling of the expanded protein would therefore place it in a compartment where its degradation could be more difficult and thus accelerate its deposition, while its toxic functions within the nucleus could further damage the cell[211].

5. A-synuclein

Synucleins (α-, ß- and γ-synuclein) are small natively unfolded proteins, ranging from 127 to 140 amino acids, encoded by three different genes: i) α-synuclein gene was mapped to chromosome 4q21.3-q22; ii) beta-synuclein to chromosome 5q35; and iii) gamma-synuclein mapped to chromosome 10q23[217]. These highly charged proteins are abundant in neurons and enriched in presynaptic terminals.

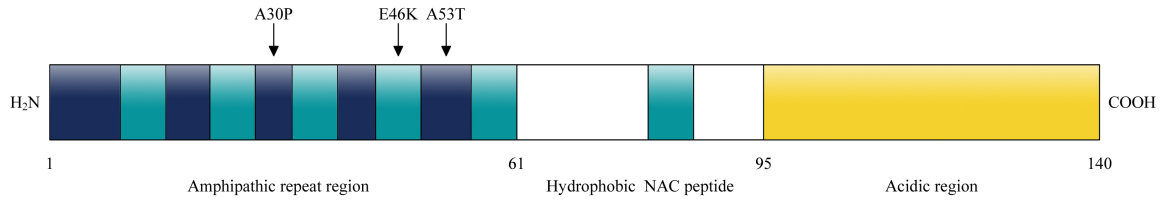

Figure 12. A-synuclein structure. The structure of α-synuclein with its three different domains and the sites for the three common mutations. (adapted from Oueslati et al. 2010[231])

A-synuclein, in particular, is a natively unstructured protein[218] with 140 amino acids of approximately 19 kDa, that can be divided into three distinct domains: i) the N-terminal domain (1-60 amino acid residues), with imperfect 11 residue repeats (KTKEFV); followed by an ii)

hydrophobic intermediate region, comprising residues 61-95 (the NAC domain); and iii) the negatively charged C-terminal domain, residues 96 to 149[219] (figure 12). The central region or NAC region is essential for α-synuclein fibrillization. The C-terminal region has been proposed to contribute to α-synulcein's thermostability and to be responsible for the majority of α-synuclein's interactions with proteins[18,20] and with dopamine[220]. This region contains most post-translational modifications and thus, increasing data suggest that stability, structure, aggregation and function of α-synuclein may be largely determined by its C-terminus[221].

A-synuclein has been identified in presynaptic terminals, cytosol, mitochondria and nucleus[147]. It localizes mostly to presynaptic terminals, where it can bind lipid membranes and regulate intracellular signaling, as it was shown to selectively inhibit phospholipase D2[147]. A-synuclein is thought to play an important role in synaptic transmission regulation and vesicle trafficking, as studies suggest that the absence of α-synuclein leads to increased rate of dopamine transmission recovery[222] and to a smaller vesicular pool[223], possibly with decreased production of synaptic vesicles[106]. Moreover, a role in vesicle fusion and docking of synaptic vesicles with the presynaptic membrane, by involvement in SNARE-complex assembly[224] and regulation of dopamine transporter (DAT), with decreased extracellular dopamine uptake by DAT[225], have been also proposed for α-synuclein. In the mitochondria, α-synuclein binds to mitochondrial respiratory complex I[226] and contributes to mitochondrial dyshomeostasis[227]. Interactions with other proteins, lipid membranes and nucleic acids, including a possible chaperone activity, are also indicated for α-synuclein[228]. Nonetheless, while there in unquestionable relationship between α-synuclein aggregation and disease (synucleinopathies), the specific role of this protein is still unknown.

Three missense point mutations have been identified - in residue 53 alanine to threonine (A53T), alanine 30 to proline (A30P) and glutamate to lysine in residue 46 (E46K)[22,229,230]. Additionally, genomic duplications and triplications of wild-type SNCA where found to increase protein expression approximately 2-fold and have concomitantly been associated to disease[175]. Moreover,

patients with SNCA triplication develop a more severe pathology than patients with duplication[175]. Apart from genetic alterations in α-synuclein, which indicate that elevated levels or abnormal function, conformation or interactions, may lead to disease; various post-translational modifications have been described for α-synuclein; these include phosphorylation, ubiquitination, nitration, truncation and sumoylation. Some of these modifications have been implicated in controlling conformation, membrane binding, aggregation and toxicity of α-synuclein[32,231]. Post-mortem studies of patients with PD, MSA, DLB and other synucleinopathies have identified α-synuclein hyperphosphorylation at serine 129 (S129)[232,233,234,235]. This residue has therefore emerged as a hallmark of α-synuclein aggregation in PD and related synucleinopathies[232,233,234,235]. Increasing evidence has supported that phosphorylation of α-synuclein at one or multiple sites may play a crucial role in regulating key features of this protein, that may ultimately lead to neurotoxicity if abnormally controlled[236,237]. Residues serine 87 (in *in vivo* and *in vitro* studies)[238,239], tyrosine 125 (*in vitro* and *in vivo*)[240,241], and tyrosines 133 and 136 (*in vitro*)[240] have been identified as additional phosphorylation sites. Studies to clarify the role of phosphorylation at Ser129 are contradictory - some show a correlation between this post-translational modification and increased toxicity to the cell[237,238], while others reach the opposite conclusion, suggesting that phosphorylation at Ser129 may be cytoprotective[236]. Moreover, whether this modification in α-synuclein has an impact in aggregation has also created a debate[151,237].

Another post-translational modification of α-synuclein is ubiquitination, which is patent in the well described hallmark of PD, Lewy bodies, with co-positivity for α-synuclein and ubiquitin of Lewy bodies[101,242]. The core of these inclusions is immunoreactive for both α-synuclein and ubiquitin, while the outer rim is apparently ubiquitin-free[242]. The co-localization of these proteins was also found in glial cytoplasmic inclusion in MSA[172]. Moreover, various E3 enzymes have been shown to influence α-synuclein, namely parkin and UCH-L1[243,244], two proteins associated to PD. Parkin overexpression was shown to reduce α-synuclein toxicity in rodents and cellular models, even

though the interaction of these proteins seems not to occur[244,245]. On the other hand, overexpression of ubiquitin had no effect on cell viability but co-expression of ubiquitin and α-synuclein were shown to diminish cell degeneration in Drosophila models[243]. Furthermore, neuroprotection was found to be potentially dependent on polyubiquitin chain linked by K48 residue, as only K48R and not K63R mutants, showed this effect[243]. Thus, neuroprotection of α-synuclein ubiquitination could arise by targeting this protein for proteasomal degradation. Nonetheless, the majority of α-synuclein species in Lewy bodies are either mono or di-ubiquitinated[232], suggesting that the ubiquitin tag in the aggregated α-synuclein may be involved in regulation of other pathways or pathophysiologic properties[219].

Truncation of α-synuclein has also been largely studied with consequent identification of C-terminal truncated species[232]. Although these species were also found in healthy individuals brains, significant differences consist in the higher levels and the almost exclusive presence of these truncated species in aggregates positive for full-length α-synuclein in PD, DLB and MSA brain tissues[246,247]. Hence, truncation of α-synuclein may be a physiologic modification, that once disrupted leads to accumulation of C-terminal truncated forms in Lewy bodies[246,247]. C-terminal truncated variants of α-synuclein show an increased tendency to fibrillization when compared to the wild-type protein[246]; in this perspective, cleavage of C-terminal domain of α-synuclein could constitute a trigger for the initiation of fibrillogenesis in PD and related synucleinopathies[219].

Conclusions from the various studies on α-synuclein post-translational modifications need to be taken critically into account, as different mechanisms, models and, most of the times, non-physiological conditions, were created. Furthermore, understanding the cross-talk between the different modifications, and the effect of cellular environment, is a demanding task that needs to be accomplished before conclusions can be readily taken.

Protein-protein interactions contribute to regulate various cellular functions and may, arguably, further amplify the damage caused by a damaging protein. Increasing evidence support that

common mechanisms may underlie neurodegeneration among different diseases - protein aggregation is an inescapable hallmark of conformational diseases and many of the proteins traditionally linked to these diseases have been shown to stimulate aggregation of other proteins prone to aggregate. Furthermore, misfolding of α-synuclein may initiate aggregation of other well-functional unfolded proteins, such as amyloid-ß peptide[248] and tau[18]. Amyloid-ß, on its turn, stimulates aggregation of α-synuclein[249].

In vitro studies have supported a direct interaction between tau and α-synuclein[18,20]. A-synuclein is able to bind tau and enhance both tau phosphorylation by protein kinase A[20] and tau fibrillization[18]. Interestingly, phosphorylated tau was identified in Lewy bodies in sporadic PD patients[250]. As described previously, Lewy bodies, PD traditional hallmark, is primarily composed of α-synuclein and ubiquitin. Other proteins that accumulate along with α-synuclein include parkin[251], synphilin-1 [252], SEPT4 and torsinA[253]. Synphilin-1 is a 90kDa protein that localizes throughout the brain, including the *substantia nigra*[254,255]. Although its role is not well characterized, it is apparently involved in synaptic vesicle pool regulation[255]. Curiously, synphilin-1 has been shown to associate with two proteins involved in PD pathogenesis, parkin and α-synuclein, and is present in α-synuclein-positive Lewy bodies[252,254,255]. The interaction between α-synuclein and synphilin-1 occurs physiologically and is influenced by α-synuclein mutations A53T and A30T[256]. Moreover, overexpression of synphilin-1 was shown to inhibit in a dose-dependent manner the degradation of α-synuclein by the UPS[257]. Lastly, α-synuclein co-aggregates with the polyQ expanded protein huntingtin[258,259] and α-synuclein overexpression induce huntingtin's aggregation[259]. Concordantly, intracellular levels exacerbated HD phenotype[260]. Recent findings suggest that α-synuclein physically interacts with huntingtin, both wild-type and expanded. Indeed, these proteins seem to influence each other patterns of aggregation and subcellular localization[261].

CHAPTER II - Interplay between α-synuclein and ataxin-3

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1. Introduction

Parkinson's disease (PD) is the most common motor neurodegenerative disorder. Since the identification of mutations in the α-synuclein (α-Syn) gene in families with hereditary PD in the late 90s[22], this protein became a target of experimental research with the objective of unraveling the mechanisms underlying PD pathogenesis. Subsequently, triplication of wild-type SNCA gene, leading to increasing levels of α-Syn, was also associated with the disease[175].

α-Syn is an ubiquitous protein known to have affinity for lipid membranes and a role on synaptic transmission[147]. It was identified as the major component of Lewy bodies, cytoplasmic protein aggregates observed in PD patient's neurons, which rapidly became the disease hallmark[101,102]. α-Syn has also been identified in other diseases, typically referred to as synucleinopathies. This group includes Multiple System Atrophy (MSA) and Dementia with Lewy Bodies (DLB) which, in addition to other clinical features, may exhibit parkinsonian features[172].

In addition to the identification of genetic mutations, several pathways, including mitochondrial dysfunction, oxidative stress and endoplasmic reticulum stress were proposed to play a role in PD pathogenesis[118,121,124]. Mechanisms involving protein aggregation and degradation are also believed to be involved in disease progression[14]. The same mechanisms have been suggested to explain the pathogenesis in other neurodegenerative diseases, namely Machado-Joseph disease (MJD), an

hereditary motor neurodegenerative disease associated with an unstable CAG expansion in the ATXN3 gene, which codes for ataxin-3 (Atx3). Interestingly, one of MJD clinical subtypes is characterized by a parkinsonian clinical phenotype[29,30,31] and L-DOPA-responsive MJD patients have been reported[192].

Apart from the characteristic involvement in synucleinopathies, α-Syn was identified as the precursor of the major non-amyloid Alzheimer's disease (AD) plaques[262.]. Succeeding data showed interaction of α-Syn with proteins involved in AD such as tau and ß-amyloid[18,20] and Huntington's disease, as is the case of huntingtin[258,259,261]. Interestingly, α-Syn has also been associated with proteins involved in PD pathogenesis, including parkin[244,245] and LRRK2[160,161].

An increasing body of evidence supports the involvement of co-aggregation of proteins in distinct conformational diseases[18,20,248,249]. Interestingly, many clinical features are shared by various of these diseases, reflecting the cellular and molecular blend of their common underlying mechanisms and the possible proteins interactions leading to disease phenotype[103,149,152]. Type 4 MJD exhibiting parkinsonism and L-DOPA response is such an example. Indeed, the report of α-Syn-positive aggregates in the *substantia nigra* of adult rats expressing expanded ataxin-3 and in the human striatum[263], led us to hypothesize an association between α-Syn and Atx3.

Focusing on the impact of α-Syn intracellular levels in disease pathogenesis and the putative role of α-Syn in MJD, we aimed to investigate in this study whether α-Syn cellular levels were influenced by Atx3 expression, both the wild-type or expanded forms. We analyzed the impact of mutant ataxin-3 in altering α-Syn intracellular levels, as well as the role of wild-type ataxin-3 in PD cellular phenotype(s). Moreover, taking into account the neurotoxicity caused by wild-type α-Syn overexpression[264,265], namely on mitochondrial dysfunction and oxidative stress, we investigated the intracellular production of reactive oxygen species (ROS) evoked by iron or rotenone (complex I inhibitor) and its modulation by Atx3.

2. Materials and Methods

Culture and transfection of SH-SY5Ywtα-syn cell line

Stable SH-SY5Y cell lines conditionally expressing wt α-Syn (SH-SY5Ywtα-syn) were kindly donated by Dr. Leonidas Stefanis (Division of Basic Neurosciences, Biomedical Research Foundation of the Academy of Athens, Soranou Efesiou, Athens, Greece), and have been previously described by Vekrellis et al. 2009[266].

SH-SY5Ywtα-syn cells were cultured at 37ºC in RMPI 1640 medium supplemented with 10% fetal bovine serum (FBS) and selection antibiotics (50 μg/ml hygromycin B and 250 μg/ml geneticin) in an atmosphere of 95% air and 5% CO_2.

α-Syn overexpression was switched off in the presence of 2 μg/mL doxycycline (DOX), a tetracycline analogue kept in the cultured medium. Maximum protein expression was achieved after 5 days without DOX.

SH-SY5Ywtα-syn cells were transfected using FuGENE® transfection reagent, previously diluted with serum-free medium (without antibiotics). Plasmids encoding for Atx3 Q20 and Q80 were added to the transfection reagent in a 6:1 ratio. The contents were mixed and the complex was incubated for a minimum of 15 minutes at room temperature. Then, the complexes were added to the cells in a drop-wise manner. The cells expressed the Atx3 plasmids for 48h.

Incubation with rotenone (100 nM) or $FeSO_4$ (2.5 mM) was performed for 2 hours at day 2 after DOX removal.

Culture and transfection of MEF cell line

MEF cells were cultured at 37ºC in DME medium with 10% FBS, maintained at 10% CO_2 as previously described by Evert et al. 1999[267]. Cells were transfected using Lipofectamine® 2000 transfection reagent, previously diluted with Opti-Mem®. Plasmids encoding for wt α-Syn, Atx3

Q23 and Q70, were diluted in Opti-Mem® and added to the transfection reagent in a 4:1 ratio. The complexes were then added to the cells. Expression was carried 24h.

Immunocytochemistry

The culture medium was removed and SH-SY5Ywtα-syn cells were washed three times in phosphate buffer solution (PBS, in mM: 137 NaCl, 2.7 KCl, 1.4 K_2HPO_4, 4.3 Na_2HPO_4, pH 7.4). Then, the cells were fixed with 4% paraformaldehyde for 10 min. Further 3 washes with PBS were made. Cell membrane was permeabilized with 0.2% Triton X-100 (in PBS) during 2 min and subsequently blocked in 3% BSA/PBS. After washing with PBS, the cells were incubated for 1h at room temperature with rabbit anti-ubiquitin (1:200), rabbit anti-ataxin-3 (1:1000), mouse anti-α-Syn (1:200) and mouse anti-ataxin-3 (1:1000) were prepared in 3% BSA/PBS. Then, three washes were carried out with PBS, followed by incubation with secondary antibodies anti-mouse IgG Alexa-fluor 594 (1:200) and anti-rabbit IgG Alexa-fluor 488 (1:200) were incubated in 3%BSA/PBS, for 1 h, at room temperature. Finally, the cells were stained with Hoechst 33342 (4µg/mL in PBS) was performed, for 15 minutes. The coverslips were mounted using DAKO solution. Cells were visualized using a confocal microscope LSM 510 Meta.

Analysis of intracellular reactive oxygen species

After washing with PBS, SH-SY5Ywtα-syn cells were incubated for 30 min with 20 µM H_2DCF-DA (2',7'-dichlorodihydrofluorescein diacetate), a stable non-fluorescent cell permeable compound, at 37ºC, in Krebs medium (in mM: 135 NaCl, 5 KCl, 0.4 KH_2PO_4, 1.8 $CaCl_2$, 1 $MgSO_4$, 20 HEPES and 5.5 glucose) pH 7.4. H_2DCFDA is incorporated by the cells and hydrolysed by esterases to form H_2DCF, which is converted to DCF by intracellular peroxides and emits fluorescence (LeBel et al., 1993). Intracellular levels of peroxides were measured by following DCF fluorescence (488 nm

excitation, 530 nm emission) at 37ºC, continuously, for 1 h, using a Microplate Spectrofluorometer Gemini EM (Molecular Devices, USA).

After the assay, cellular protein content was quantified using the Bradford assay (Bio-Rad).

Western Blotting - MEF cells and mice brain

Soluble protein for western blotting was harvested from MEF lysates cells and total brains of transgenic mice in 50 mM Tris-HCl, pH 8.0, on ice, containing 120 mM NaCl, 5 mM EDTA, 0.5% NP-40, 2 µg/ml aprotinin, 100 µg/ml PMSF and 10 µg/ml leupeptin, followed by high speed centrifugation at 4ºC. Supernatants were removed and protein concentration was determined using the Bradford assay (Bio-Rad). For electrophoresis, 40 µg protein were heat-denatured by 8 minutes boiling in Laemmli loading buffer and separated by 10% SDS-PAGE and electroblotted to nitrocellulose membranes. The membranes were blocked for 1 h in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5% skim milk (Difco, Detroit, MI), 2% BSA and 0.1% Tween 20 and subsequently incubated with the primary antibodies mouse 1H9 (Chemicon, 1:1000 in blocking buffer) and mouse α-synuclein was performed overnight, at 4ºC. In the next day the membranes were incubated with the secondary antibody anti-mouse IgM (1:20000 in PBS, 0.1% Tween 20) for 1 h, at room temperature. Detection was performed using enhanced chemiluminescence (ECL) detection kit (Amersham, Braunscheig, Germany).

Generation of transgenic mice and mouse genotyping was done as described in Bichelmeier et al. 2005[268]. The transgene expression (human atx3 with 15 or 70 repeats) is regulated through the mouse prion promoter (C57BL/6J strain).

Western Blotting - SH-SY5Ywt-α-syn

Total extracts from SH-SY5Ywt-α-syn were collected, separated on SDS-PAGE 10% gels and electroblotted onto polyvinylidene difluoride (PVDF) membrane in CAPS/methanol 10% at 320A. The membranes were blocked with 5% non-fat milk solution for 60 minutes and incubated with Ataxin-3 mouse antibody (Chemicon MAB5360, 1:1000), α-synuclein rabbit anti-body (Cell Signaling, 1:1000), tubulin mouse anti-body (Sigma T-6199, 1:2000) and ubiquitin rabbit anti-body (DakoCytomation, 1:200), overnight, at 4ºC. Incubation with alkaline phosphatase-conjugated secondary antibody (1:20000) was performed during 1 h at room temperature. Detection was done using an enhanced chemifluorescent reagent (ECF) and BioRad VersaDoc 3000 Imaging System.

Statistical analysis

Data were expressed as mean ± SEM of the number of experiments indicated in the figure legends. Comparisons among multiple groups were analyzed with one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. Significance was defined as $p<0.05$

3. Results

3.1. *Correlation between α-synuclein intracellular levels and ataxin-3 expression*

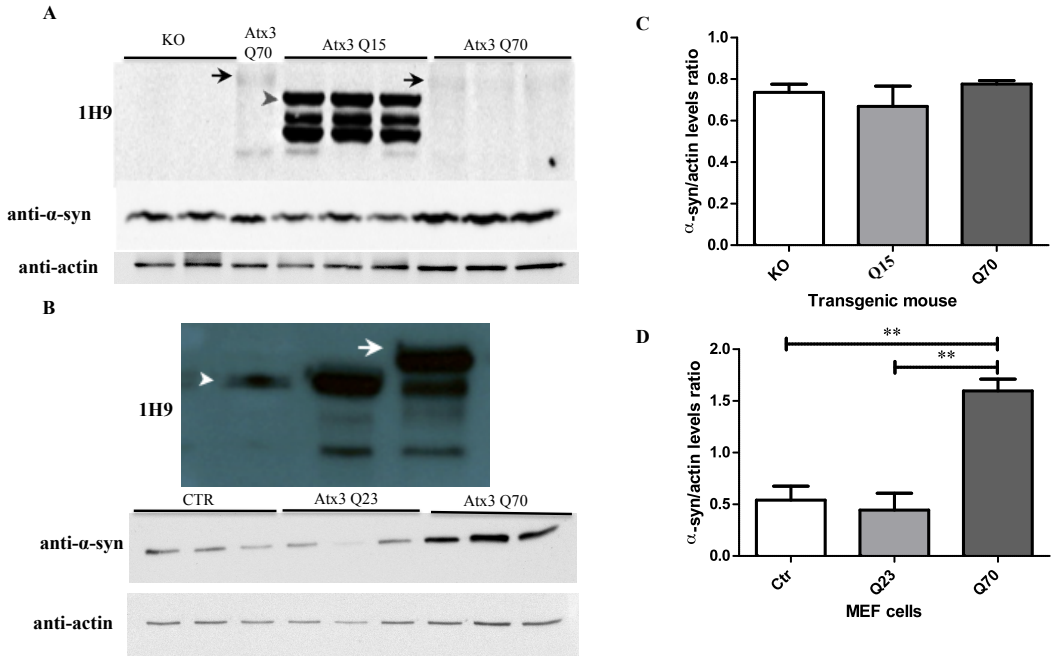


Figure 1. Analysis of α-Syn levels and Atx3 levels in co-transfected MEF cells and brains of transgenic and knock-out mice: Atx3, α-Syn and actin was immunostained in mice total brain **(A)** and MEF cells co-transfected with α-Syn and Atx3Q23 or Q70 **(B)** using 1H9 antibody, anti α-Syn and anti-actin antibodies, respectively (➔ shows the expanded band of Atx3, while ➤ the normal length band). **(C)** Graph shows the ratio between α-Syn levels and actin levels in knock out (KO) and wild type (Q15) or expanded (Q70) transgenic mice. Data represents the mean±SEM of 3 independent experiments. **(D)** Graph shows the ratio between α-Syn levels and actin levels in MEF cells co-trasnfected with α-Syn and Q23 or Q70 Atx3. Data represents the mean±SEM of 3 independent experiments Statistical analysis: **p<0.01 significantly different using One-way ANOVA followed by Tukey multiple comparison test.

In order to study the effect of Atx3 in α-Syn intracellular levels, we first transfected MEF cells (previously described by Evert et al. 1999[267]) with plasmids coding for eGFP, Atx3 Q22 or Atx3 Q70,which were co-transfected with α-Syn, and expression was carried out for 24h.

Co-transfection of α-Syn proteins and Atx3 was regularly achieved in MEF cells (Figure 1B). α-Syn levels were significantly increased in cells co-transfected with Atx3 Q70 in comparison to cells co-transfected with Atx3 Q23 and non-transfected cells (control, retaining Atx3 endogenous levels)

(Figure 1B) Additionally, although not significant, cells co-transfected with α-Syn and Atx Q23 apparently displayed slightly lower levels of expression of α-Syn when compared to the control (Figure 1B).

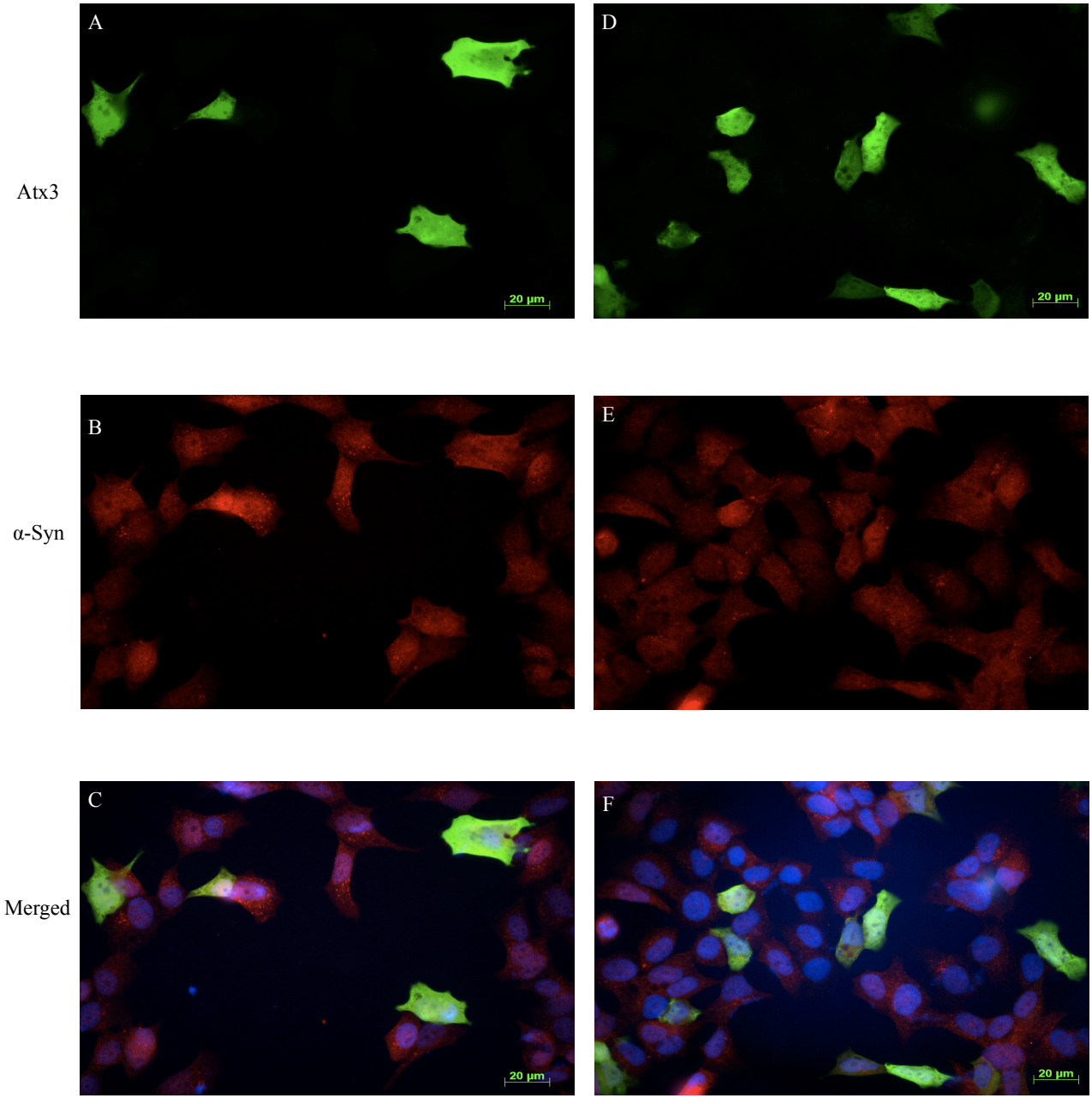
The Atx3 staining in mice brain samples is accordingly to what has been previously described[266] (Figure 1A). In this experiment we used transgenic knock-out mice for Atx3 or transgenic mice expressing Atx-3 Q15 or Atx3 Q70 regulated by the mouse prion promoter (Bichelmeier et al. 2005)[266]. Endogenous α-Syn was readily visible in all samples (Figure 1A), but no differences were found in α-Syn levels of knock-out mice and wild-type/expanded Atx3 transgenic mice.

3.2. Analysis of α-synuclein and ubiquitin cellular levels in transfected SH-SY5Ywtα-syn cells

Immunocytochemical analysis of transfected SH-SY5Ywtα-syn cells evidenced positive Atx3-transfected cells (Q22 or Q80). Cell conditionally overexpressing wild-type α-Syn through DOX removal from the growth medium showed a higher staining for this protein (Figure 2E,F and 2K,L).

There were no significant changes in α-Syn levels between cells expressing Atx3 at endogenous levels and cells overexpressing Atx3 Q22 or Q80 for 48 h (Figure 2). Moreover, no aggregates of α-Syn or Atx3 were observed in any of these cell lines.

Nonetheless, in the majority of Atx3 transfected cells, the ubiquitin staining appeared to be higher when compared to neighboring non-Atx transfect cells (Figure 3). A slight increase in ubiquitin levels is also observed after α-Syn overexpression (compare figure 2B and 2H to 2E and 2K). Again, no aggregates were observed in these cells that co-localized for Atx3 and/or ubiquitin.


Atx3
α-Syn
Merged
A
B
C
D
E
F
20 µm
20 µm
20 µm
20 µm

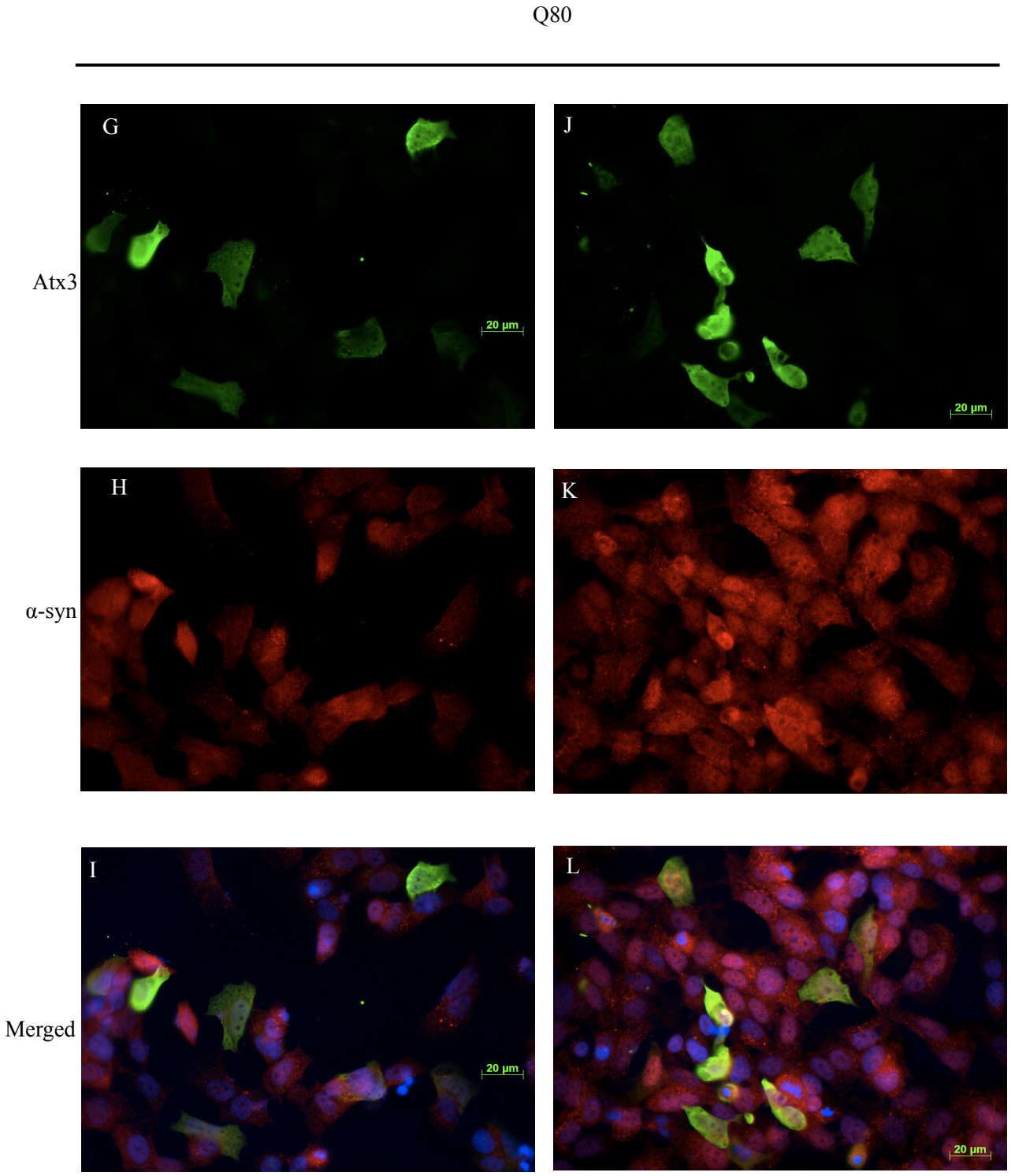
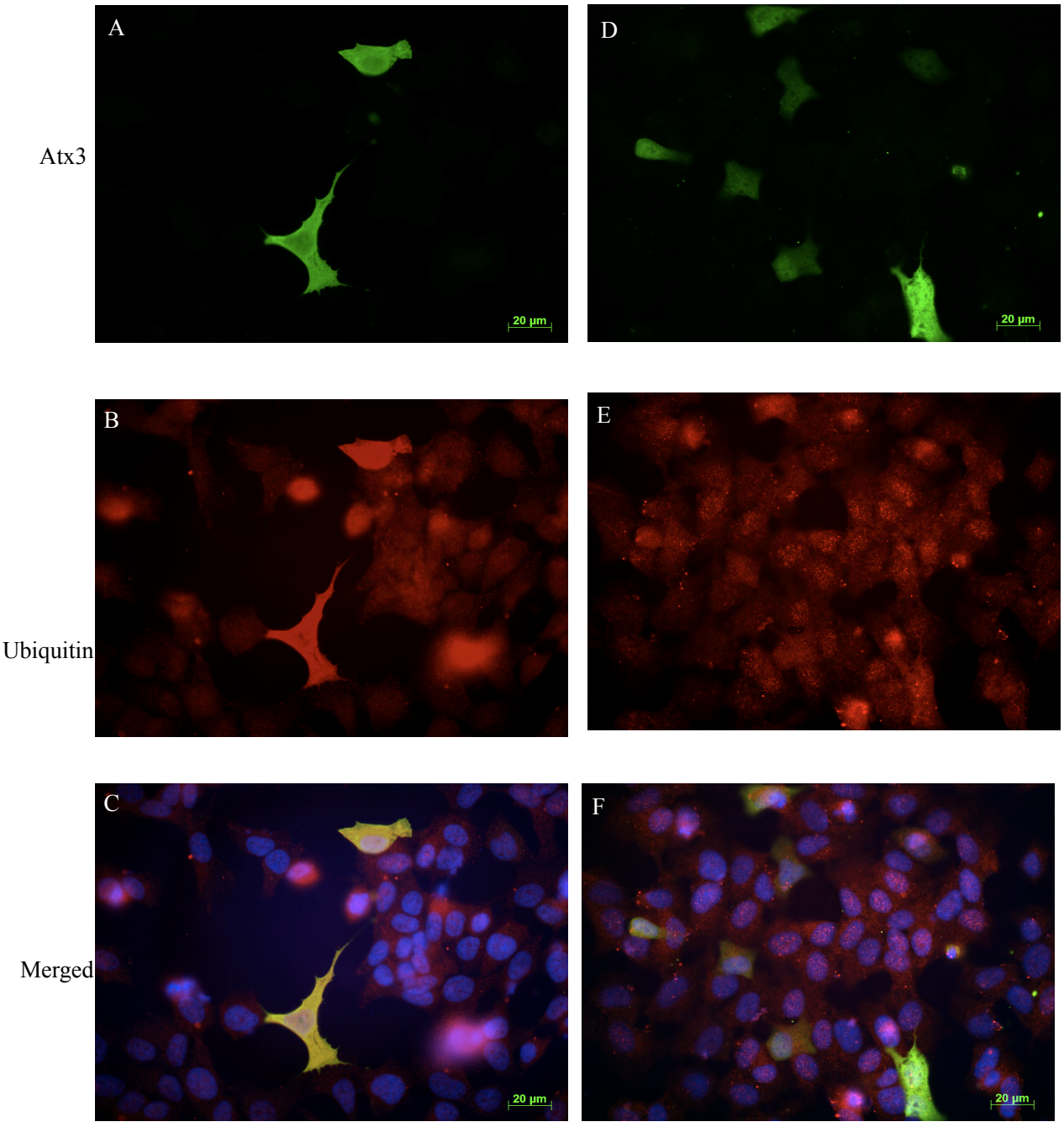


Figure 2. α-Syn cellular levels in transfected SH-SY5Ywtα-syn cells. A,D: Atx3 Q22; B,E: α-syn; C,F: Merged (Green Atx3 Q22, red α-Syn, blue Hoechst)); G,J: Atx3 Q80; H,K: α-Syn; I,L: Merged (Green: Atx3 Q80, red α-Syn, blue Hoechst); Images A-C and G-I correspond to cells expressing endogenous α-Syn, while images D-F and J-L correspond to cells overexpressing α-Syn for 48 h. Cells were transfected with Atx3 Q22 or Atx3 Q80 for 48 h.

Q22



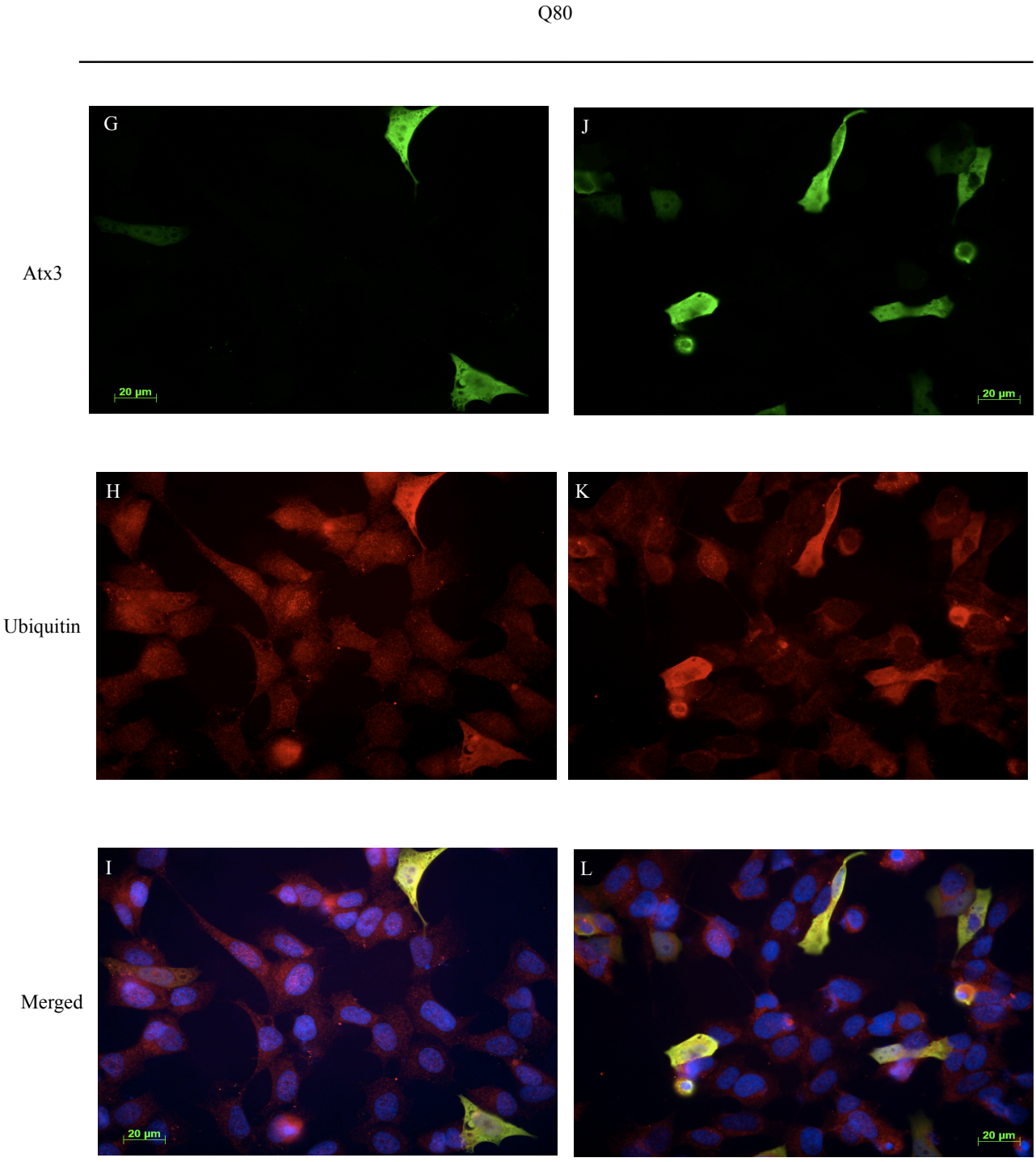


Figure 3. Ataxin-3 and α-synuclein overexpression appear to influence ubiquitin cellular levels in transfected SH-SY5Ywtα-syn cells. A,D: Atx3 Q22; B,E: ubiquitin; C,F: Green Atx3 Q22, red ubiquitin, blue Hoechst (merged); G,J: Atx3 Q80; H,K: ubiquitin; I,L: Green: Atx3 Q80, red ubiquitin, blue Hoechst (merged); Images A-C and G-I correspond to cells expressing endogenous α-Syn, while images D-F and J-L correspond to cells overexpressing α-Syn for 48 h. Cells were transfected with Atx3 Q22 or Atx3 Q80 for 48 h.

3.3. *Intracellular ROS formation in transfected SH-SY5Ywtα-syn cells*

Oxidative stress is constantly suggested as a etiologic factor for various diseases, namely PD and MJD[121,269]. Thus, we evaluated the influence of α-Syn and Atx3, two proteins widely associated to these diseases, in ROS formation.

In the absence of exogenous stimuli, Atx3 Q22 produced similar hydroperoxide levels to control, while both Atx3 Q80 and α-Syn exhibited a statistically significant increase in the production of ROS species (figure 4). Moreover, Atx3 Q22 seems to slightly decrease ROS production in the presence of α-Syn. However, this result was not statistically significant.

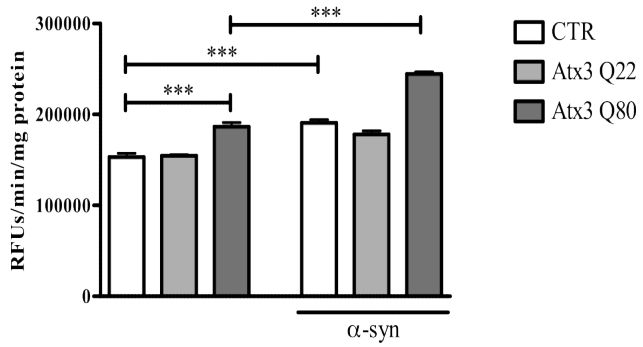


Figure 4. Analysis of hydroperoxide production by H_2DCFDA. Cells were transfected with eGFP (control), Atx3 Q 22 or Q80 for 48 h. All cell lines overexpressed α-Syn for 48h. Data are the mean ± SEM of triplicates from 3 independent experiments. Statistical analysis: ***p < 0.001 significantly different using One-way ANOVA followed by Tukey multiple comparison test.

Strikingly, besides the α-Syn and atx3 Q80 individual increase in the intracellular levels of hydroperoxides, cells overexpressing both α-Syn and transfected with atx3 Q80 exhibited significantly higher levels of hydroperoxides in comparison to Atx3 Q80 cells expressing α-Syn at endogenous levels (figure 4)

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3.4. *Intracellular ROS formation in transfected SH-SY5Ywtα-syn cells after exposure to iron*

SH-SY5Ywtα-syn cells were exposed to iron (2.5 mM of $FeSO_4$) for 2 h and before the evaluation hydroperoxide formation. The cumulative effect of Atx3 Q22 or Q80 over iron-induced hydroperoxide production was assessed in cells under endogenous or overexpression levels of α-Syn (figure 5).

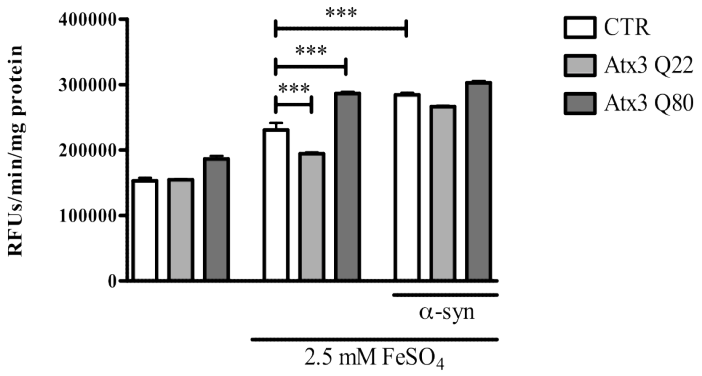

Figure 5. Analysis of hydroperoxide production by H_2DCFDA after exposure to iron. Cells were transfected with enhanced Green Fluorescent Protein (eGFP)/CTR, Atx3 Q22 or Atx3 Q80 for 48 h. All cell lines overexpressed α-Syn for 48h. Data are the mean ± SEM of 3 independent experiments run in triplicates. Statistical analysis: ***$p < 0.001$ significantly different using one-way ANOVA followed by Tukey multiple comparison test.

Exposure of SH-SY5Ywtα-syn cells to iron for 2h augmented the levels of DCF fluorescence in all conditions tested, suggesting an iron- induced increase in intracellular peroxides formation (figure 5).

Cells under endogenous expression of α-Syn showed significantly less fluorescence exposed to iron in comparison to cells overexpressing this protein, suggesting that overexpression of α-Syn exacerbates iron induced-hydroperoxide formation. Transfection of Atx3 Q80 largely increased DCF fluorescence following exposure to iron, when compared to cells expressing endogenous Atx3 or Atx3 Q22. On the other hand, cells transfected with Atx3 Q22 and exposed to iron showed significantly lower levels of fluorescence when compared to iron exposed cells expressing

endogenous atx3. Furthermore, and although not statistically significant, the transfection of Atx3 Q22 in cells exposed to iron and overexpressing α-Syn seems to result in lower levels of fluorescence when compared to cells transfected with eGFP and expressing endogenous levels of wild-type ataxin-3, which may indicate a protective and possibly antioxidant effect induced by Atx3 Q22.

These results clearly reveal a cumulative effect of Atx3 Q80 and α-Syn on ROS generation upon exposure to iron.

3.5. *Intracellular peroxide formation in transfected SH-SY5Ywtα-syn cells after exposure to rotenone*

In order to correlate our results of increased ROS formation with mitochondrial dysfunction, another well-established pathogenic mechanism in PD[107, 108, 121] and also associated to MJD[269], we evaluated whether hydroperoxide formation was influenced by the exposure to rotenone, a mitochondrial complex I inhibitor.

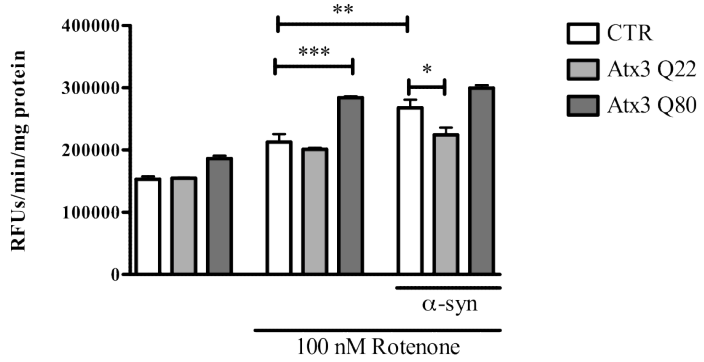

Figure 6. Analysis of hydroperoxide production by H_2DCFDA after exposure to rotenone. Cells were transfected with eGFP (CTR), Atx3 Q 22 or Atx3 Q80 for 48 h. All cell lines overexpressed α-Syn for 48h. Data are the mean ± SEM of 3 independent experiments run in triplicates. Statistical analysis: * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ significantly different using One-way ANOVA followed by Tukey multiple comparison test.

Exposure to rotenone (100 nM rotenone) for 2h increased DCF fluorescence in all conditions tested, when compared to the respective controls (figure 6). Atx3 Q80 overexpression in cells exposed to rotenone induced a significant increase in ROS production, when compared to rotenone control and cells transfected with Atx3 Q22 and incubated to rotenone. Additionally, a significant increase in ROS production was also observed when cells were exposed to rotenone and overexpressing α-Syn in comparison to cells exposed to rotenone but expressing a-syn at endogenous levels (figure 6). Interestingly, transfection of Atx3 Q22 induced a significant decrease in ROS formation in cells overexpressing α-Syn and exposed to rotenone (figure 6).

4. Discussion

Apart from the missense mutations previously identified (A30P, A53T and E46K), SNCA locus duplications and triplications have all been implicated in rare forms of familial PD[22,229], although the underlying pathways linking them to neuronal cell loss are still unclear. Studies have shown a role for α-Syn in ubiquitin-proteasomal processing, oxidative injury and mitochondrial dysfunction[243,264,265]. On the other hand, Atx3 has a deubiquitinating activity, with ability to bind and cleave polyubiquitin chains, and, consequently, to target proteins for proteasomal degradation[62]. The expanded form of the protein has been shown to promote protein misfolding leading to protein aggregation in MJD[49].

Our results suggest that higher levels of intracellular α-Syn may be influenced by expression of expanded ataxin-3. The accumulation of α-Syn could result, in part, from the loss of function of normal ataxin-3 and particularly its deubiquitinating activity, which would therefore be committed with tagging α-Syn for proteasomal degradation. Another explanation relies on a possible gain of function of the expanded protein, with disruption of cellular mechanisms contributing for α-Syn clearance within the cell. Furthermore, polyQ proteins have been associated to dysfunction of the two major protein degradation systems within the cell, the UPS[270] and autophagic pathways[271]. Taking these reports into account, disruption of the normal pathways that eliminate altered proteins by expanded Atx3 could therefore lead to accumulation of α-Syn, explaining the higher intracellular levels of α-Syn reported by us.

The role of wild-type Atx3 in reducing expanded protein toxicity has been defended and contradicted. Supporters suggest that the normal activity of ataxin-3 and its involvement in the UPS may target the expanded protein for degradation, thus decreasing its toxicity[272]. This would also explain the presence of wild-type Atx3 in aggregates positive for the expanded protein[55]. The protective effects of wild-type Atx3 could also contribute to explain why milder phenotypes are presented by heterozygous individuals, although gene dosage has been used as an explanation[57].

Contradicting results in a mice have suggested that similar phenotypes arise from co-expressing both wild-type compared to expression of only the expanded form of the protein[273]. Additionally, overexpression of wild-type Atx3 in a lentiviral rat model of MJD did not prevent development of MJD typical phenotype[274]. The wild-type form of Atx3 was shown to be present in aggregates in human *substantia nigra*, suggesting that Atx3 may help to promote α-Syn proteolysis[275]. Our data further suggest that increased expression of wild-type Atx3 might decrease α-Syn levels, when compared to the mutant protein and, arguably, when compared to cells expressing endogenous wild-type ataxin-3. Nonetheless, we did not achieve similar results with endogenous α-Syn expression when tested in the mice model of MJD, suggesting that such protein changes may require significant levels of the proteins. Additionally, we show that cells transfected with wild-type Atx3 exhibit lower ROS levels when compared to the expanded protein, and suggest a role for wild-type Atx3 in dealing with rotenone and iron-induced oxidative stress.

Increasing data suggests that misfolding or aggregation of one protein could induce the deposition of other aggregated-prone proteins[18,20,248,249]. Concordantly, co-aggregation of α-Syn and Atx3 have been reported in a rat *substantia nigra* and human striatum after expression of expanded Atx3. Moreover, these aggregates were not detected upon exposure of wild-type Atx3[263]. Apart from α-Syn and Atx3, ubiquitin was detected in the aggregates, such as seen in typical α-Syn-positive aggregates - the Lewy bodies. Nonetheless, the presence of ubiquitin does not necessarily reflect α-Syn ubiquitination, but of any protein passible of being ubiquitinated, including Atx3[276]. The presence of ubiquitinated α-Syn could otherwise reflect the interaction with the expanded Atx3 ubiquitin-interacting motifs (UIM), culminating in its recruitment to Atx3-positive aggregates[263].

Following 48 h of expression of both proteins (α-Syn and Atx3, Q22 or Q80) our results showed no co-aggregation between α-Syn, ubiquitin or Atx3, and no proteinaceous aggregates associated with expanded Atx3 expression were detected. Nonetheless, ubiquitin cellular levels seem to be increase by Atx3 and α-Syn expression.

In addition to the influence in the UPS and the autophagic pathway, polyQ proteins have been described to have an impact on the crosstalk between the proteasome and the mitochondria[270]. Altered mitochondrial structure and deregulated mitochondrial activity, including increased caspase pathway activation and mitochondrial swelling, have been associated to polyQ disorders[277]. Reduction of enzymes involved in antioxidant defenses with increased mitochondrial DNA damage and increased activation of caspase pathways have been also described in cellular models of MJD[278]. Additionally, decreased activity of mitochondrial complex II and increased tendency towards its impairment have been reported to be associated with expanded Atx3[269].

As for MJD, PD has been thoroughly linked to mitochondrial dysfunction and related oxidative stress. Moreover, α-Syn is suggested to localize to the mitochondria, where it may interact with mitochondrial complex I[264,265].

In the present study we demonstrate a clear correlation between ROS formation and expression of both α-Syn and expanded Atx3. In fact, greater ROS formation is achieved after expression of these two proteins, in the absence of exogenous stimulus. Moreover, iron, an oxidative stress inducer, and rotenone, a complex I inhibitor, largely increased the oxidative stress of α-Syn and expanded Atx3.

Augmented brain iron accumulation beyond observed in controls of similar age was previously observed in PD patients' brains[279], and postmortem and imaging studies have corroborated the increased iron deposits in selected brain regions, namely the *substantia nigra*[28,103]. The extent of *substantia nigra* deposits correlates with disease severity in PD[280]. Additionally, increased levels of ROS production have been described after exposure to iron[235]. Numerous studies have associated α-Syn to increased neuronal susceptibility to iron-induced toxicity, with significant disruption of normal mitochondrial membrane potential[281]. Iron directly binds α-Syn and this interaction may result in a conformational change of α-Syn that may further promote its aggregation[282].

On the other hand, as various reports show[283] and our results support, α-Syn exacerbates iron-induced oxidative stress. Importantly, the expanded Atx3 also seems to exacerbate iron-induced ROS formation, a finding that may be of significant importance due to the compromise of *substantia nigra* in some MJD patients[194].

Another common key pathogenic mechanism in PD and MJD is mitochondrial dysfunction. Despite the fact that no evidence has been reported for mitochondrial complex I involvement in MJD pathogenesis, we show that upon exposure with rotenone, a potent complex I inhibitor, there is increased susceptibility to ROS formation in cells expressing expanded Atx3.

Taken all together, our studies show that α-Syn and expanded Atx3 exhibit increased susceptibility to toxic stimuli, as evidenced through the formation of ROS, when compared to cells expressing endogenous levels of the respective wild-type proteins. Furthermore, α-Syn levels might be influenced by expanded Atx3 expression.

Concluding Remarks

In this thesis, we reviewed some of the mechanisms that contribute to proteostasis and discuss how their deregulation can lead to conformational diseases, namely PD and MJD. Additionally, we investigated the association between α-synuclein and ataxin-3.

Various neurodegenerative diseases have been described and characterized by the most common set of symptoms that patients displayed. Nonetheless, they often share many features. Motor neurodegenerative diseases, as both PD and MJD, may present neuropsychiatric symptoms such as dementia or depression, while there are reports of AD patients presenting with parkinsonism. Moreover, various neurodegenerative disorders enter the clinical differential diagnosis of PD, as they may present with an association of clinical features, traditionally described for PD.

Accompanying the miscellaneous clinical presentations of these diseases, many underlying mechanisms have been shown to contribute for the pathogenesis of distinct neurodegenerative disorders. The most obvious and undeniable factor contributing for the pathogenesis of this set of diseases is age. Some authors believe that a possible way through which age may influence pathogenesis is through progressive decline of the mechanisms contributing for cellular well-functioning or, through progressive overwhelming of the same mechanisms.

In this thesis, we chose to specifically focus in PD and MJD, as they are predominantly motor disorders and type 4 MJD may present with a clinical syndrome indistinguishable from PD. Furthermore, both diseases have been associated with *substantia nigra* depigmentation, a finding that is characteristically correlated to the compromise of the nigrostriatal pathway.

The mechanisms underlying these disorders include deregulation of the pathways involved in the clearance of aggregate-prone proteins, oxidative stress and mitochondrial dysfunction, among others.

Studies on ataxin-3 and α-synuclein have clarified some functions and interactions of these proteins, although much is left to be discovered.

Ataxin-3 is a DUB enzyme and lower levels or impairment of its catalytic site have been associated with accumulation of misfolded proteins within the cell. Other roles have been described for ataxin-3, including transcription regulation. The expanded ataxin-3 has been shown to interact with some of the wild-type protein substrates and to be able to be shuttled to the nucleus. Additionally, although wild-type ataxin-3 is considered an aggregate-prone protein, the polyQ expansion further predisposes ataxin-3 for deposition. Wild-type ataxin-3 has been identified in aggregates positive for the expanded form of the protein, suggesting that the expanded form may be a substrate of the wild-type protein.

Another aggregate-prone protein is α-synuclein. Mutations have been described for α-synuclein, however studies show that overexpression of the wild-type form of this protein is toxic for the cell as it increases oxidative stress and induces mitochondrial dysfunction.

Moreover, we reviewed increasing evidence associating α-synuclein in other diseases apart from PD. These, not only include the predicted synucleinopathies, but other diseases such as AD and HD. Thus, it is conceivable that a meshwork of mechanisms, and particularly protein interactions, underlie a common pathogenic basis for neurodegeneration, rather than a single protein inducing disruption of different pathways within the cell.

Therefore, on the second part of this thesis we studied the interplay between α-synuclein and ataxin-3. We hypothesized that the normal function of ataxin-3 could prevent α-synuclein-associated toxicity by promoting its degradation and that expanded ataxin-3 could further increase α-synuclein effects.

Our results show a detrimental role for the expanded ataxin-3 in α-synuclein levels. Furthermore, we show that both α-synuclein and expanded ataxin-3 *per se* increase oxidative stress levels within

the cell, and that they may have a synergistic effect on increasing ROS formation. Moreover, our results suggest a putative protective role for ataxin-3 in dealing with oxidative stress induced by iron and rotenone, which may be related with decreased protein clearance.

To the best of our knowledge these are the first published studies committed to understand the role of the interplay between α-synuclein and ataxin-3.

Any process that leads to disruption of the redox potential within the cell can have drastic effects, including cell death. Decreased glutathione levels and deficiencies in brain antioxidant enzymes have been associated to PD pathogenesis. Thus, further studies are required to evaluate whether the toxicity of the expanded ataxin-3 and α-synuclein involve the decrease in these protective enzymes, and whether the wild-type protein induces to an increase in antioxidant activity. Understanding the toxicity of this interaction and the role played by the cellular mechanisms that clear up aggregate-prone proteins, the UPS and the autophagy pathways, could further clarify the effects of this interaction. Moreover, it is still unclear whether the two proteins (α-synuclein and ataxin-3) directly interact. Additionally, and taking into account the effects exerted by rotenone (a complex I inhibitor), it would be interesting to analyze whether the other complexes of the mitochondrial chain are affected by the expression of these proteins and whether this is linked to mitochondrial dysfunction.

Acknowledgments

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Esta tese é por vocês.

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