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Mitochondrial fusion and fission regulation in Parkinson's Disease

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Professora Doutora Sandra Morais Cardoso (Universidade de Coimbra) e supervisão da FCTUC pela Professora Doutora Emília Duarte (Universidade de Coimbra).

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Contents

Abbreviations

6-OHDA, 6-Hydroxydopamine

ASYN, α-synuclein

ATP, adenosine triphosphate

BSA, bovine serum albumin

CCCP, Carbonyl cyanide m-chlorophenyl hydrazone

CMT2A, Charcot-Marie-Tooth subtype 2A

CXI, complex I

Cybrid, cytoplasmatic hybrid

DHE, dihydroethidium

Drp1, Dynamin related protein 1

DTT, Dithiothreitol

ETC, electron transport chain

FCCP, carbonylcyanide-p-trifluoromethoxyphenylhydrazone

LB, Lewy bodies

Leucine-rich repeat kinase 2, LRRK2

Mff, mitochondrial fission factor

MMP, mitochondrial membrane potential

MPP^+, 1-methyl-4-phenylpyridinium

MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

Mfn1, mitofusin-1

Mfn2, mitofusin-2

mtDNA, mitochondrial DNA

NURR1, Nuclear receptor related 1 protein

OMI/HTRA2, Heat transfer requirement 2

Opa1, Optic atrophy 1

OXPHOS, Oxidative phosphorylation

PBS, phosphate-buffered saline

PINK1, PTEN-induced putative kinase 1

PD, Parkinson's disease

ROS, reactive oxygen species

SNpc, substantia nigra pars compacta

Resumo

A doença de Parkinson é a segunda doença neurodegenerativa mais comum e a doença neurodegenerativa associada ao movimento mais comum. É causada pela perda dos neurónios dopaminérgicos na *substantia nigra pars compacta* levando a um défice de dopamina no estriado. Perceber a base molecular da doença de Parkinson tem se revelado um grande desafio no campo das doenças neurodegenerativas. Apesar de terem sido propostas várias hipóteses para explicar os mecanismos subjacentes a patogenia da doença de Parkinson, um crescente corpo de evidencias tem enfatizado o papel da disrupção da dinâmica mitocôndrial como um grande contribuidor para a etiopatogenia da doença de Parkinson. Tem se vindo a acumular dados que sugerem que uma dinâmica mitocondrial anormal se encontra envolvida na disfunção mitocôndrial ou medeia a morte neuronal em diferentes modelos da doença de Parkinson. Aliás, a integração da fissão, fusão e autofagia mitocondrial forma um mecanismo de manutenção de qualidade mitocôndrial da homeostase mitocôndrial na qual defeitos na função mitocôndrial têm sido associados à doença de Parkinson. A maioria dos casos surge como condição esporádica e os restantes são herdados com mutações em vários genes que tem sido ligados a essas formas genéticas da doença. Os estudos de duas das proteínas ligadas as formas familiares da doença, nomeadamente a PINK1 e a Parkin, forneceram evidências que estas duas proteínas actuam na mesma via regulando a fissão e fusão mitocôndria e a mitofagia. Uma vez que uma dinâmica mitocôndrial anormal tem sido cada vez mais implicada na patogenia da doença de Parkinson, nesta tese, investigámos a regulação da dinâmica mitocôndrial em diferentes modelos celulares da doença de Parkinson. Nos observamos que uma diminuta localização mitocôndrial da OPA1 leva a um dano da dinâmica mitocôndrial na maioria dos modelos celulares de

Parkinson. Para além do mais, a clivagem das isoformas longas da Opa1 parecem ser responsáveis pelo padrão de fragmentação observado nos modelos celulares de PD esporádicos derivados da mitocôndria. A sobrexpressão da alpha-synucleina induz a tubulação da rede mitocondrial com estruturas elongadas que são o oposto do observado em modelos celulares de PD. Inesperadamente, a sobrexpressao da Opa1 não resgatou o aumento de produção de espécies reactivas de oxigénio induzida pelo MPP^+. Globalmente, as nossas observações sugerem que uma fusão mitocondrial dependente da Opa1 desempenha um papel crucial na mediação nas anormalidades mitocôndriais e disfunção celular induzida MPP^+/mtDNA.

Estes estudos sugerem que a dinâmica mitocôndrial desempenha um papel importante na patogenia da doença de Parkinson, e um melhor entendimento destes mecanismos podem levar à descoberta de novos alvos terapêuticos para esta doença.

Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disorder and the most common neurodegenerative movement disorder. It is caused by the loss of dopaminergic neurons in the substantia nigra pars compacta leading to a dopamine deficit in the striatum. Understanding the molecular basis of PD has proven to be a major challenge in the field of neurodegenerative diseases. Although several hypotheses have been proposed to explain the molecular mechanisms underlying the pathogenesis of PD, a growing body of evidence has highlighted the role of mitochondrial dynamics disruption as a major contributor to PD etiopathogenesis. Accumulating data suggests that abnormal mitochondrial dynamics is involved in mitochondrial dysfunction or mediates neuronal death in different PD models. Moreover, integration of mitochondrial fusion, fission and mitochondrial autophagy forms a quality maintenance mechanism of mitochondrial homeostasis that defects in mitochondrial function have been associated with PD. Most of the cases arise as sporadic conditions and the others are inherited with mutations in several genes being linked to these genetic forms of PD. Studies of two of the proteins linked to the familial forms of the disease, namely PINK1 and Parkin, provided evidence that these two proteins act in the same pathway regulating mitochondrial fusion, fission and mitophagy. Because abnormal mitochondrial dynamics are increasingly implicated in the pathogenesis of PD, in this thesis, we investigated the regulation mitochondrial dynamics in different PD cellular models. We observed that a decreased OPA1 mitochondrial localization-drive mitochondrial dynamics impairment in most cellular PD models. Moreover, OPA1 long isoforms cleavage seems to be responsible for mitochondrial fragmented pattern observed in sporadic mitochondrial-driven cellular PD models. Alpha-synuclein overexpression induces a tubular mitochondrial network

with elongated structures that is the opposite of what was observed in mitochondrial PD cellular models. Unexpectedly, OPA1 overexpression did not rescued MPP+-induced increase in reactive oxygen species (ROS). Overall, our findings suggest that OPA1-dependent mitochondrial fusion plays a crucial role in mediating MPP+/mtDNA induced mitochondria abnormalities and cellular dysfunction.

These studies suggest that mitochondrial dynamics can play an important role in PD pathogenesis, and a better understanding of these mechanisms can lead to the discover of new therapeutical targets for this disease.

Keywords: Parkinson's disease; mitochondrial dysfunction; mitochondrial dynamics; mitochondrial fission; mitochondrial fusion; mitophagy

Chapter 1
Introduction

Mitochondria, the “power house” of living cells as described before (Banerjee et al., 2009), are found virtually in every eukaryotic cell. These are especially complex organelles that are involved in a number of cellular functions and are essential for both life and death. The main of those functions include: the production of cellular ATP; participation in the synthesis of key metabolites; the regulation of apoptosis; calcium buffering; and the primary source of endogenous reactive oxygen species (ROS) (Delettre et al., 2000, Benard et al., 2007). As there are several assaults, either generated *in situ* or those imposed from extracellular environment, mitochondria are easy targets and their functions could be compromised. Therefore, a mitochondrial dysfunction results in a smaller supply of cellular energy, a failure in maintaining cellular homeostasis, and activation of cell death pathways which could underlie selective dopaminergic neurodegeneration in Parkinson’s disease (PD) (Beal, 2005).

PD is the second most common neurodegenerative disorder after Alzheimer’s disease (AD), and the most common neurodegenerative movement disorder. Pathologically, it is classically characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), leading to a dopamine deficit in the striatum, and the presence of proteinaceous cytoplasmatic inclusions called Lewy bodies (LBs) in surviving neurons (Forno, 1996). Clinically, PD patients manifest symptoms of progressive rigidity, bradykinesia, tremor, and postural instability, as well as symptoms involving non-motor brain functions, including cognitive and autonomic functions (Lang and Lozano, 1998a, b, Weintraub and Stern, 2005, Barbas, 2006). In PD it is known that the loss of the nigral dopaminergic neurons and terminals are responsible for movement alterations, although there are indications that several other neuronal populations throughout the brain are also affected in this disease (Braak et al., 2004).

Nowadays there is no cure for PD, as little is known about the etiopathogenesis of PD, and so the pursuit to define disease mechanisms continues.

1.1. Parkinson's disease and mitochondrial involvement

In almost 90-95% of all occurrences PD arises essentially as a sporadic condition, i.e. in absence of any apparent genetic linkage, and, over the years, several theories have been proposed for the etiology of the disease in an attempt to explain the why and the how of neurodegeneration in PD. There are several evidences for the relation between mitochondrial dysfunction and PD pathogenesis however it is debatable if it is the cause or an effect. Even though, it was recently proposed the mitochondrial cascade hypothesis for PD (Cardoso, in press).

1.1.1 Critical role of OXPHOS dysfunction in PD

The discovery that exposure to MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a selective inhibitor of mitochondrial complex I (CXI) of the electron transport chain (ETC), led to a development of progressive and irreversible parkinsonism provided strong evidence that mitochondrial dysfunction may be involved in the pathogenesis of PD (Langston et al., 1983). This direct relation was further established when it was (Schapira et al., 1989) described a CXI deficiency in the substantia nigra of PD patients postmortem brains, which was also seen in skeletal muscle and platelets (Bindoff et al., 1989, Parker et al., 1989). In agreement, some data suggests that sporadic PD is characterized by a systemic decrease in CXI (Penn et al., 1995, Keeney et al., 2006). Indeed, these authors were able to see that there was an

increase in the levels of protein carbonyls (a marker of an oxidative modification of proteins) in several catalytic subunits of CXI, which was in agreement with reduced electron transfer rates, indicating that the oxidative damage of CXI subunits may lead its misassembly and dysfunction. The reason why substantia nigra is more vulnerable to this impairment of CXI activity is possible due to increased generation of ROS from dopamine metabolism and iron content from dopaminergic neurons (Chinta and Andersen, 2008).

1.1.2. Oxidative stress and PD

Mitochondria are the main source for endogenous ROS production (reviewed in (Starkov, 2008, Murphy, 2009) and there are evidence of a linkage between oxidative stress and neurodegeneration in PD. In addition, the levels of antioxidants and oxidized targets are known to be altered in PD, as reviewed before (Jenner, 2003). Together with the fact that inhibition of mitochondrial complexes increases free radicals production (Orth and Schapira, 2002, Turrens, 2003), it is becoming clear a relation between mitochondrial dysfunction and PD. Indeed, an unbalance between exacerbated ROS production and/or defective ROS removal results in oxidative damage to mitochondrial DNA (mtDNA), proteins and lipids. According to the mitochondrial protein expression and/or abundance, it was reported that subunits of CXI was differentially expressed in mitochondria enriched fractions from post mortem PD substantia nigra, when compared to control (Jin et al., 2006). A reduction of the immunostaining for a mitochondrial protein alpha-ketoglutarate was also noted in post mortem PD brain (Mizuno et al., 1994). Oxidative damage to mtDNA may compromise respiratory chain subunits, which are encoded by mtDNA, establishing a vicious circle of oxidative stress and

bioenergetic failure, which has been the rationale for the mitochondrial theory of aging (Linnane et al., 1989).

1.1.3. The role of mitochondrial DNA in PD

Mitochondria house their own DNA, and it is strongly believed that the proximity of mtDNA to ROS generation as a consequence of normal respiratory chain function could increase mtDNA mutations (Richter et al., 1988, Ozawa, 1997). Thus, the alleged CXI defect in PD brain, and consequent increase in ROS production, may affect the mtDNA which encodes 7 of the 46 protein subunits of CXI, making mutations in the mtDNA an obvious trigger of PD pathology. Studies with cytoplasmatic hybrids (cybrids) cell lines supported this idea of a relation between mtDNA encoded defects and PD, showing that CXI defect from PD platelets is transferable into mtDNA deficient cell lines (Swerdlow et al., 1996, Gu et al., 1998). Such approach, the use of cybrids to investigate the potential of mtDNA from PD to determine the origin of the CXI defect, led the authors to suggest that mtDNA in those patients cause the CXI deficiency through inherited or somatic mutations. Indeed, these defects were associated with increased free radical production, impaired mitochondrial calcium buffer and increase susceptibility to MPP^+ (Sheehan et al., 1997). With these results it could be hypothesized that CXI deficit in PD are inherited either from mitochondrial genome or from alteration on somatic mtDNA. Moreover, Trimer et al., (2004) using a cybrid model of PD reported the formation of fibrillar and vesicular inclusions that replicated most antigenic and structural features of LBs (Trimmer et al., 2004). It is important to underline that there was no need in this experiment for exogenous protein expression or

inhibition of mitochondrial or proteasomal function. In PD, the maternal inheritance pattern of mtDNA mutation is rare and may be a mere coincidence (Wooten et al., 1997, Swerdlow et al., 1998, Thyagarajan et al., 2000). Although, some studies indicated that mtDNA abnormality may contribute to PD, as a mitochondrial 12SrRNA point mutation was found in a pedigree with Parkinsonism, deafness and neuropathy (Thyagarajan et al., 2000). Another evidence of mtDNA relevance in PD arose when the ablation of the mitochondrial transcription factor A gene, which regulates transcription of mtDNA and copy number, in nigrostriatal dopaminergic neurons of mice caused the slow progressive degeneration of dopaminergic neurons seen in PD (Ekstrand et al., 2007). Moreover, this mouse model showed reduced mtDNA expression, respiratory chain deficit and neuronal death, leading to progressive, L-dopa-responsive impairment of motor functions. Therefore, the role of mtDNA cannot be overruled and further investigations are warranted to better understand its role in PD.

1.2. Familial PD genes and mitochondrial homeostasis

As mentioned above, 90-95% of the cases of PD arise as sporadic conditions. The others 5-10% of the cases are inherited, and linkage analysis has identified a number of PD-associated genes. They usually, but not exclusively, show an early onset of symptoms and are in essence clinically indistinguishable from sporadic PD (Bonifati, 2007, Gasser, 2009). Inherited forms of PD have been linked to 16 familial PD-linked genetic loci (PARK1-16), including a–syn, parkin, DJ-1, PINK1 (PTEN-induced kinase 1), NURR1 (Nuclear receptor related 1 protein), OMI/HTRA2 (Heat transfer requirement 2), LRRK2 (Leucine-rich repeat kinase 2), were shown to locate in or

interact with mitochondria under certain conditions (Fig. 1) (Arduino et al., 2011, Esteves et al., 2011).

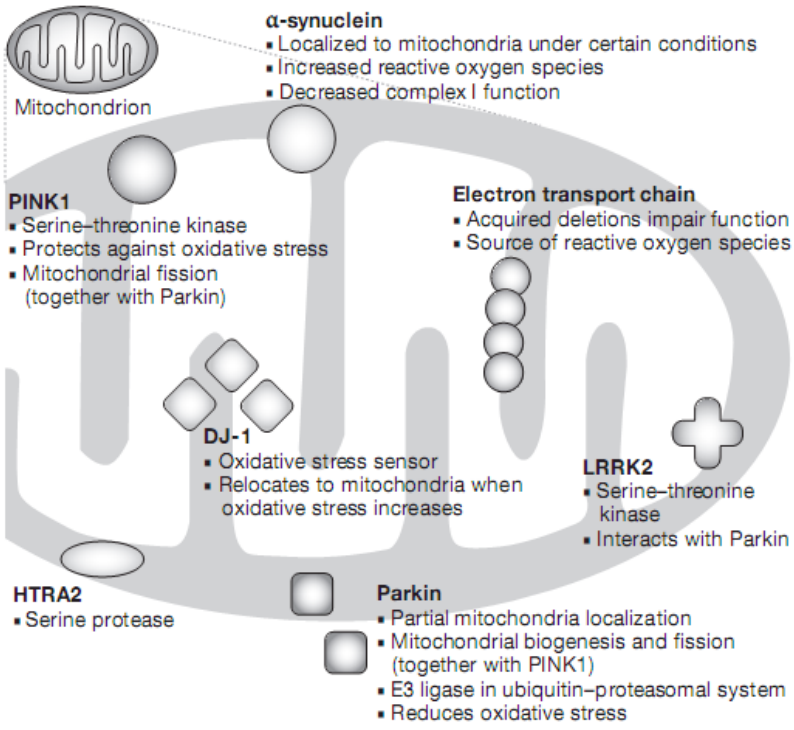


Figure 1 - Products of PD-associated genes and their role in maintaining mitochondrial homeostasis through modulation of mitochondrial dynamics and oxidative stress. Adapted from (Henchcliffe and Beal, 2008).

The study of the function and dysfunction of PD genes confirms us the relevance of the biochemical alterations found in sporadic PD, i.e., mitochondrial dysfunction, oxidative stress and an imbalance in protein homeostasis characterized by an increase in protein misfolding and aggregation accompanied by an impaired removal of misfolded proteins. Indeed, several of the proteins encoded by them carry out important function within mitochondria and/or act to reduce oxidative stress, providing strong evidences for a casual involvement of mitochondrial dysfunction and oxidative stress in PD pathogenesis. Thus, insight into the function of PD genes can promote our understanding of the molecular causes of PD and help to focus research on key

biochemical pathways. This also suggests that the mechanisms underlying sporadic and familial PD are similar. We will briefly focus on α-synuclein, Parkin and PINK1 proteins due to their relevance to mitochondrial dynamics.

1.2.1. Parkin

Mutations in the Parkin gene (PARK2) were reported to cause early onset juvenile form of autosomal recessive parkinsonism (Kitada et al., 1998). It is suggested that Parkin is a RING finger containing protein that acts as an E3 ubiquitin protein ligase in the proteasome-mediated degradation of several proteins in vitro (Shimura et al., 2000). The loss of the E3 ligase activity has been suggested to result in accumulation of toxic substrates leading to the autosomal recessive form of PD (Dawson, 2006). It was seen in a Parkin knockout mouse line an increase in striatal extracellular dopamine concentrations, reduced synaptic excitability, and a mild, non-progressive motor deficit at 2–4 months (Goldberg et al., 2003). Surprisingly, no loss of dopaminergic neurons was described and, like in most patients with Parkin mutations, no inclusion formations were noted in these mice (Goldberg et al., 2003). In a study conducted by Palacino and co-workers (2004) Parkin-deficient mice revealed decreased levels of several subunits of complexes I and IV. In addition, striatal cells from the mice exhibited reduced mitochondrial respiratory capacity and decreased antioxidant capacity (Palacino et al., 2004). Recently, it was proposed the hypothesis that Parkin could be a key player in the turnover of damaged mitochondria by mitophagy (McBride, 2008), as it was shown that Parkin can be recruited to depolarized mitochondria targeting them to the destruction by autophagy (Narendra et al., 2008).

1.2.2. PINK1

Mutation in phosphatase and tensin homologue (PTEN)-induced putative kinase 1 (PINK1) are associated with hereditary early-onset of PD (Valente et al., 2004a), as certain heterozygous PINK1 mutations may pre-dispose to the development of sporadic early-onset PD (Valente et al., 2004b). PINK1 is a serine/threonine kinase which is translated in the cytoplasm and imported into mitochondria through an N-terminal mitochondrial targeting sequence (Valente et al., 2004a, Silvestri et al., 2005). PINK1 mutations cause PD possibly due to impairment on the phosphorylation of its substrates, probably in mitochondria. These mutations have been reported within and outside the kinase domain (Hatano et al., 2004, Valente et al., 2004a), however, the localization of PINK1 to the mitochondria is not affected by these mutations (Zhou et al., 2008b). Although it is known that PINK1 is imported to mitochondria, as said before, its sub-mitochondrial localization remains intensely debatable. This topic is crucial for identifying its physiological substrates and its mode of action in the context of PD pathogenesis. It seems that the majority of the studies demonstrates that PINK1 localizes in the inner mitochondrial membrane (Silvestri et al., 2005, Gandhi et al., 2006, Pridgeon et al., 2007), while other suggest that it associates with the intermembrane space (Silvestri et al., 2005, Plun-Favreau et al., 2007, Pridgeon et al., 2007) or even the outer mitochondrial membrane (Gandhi et al., 2006) with the kinase domain facing the cytoplasm (Zhou et al., 2008b). The depletion of PINK1 in cultured cells results in abnormal mitochondrial morphology and membrane depolarization. Similar mitochondrial alterations are present in primary cells from patients with PINK1

mutations (Exner et al., 2007) suggesting that PINK1 plays an important role in mitochondrial maintenance. There is also a study in which seems to exists an important role for PINK1 protecting mitochondria against oxidative stress by phosphorylating the mitochondrial chaperone protein TRAP1 (Pridgeon et al., 2007). There is no doubt that more studies in this area are needed, although there is a certainly that both proteins modulate mitochondrial dynamics including fusion/fission which will be further mentioned in detail concerning PD pathogenesis.

1.2.3. α-synuclein

Three different missense mutation in α-synuclein (ASYN) gene (PARK1& 4 locus), a duplication and triplication of the ASYN gene locus lead to dominantly inherited early-onset of PD (Polymeropoulos et al., 1997, Kruger et al., 1998, Singleton et al., 2003, Zarranz et al., 2004). Although its physiological function is still quite unknown, it is known that ASYN is a presynaptic protein that is present in an aggregated form in LB's in PD due to the presence of its hydrophobic non-amyloid beta component domain. It has been described that familial mutation and over-expression of this protein accelerates this so-called ASYN protofibrilis (Conway et al., 1998, Conway et al., 2000, Fredenburg et al., 2007). The selective toxicity of ASYN to dopamine neurons (Xu et al., 2002) maybe in part explained by the stabilization of the toxic ASYN protofibrilis by dopamine adducts (Conway et al., 2001). There are various mechanisms proposed to explain ASYN toxicity, one of those includes mitochondrial dysfunction. However both are hallmarks implicated in PD pathogenesis it was not clear until recently that these two processes are interrelated and complement each other in

disease pathogenesis. Indeed, recent evidences suggests this interplay in which mice harboring human A53T ASYN mutant shown mitochondrial accumulation of human ASYN and exhibit mitochondrial degeneration associated with increased mtDNA damage and impaired activity of the electron transport chain complex IV cytochrome oxidase (Martin et al., 2006). Studies in yeast, where abrogation of mtDNA inhibited α-synuclein-induced ROS formation and apoptosis (Buttner et al., 2008), highlighted the requirement of mitochondria in mediating the toxicity of α-synuclein. Mitochondrial association of ASYN in cells was also linked to oxidation of mitochondrial proteins and increased levels of calcium and nitric oxide (Parihar et al., 2008). It was interesting to note that mice lacking ASYN exhibited alterations in membrane lipids and reduced activities of the electron transport complexes I and III in mitochondria (Ellis et al., 2005), suggesting that ASYN has a physiological role in mitochondria, and that the mitochondrial deficits observed in transgenic animals may be due to toxic gain-of-function of over-expressed or mutated ASYN present in mitochondria. Recently, is was shown that human ASYN N-terminal 32 amino acids contain a cryptic mitochondrial targeting signal, which is important for mitochondrial targeting of ASYN (Devi et al., 2008). It was also seen, in human dopaminergic neurons, that accumulation of wild-type ASYN in mitochondria caused reduced mitochondrial CXI activity and increased ROS production. Although, these defects occurred in an early time point in dopaminergic neurons expressing familial with A53T mutation as compared with wild-type ASYN (Devi et al., 2008). In this work it was also shown that ASYN interacts with CXI in the inner mitochondrial membrane. In fact, the content of ASYN in purified mitochondria from substantia nigra were several-fold higher in PD compared to healthy individual, suggesting that mitochondrial accumulation of ASYN may contribute to the CXI defects of the disease (Devi et al., 2008). Therefore, the interaction process of ASYN with

mitochondria is quite complex and impacts mitochondrial physiology to affect normal functioning of dopaminergic neurons.

1.3. Mitochondrial dynamics

1.3.1. Mitochondria as an organelle with a tightly controlled dynamic

What was once thought to be an isolated and rigid structure organelle, mitochondria became now highly dynamic organelle that constantly divides (fission) and fuse (fusion) with each other (Chan, 2006). Indeed, this highly dynamic and orchestrated process of mitochondrial fusion and fission affects, besides morphology, the size, number, length and even mitochondrial function and distribution. This phenomenon regulated by a delicate balance between these two opposing processes is important in order to maintain the integrity of mitochondria, electrical and biochemical connectivity, in mitochondrial turnover, and in aggregation, stabilization, and protection of mtDNA (Westermann, 2002).

1.3.1.1 Mitochondrial fission

For the mitochondrial division, both the inner and outer membrane need to fise and the mtDNA and other critical mitochondrial contents need to be redistributed to the daughter mitochondria, so that the parental gives rise to two isolated, small, round

organelles. Although the precise mechanism of mitochondrial fission is not fully elucidated, it involves at least two proteins in mammals: a large GTPase, dynamin-related protein 1 (Drp1, also referred to as DLP1) and a small molecule, Fis1 (Chan, 2006, Knott et al., 2008) (Fig. 2). Drp1, a protein that controls membrane tabulation and fission, although primarily cytosolic is recruited to the outer mitochondrial membrane. Once there, Drp1 has the ability to oligomerize and form large complexes with other proteins (like Fis1) (Hoppins et al., 2007). It is believed that once a ring-like complex structure is formed along the mitochondrial surface, Drp1 uses GTP hydrolysis to constrict and twist tubule to initiate fission (Smirnova et al., 2001). As mentioned before, Fis1 is involved in mitochondrial fission as it acts as a receptor to recruit Drp1 to mitochondria. This mitochondrial outer membrane protein, resident on the surface of mitochondria (James et al., 2003), appears to be a limiting factor during the process of mitochondria fission as, in mammalian cells, knockdown of Fis1 blocks mitochondrial fission without affecting Drp1 localization to mitochondria (Lee et al., 2004). Recently, another protein, Mff, was suggested to be involved in mitochondrial fission as the knockdown of the same caused mitochondrial elongation and resistance to CCCP-induced fragmentation (Gandre-Babbe and van der Bliek, 2008). However, further studies are necessary to elucidate the potential role of this protein in the recruitment of Drp1 to mitochondria. Therefore, this process of mitochondrial fission seems to be very useful and crucial for mitochondrial function as it facilitates equal segregation of mitochondria into daughter cells in cellular division and to enhance

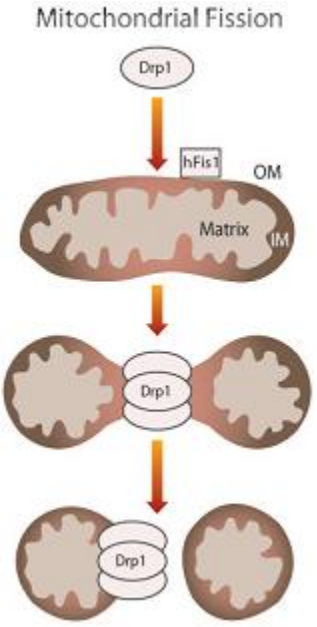


Figure 2 – Drp1 and Fis1 function and localization during mitochondrial fission. Adapted from (Knott et al., 2008).

distribution of mitochondria along cytoskeletal tracks. It also has an important role in the targeting of damaged segments of mitochondria to the autophagic process, playing a housekeeping role in the cell, as it will be mentioned later.

1.3.1.2 Mitochondrial fusion

On the other side, mitochondrial fusion, in mammalian cells, is mediated in part by the outer membrane dynamin-like GTPases mitofusin-1 and -2 (Mfn1 and Mfn2) and the inner membrane optic atrophy protein (Opa1) (Fig. 3) (Cerveny et al., 2007, Detmer and Chan, 2007b). Although both Mfn1 and Mfn2 have the same location and appear to play similar roles in mitochondrial fusion, they function independently of each other, even having different rates of GTP hydrolysis (Ishihara et al., 2004) (Fig. 4). Indeed, it was reported that these two proteins could form homo-oligomeric and hetero-oligomeric complexes, during mitochondrial fusion, and thus tether outer membranes of neighboring mitochondria together (Ishihara et al., 2004, Zuchner et al., 2004). The inner membrane protein OPA1, that faces intermembrane space, has been propose to be primarily involved in the inner membrane fusion in a process that requires Mfn1, but not Mfn2 (Cipolat et al., 2004). Like mitochondrial fission, fusion seems to be required for a normal mitochondrial function as this process is likely to protect function by providing

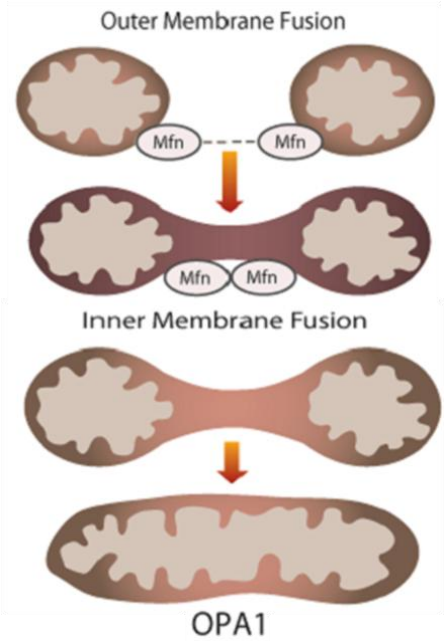


Figure 3 – Mitochondrial outer and inner membrane fusion controlled by Mfn1/2 and OPA1 respectively. Adapted from (Knott et al., 2008)

a chance for mitochondria to mix their contents, thus enabling protein complementation, mtDNA repair and equal distribution of metabolites.

Gene product	Role in mitochondrial dynamics	Localization
Mfn1/2	outer membrane fusion (GTPase)	outer mitochondrial membrane with the N- and C-termini facing the cytosol (U-shaped transmembrane domain)
OPA1	inner membrane fusion (GTPase)	inner mitochondrial membrane facing the intermembrane space
Drp1	fission (GTPase)	cytosol, during fission recruited to punctae at the outer mitochondrial membrane
Fis1	fission	outer mitochondrial membrane (transmembrane protein)

Figure 4 – Proteins reponsable for the modulation of mitochondrial dynamics and cellular localization of the same. Adapted from (Winklhofer and Haass, 2010)

1.3.1.3. Mitophagy

As mentioned before, mitochondrial dysfunction can be a key determinant in cell death as this organelle provides critical functions for cell survival. Indeed, it is necessary an orchestrated mechanism capable of selectively remove damaged mitochondria that are functionally impaired or have accumulated mtDNA mutation that potentiates ROS generation. The process responsible for this is denominated autophagy. It is a process in which cellular components are isolated by engulfment into autophagosomes. Further these autophagosomes fuse with lysosomes which contain hydrolytic enzymes that break down cellular components. Generally this process is activated during nutrient deprivation so that the products can be recycled into more urgently needed molecules. However, autophagy can have a housekeeping role in

maintaining quality control by turning over organelles, like mitochondria, and degrading protein aggregates. So mitochondria can be targeted for degradation in a process called mitophagy. Although this process is not new, it had remained unclear whether mitophagy can selectively degrade dysfunctional mitochondria. In a study performed by Kanti and Klionsky it was reported that selective mitophagy was regulated independently from bulk autophagy (Kanki and Klionsky, 2008). It was also described that mitochondrial fission could target mitochondria to degradation. Indeed, it was shown in pancreatic b-cells and COS7 cells that mitochondrial fission can yield uneven products in which one of the daughter mitochondrion was depolarized, much less like to fuse and had reduced levels of OPA1 protein, and eventually was autophagocytosed (Twig et al., 2008). Supporting this find it was also stated that to mitophagy occur there must be a loss of fusion and the presence of fission. In the base of this theory is the fact that the overexpression of OPA1, Fis1 RNAi, and Drp1 dominant-negative expression all reduced level of mitophagy. Although mitochondrial fragmentation is suitable for mitophagy, it is not a sufficient signal for this process (Narendra et al., 2008, Twig et al., 2008).

1.4. Why is mitochondrial dynamics important in neurons?

Changes in the mechanisms that mediate mitochondrial dynamics can lead to different defects in neurons (Fig. 5). Some of the reasons for the particular importance of mitochondria fusion and fission in neurons possible rely on their unique features such as their post-mitotic state and their long processes with higher energy requirements. Indeed, this high energy demands to support their survival and specialized function may

lead this type of cell vulnerable to mitochondrial dynamics defects. In agreement with this theory specific mutations in fusion related genes result in human neuropathies (Delettre et al., 2000, Zuchner et al., 2004), while mutations in both fission and fusion related genes have been associated with central nervous system disease (Waterham et al., 2007, Brockmann et al., 2008). Indeed, these neurons with long axons (like peripheral sensory neurons and motor neurons) are preferential degenerated when

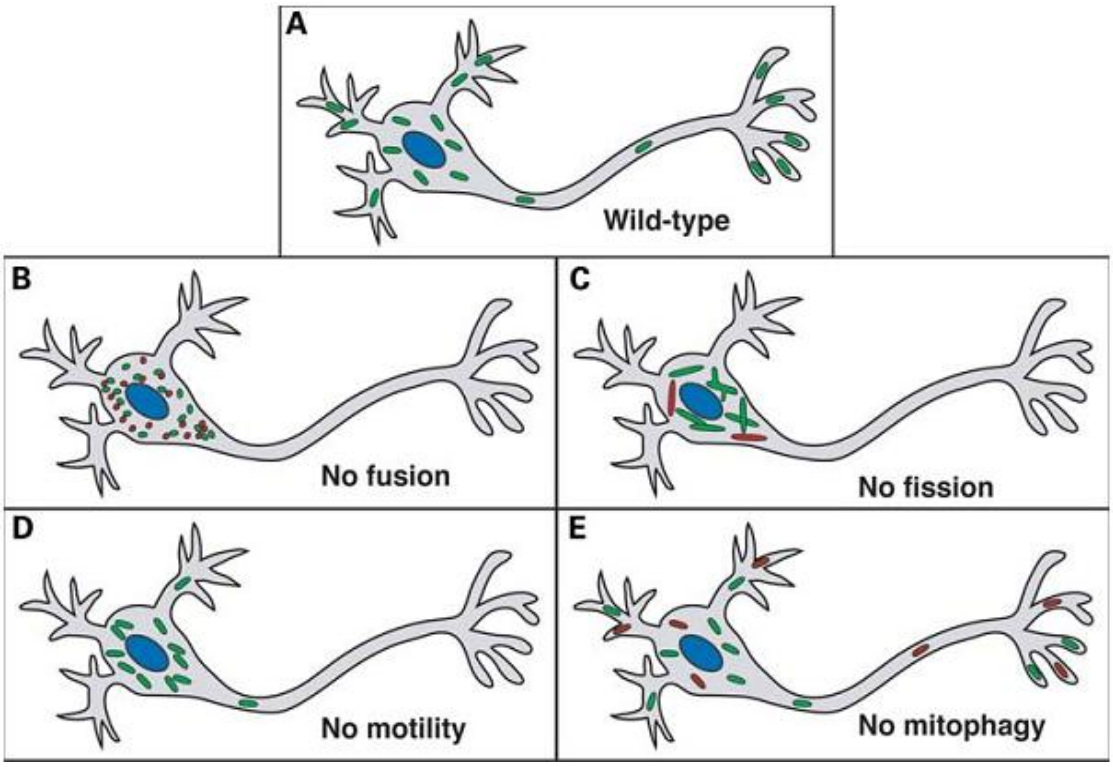


Figure 5 – Neuronal dysfunction as consequence of defects in mitochondrial dynamics. **(A)** In wild type neurons, mitochondria are distributed along the neurons and function normally with a normal performance of their function. **(B)** In neurons where there is no fusion there is an increase in fragmented mitochondria, some of those dysfunctional, which are not distributed along the cytokeletal tracks. **(C)** When there is no fission mitochondria forms a tubular network difficult to transport to axons and dendrites, with accumulation of damaged mitochondria in the soma. **(D)** When the motility is affected there is not a good distribution of these organelles along the neurons. **(E)** Without the process necessary for the turnover of mitochondria there is an accumulation of damaged mitochondrial along the neurons. Addapted from (Chen and Chan, 2009).

mitochondrial dynamics are compromised leads due to their high energy requirement for several aspects of neuronal physiology. In fact, synaptic transmission requires high level of energy and a sufficient number of mitochondria to be present at synaptic sites

so that the plasma-membrane-potential could be maintained, synaptic neurotransmitter could be released and reuptake, and to build-up a reserve pool of vesicles for prolonged or high-frequency firing. The dynamic processes of mitochondrial fission/fusion are intimately and critically involved in the formation of synapses and dendritic spines, where exist a high demand of ATP. While the unbalance of mitochondrial fission/fusion towards fission leads to an increase in synapse formation, a decrease in the same leads to a loss of mitochondria from dendritic spines and a reduction of synapse formation (Li et al., 2004). The absence of the mitochondrial fission protein Drp1 has been shown to prevent mitochondria from distributing to synapses and to lead to synaptic dysfunction (Verstreken et al., 2005).This might be linked to the need of smaller mitochondrial units which presumably are more easily transported over long distances as, e.g., required in axonal transport. Other mitochondrial feature that is very important for normal neuronal physiology is the Ca^{2+} buffering that prevents excitotoxicity by an excessive free-Ca^{2+} load in neurons. On the other hand, the process of fusion is crucial for the maintenance of mtDNA that cannot take place without this process (Rapaport et al., 1998). In fact, fusion of mitochondria has been directly implicated in preventing the accumulation of damaged mtDNA (Nakada et al., 2001, Ono et al., 2001). As it was shown recently, mitochondrial fission appears to be critical to mtDNA maintenance (Parone et al., 2008) and so we can find that in mitochondrial dynamics a delicate balance is very important as the coordination of these processes maintain and protect mtDNA. This is very important in neurons and neurodegenerative diseases as it was shown that mtDNA mutations accumulate in the brain with age (Corral-Debrinski et al., 1992). Neurons are also particularly sensitive to the impairment of the transport of mitochondria through the axons and dendrites so that these could reach sites of increased energy requirement. They are transported both in anterograde and retrograde directions, in a process

regulated in part by cytoskeletal and mitochondrial proteins, and several intrinsic component of mitochondrial bioenergetics, like ATP synthesis or changes in mitochondrial membrane potential, can impair this transport (Rintoul et al., 2003, Miller and Sheetz, 2004). This suggests a link between mitochondrial bioenergetic functions and mitochondrial transport and distribution. In addition, disruption of mitochondrial movement has been noted in excitotoxicity and oxidative stress conditions (Rintoul et al., 2003), two factors implicated in cellular stress and neuronal death.

1.4.1. Mitochondrial dynamics in neurodegenerative disorders rather than PD

There are several mutations in mitochondrial fission/fusion genes in humans that result in very specific neurodegenerative diseases. Regarding the mitochondrial fusion proteins, mutations in Mfn2 cause Charcot-Marie-Tooth subtype 2A (CMT2A), which involves axonal degeneration of motor and sensory neurons (Zuchner et al., 2004, Kijima et al., 2005). Mutations in Opa1, other mitochondrial fusion protein, cause the most common form of optic atrophy, autosomal dominant optic atrophy (ADOA) (Alexander et al., 2000). Concerning the mutations in mitochondrial fission proteins, a dominant negative mutation in the human Drp1 gene resulted in elongated and tangled mitochondria concentrated at perinuclear region (Waterham et al., 2007). The patient carrying this mutation died shortly after birth and displayed some symptoms resembling those of ADOA and CMT2A, emphasizing the importance of the fission machinery in neuronal maintenance.

It is interesting to note that there are some human genetic mutations that do not affect mitochondrial morphogenesis, as the others described above, but cause disease

through a fission/fusion-independent manner. Examples of this are some Mfn2 mutants that retain the ability to promote mitochondrial fusion (Detmer and Chan, 2007a). This could indicate that Mfn2 has other pathogenic roles not related to mitochondrial fusion. There are several studies suggesting other functions for this protein, like mitochondrial trafficking (Baloh et al., 2007) and regulating Ca^{2+} uptake and signaling by tethering mitochondria to endoplasmatic reticulum (de Brito and Scorrano, 2008), which disruption could lead to peripheral axon degeneration and neuronal death. Although, it remains necessary the creation of animal models so it could be distinguishable the contribution of the various functions of the fission/fusion genes in disease pathogenesis.

1.4.2. Mitochondrial dynamics in Parkinson's disease

One of the most accepted reasons for the vulnerability of the dopaminergic neurons from substantia nigra to changes in mitochondrial dynamics is the lower basal mitochondrial content compared to other midbrain neurons (Liang et al., 2007). This evidence could contribute to the selective vulnerability of these neurons to external or internal toxic stimuli that affect mitochondrial fission, fusion and mitophagy, compromising mitochondrial homeostasis during PD pathogenesis.

1.4.2.1. Available PD models and mitochondrial dynamics

Through the widely used models to mimic PD pathogenesis it was already described the existence mitochondrial dynamics imbalance. Indirect observations using a cytoplasmatic hybrid (cybrid) model of PD showed that PD cybrid cells contained a

significantly increased percentage of mitochondria that were enlarged or swollen and had a pale matrix with few remaining cristae (Trimmer et al., 2000). This mitochondrial morphology in PD cybrids, whose mitochondria are proceeding from platelets of PD patients, was also seen by Esteves and coworkers in a similar cybrid model, suggesting that there could be impairment in mitochondrial fission (Esteves et al., 2008).

The first evidence demonstrating mitochondrial fragmentation induction by toxins used to study PD, was the finding that both CXI inhibitors rotenone and MPP^+ induced mitocondrial fission in rat dopaminergic cell line N27 (Barsoum et al., 2006). Following studies continued to report mitochondrial fragmentation following administration of CXI inhibitors like 6-Hydroxydopamine (6-OHDA)l(Gomez-Lazaro et al., 2008), MPP^+ (Meuer et al., 2007, Endo et al., 2009, Wang et al., 2011c) and rotenone (Benard et al., 2007, Mortiboys et al., 2008, Plecita-Hlavata et al., 2008, Sandebring et al., 2009, Thomas et al., 2011). Interestingly, some of these authors went further and showed that the CXI inhibition-induced mitochondrial fragmentation was Drp1-dependent, as Drp1 silencing or its dominant-negative mutant expression prevented the mitochondrial morphological changes and the cell death induced by the toxins (Benard et al., 2007, Meuer et al., 2007, Gomez-Lazaro et al., 2008, Wang et al., 2011c). Together, these results highlight mitochondrial fragmentation as an early and upstream event mediating CXI inhibition-induced toxicity. However, it was reported that chronic exposure to rotenone significantly increased mitochondrial branching and length in human skin fibroblasts (Koopman et al., 2005). The discrepancy between these results may rely in the different duration of the toxic stimuli, e.g. acute *vs.* chronic, toxin concentration, or different cell lines, which may affect differently mitochondrial morphology. Therefore, in further necessary studies using PD-linked toxins to study mitochondrial morphology, careful experimental design should be performed in order to

accurately mimic PD pathology and enable a proper analysis of the mitochondrial network in this disease.

1.4.2.2 Role of PINK1 and Parkin in mitochondrial dynamics in PD

Until now, the strongest evidence of the role of mitochondrial dynamics in PD aroused from studies in which the manipulation of some genes associated with familial PD affected mitochondrial dynamics in several models and tissues. As previously described, PINK1 and Parkin are proteins that have an important role in mediating mitochondrial function and morphology.

1.4.2.2.1 Modulating mitochondrial shape

Back in 2003, the first studies describing mitochondrial structural alterations associated with parkin mutants arouse. Using *Drosophila* Parkin mutant flies it was reported that muscle mitochondria had abnormal morphology, including swollen or fragmented mitochondria with disrupted cristae, a phenotype that was rescued by overexpression of wild-type Parkin (Greene et al., 2003, Pesah et al., 2004). Surprisingly, no major morphological abnormalities in striatal mitochondria of parkin -/- mice were later reported (Palacino et al., 2004), an observation that would serve as a template for the discrepancies of the results arising from the models to study PINK1 and Parkin effect on mitocondrial morphology, that would then emerge.

The first detailed studies unraveling PINK1 and Parkin role in mitochondrial morphology regulation were preformed through genetic manipulations in *Drosophila*. Initially, loss of PINK1 or Parkin function in *Drosophila* resulted in similar

mitochondrial morphological abnormalities, including enlarged mitochondria with fragmented cristae. Surprisingly, Parkin overexpression was able to rescue PINK1 mutant mitochondrial morphology defects, but not vice versa, suggesting that PINK1 and Parkin take part in the same pathway in which PINK1 acts upstream of Parkin in regulation of mitochondrial morphology (Clark et al., 2006, Park et al., 2006, Yang et al., 2006). Latter findings, still in *Drosophila*, revealed how PINK1/Parkin pathway exerts its effect in mitochondrial morphology. It was shown that the Drp1 loss of function was lethal in PINK1 or Parkin mutants, as Drp1 overexpression suppressed the mitochondrial morphological abnormalities described before (Deng et al., 2008, Poole et al., 2008, Yang et al., 2008, Park et al., 2009). Interestingly, loss of function of the proteins involved in the mitochondrial fusion process, like Mfn and Opa1, were also able to restore mitochondrial morphology in PINK1 and Parkin mutants, suggesting that PINK1/Parkin pathway stimulates mitochondrial fission and/or inhibits mitochondrial fusion.

However, conformity between the results reported in *Drosophila* and findings in vertebrates was not achieved. In HeLa cells PINK1 knockdown induced mitocondrial fragmentation and reduced/altered mitochondrial cristae in mammalian cells, abnormalities that were rescued by wild type PINK1 and Parkin overexpression, but not Parkin mutant's (Exner et al., 2007). Observations in primary fibroblasts from patients with PINK1 mutations have also shown mitochondrial fragmentation. Moreover, PINK1 or Parkin down-regulation in mammalian cells was again reported to induce comparable mitochondrial fragmentation (Dagda et al., 2009, Lutz et al., 2009). As Drp1 knockdown was able to reverse this phenotype, demonstrating that increased mitochondrial fission is associated with PINK1/parkin depletion, Mfn2 and Opa1 overexpression could also rescue mitochondrial phenotype (Lutz et al., 2009). Further

studies reported again that PINK1 loss of function increased mitochondrial fission (Wood-Kaczmar et al., 2008, Cui et al., 2010, Heeman et al., 2011, Wang et al., 2011b), as human wild type PINK1 overexpression produces a pro-fusion effect in the mitochondrial network (Cui et al., 2010).

Strikingly, even between mammalian models there's still some controversy as in recent studies PINK1 or Parkin overexpression resulted in mitochondrial fragmentation, and inactivation of these proteins resulted in elongated mitochondria (Gautier et al., 2008, Sandebring et al., 2009, Cui et al., 2011, Pacelli et al., 2011, Yu et al., 2011), in agreement with the former *Drosophila* studies. Overexpression of Drp1 or the knockdown of Opa1 reversed the mitochondrial elongation induced by PINK1 RNAi, as overexpression of PINK1 or Parkin were also suppressed mitochondrial elongation induced by Drp1 knockdown (Yu et al., 2011)**.**

1.4.2.2.2. Governing mitochondrial fate

Beyond the "simple" regulation of mitochondrial morphology, through interactions with the mitochondrial fission/fusion machinery, an important role for the PINK1/Parkin pathway has been attributed at the mitochondrial quality control level.

Upon mitochondrial damage, PINK/Parkin pathway have been reported to promote poly-ubiquitination Mfn1 and Mfn2, through Parkin interaction, leading to its turnover by UPS, independently of the autophagic pathway (Chan et al., 2011, Glauser et al., 2011). The degradation of these mitochondrial outer membrane fusion proteins leads to an impairment of mitochondrial fusion which, upon physiological conditions, can exert a selective removal of damaged mitochondria by autophagy (mitophagy). Indeed, Parkin was suggested to prevent refusion of depolarized mitochondria, product

of a division process, through elimination of the proteins involved in the outer mitochondrial membrane fusion, Mfn1 and Mfn2 (Tanaka et al., 2010).

The dependence on this pathway to promote mitophagy has been extensively described as PINK1 and Parkin silencing leads to lower ubiquitination of the mitochondrial proteins Mfn1 and Mfn2 upon mitochondrial damage, leading to accumulation of defective mitochondria (Gegg et al., 2010). Mutations in PINK1 and Parkin also impairs ubiquitination of mitofusins in human fibroblasts (Rakovic et al., 2011). Moreover, a study in *Drosophila* has shown that Parkin requires functional PINK1 to correctly be recruited to dysfunctional mitochondria and promote their degradation, through Mfn1/2 degradation (Ziviani et al., 2010).

The parkin-mediated autophagy was shown to be prevented upon inhibition of Drp1-mediated mitochondrial fission, emphasizing the importance of these fission/fusion proteins in mitochondrial quality control (Tanaka et al., 2010). Importantly, it was recently revealed that Parkin also interacts and ubiquitinates Drp1, signaling this protein for degradation at the UPS (Wang et al., 2011a)

1.4.2.3. α-synuclein and mitochondrial morphology

A relation between mitochondrial morphology and ASYN was early described as overexpression of ASYN resulted in abnormal large mitochondria displaying vacuolization of the cristae (Hsu et al., 2000). Still, at the time, no direct interaction was established between them. Nowadays it is clear that ASYN interacts with mitochondria. It is extensively described its transport to this organelle and its accumulation in the same (Martin et al., 2006, Li et al., 2007, Cole et al., 2008, Devi et al., 2008, Nakamura et al., 2008, Parihar et al., 2008, Shavali et al., 2008, Liu et al., 2009, Chinta et al., 2010, Zhu et al., 2011). Some of these reports also highlight ASYN ability to associate with

proteins like complex I and the adenylate translocator, impairing their activity. ASYN transgenic mice developed mitochondrial pathology, as mitochondria contained ASYN and were shrunken, swollen, or vacuolated (Martin et al., 2006). Moreover it has been recently suggested that this association between ASYN and mitochondria has a critical role in the regulation of mitochondrial morphology. Indeed, according to two recent studies, it seems that this association drives mitochondrial fission by inhibition of the fusion process (Kamp et al., 2010, Nakamura et al., 2011). This ability of ASYN was demonstrated to be independent of the fusion/fission machinery and seems to rely simply in its direct interaction with mitochondrial membranes. In other study, overexpression of ASYN has been reported to induce mitochondrial morphological deformation as swollenness, cristae loss and vacuolation (Zhu et al., 2011).

1.5. Objectives

Taken together, the studies reviewed here provide several evidences that perturbations in mitochondrial dynamics could underline the selective degeneration observed in PD pathogenesis. However it is important to note that the processes that mediate mitochondrial dynamics - fission, fusion and mitophagy - are not isolated and independent, and there are several aspects of these machinery that need to be elucidated in order to get a better understanding in the mechanisms that are responsible for the imbalanced mitochondrial dynamics that are known to occur in PD. In this thesis we performed several studies in order to get a clear understanding of this pathway. We used different cellular PD models that mimic sporadic (cybrids), familial PD (α-synuclein overexpressing cells), or PD patients lymphocytes in order shade a light and elucidate the conflicting data in PD. We also direct manipulated the fusion machinery (OPA1

overexpression) in a way that could rescue mitochondrial function in a PD model. Our work allowed us to gain a better understanding of the pathway mediating mitochondrial dynamics in PD.

Chapter 2
Materials & Methods

2.1 Biological Material

2.1.1 Cell lines

2.1.1.1 mtDNA depleted NT2 ρ0 cells

NT2 human neuroblastoma cell line containing mtDNA (ρ+), purchased from Stratagene (La Jolla, CA) was depleted from mtDNA by long-term ethidium bromide exposure as described by Swerdlow and co-workers (1997). Cells were grown in 75 cm2 tissue culture flasks maintained in a humidified incubator at 37ºC and 5% CO2.

2.1.1.2 NT2 cybrid cell lines creation

To create the cybrid cell lines for this study, we used the teratocarcinoma cell line lacking mtDNA (NT2-Rho0 cell line) that does not possess a functional ETC and is autotrophic for pyruvate and uridine (Swerdlow et al., 1997). In brief, NT2-Rho0 cells were repopulated with platelet mitochondria from either healthy individuals or PD patients (CT cybrids and PD cybrids, respectively). Seven days after fusion, cybrid cells were placed in selection medium containing 10% dialyzed fetal calf serum and lacking pyruvate and uridine, as previously described (Swerdlow et al., 1997, Cardoso et al., 2004).These conditions result in the selective death of Rho0 cells that have not been repopulated with donor mitochondria. After selection was complete, cybrid cells were switched to cybrid expansion medium. Cells were grown in 75 cm2 tissue culture flasks maintained in a humidified incubator at 37ºC and 5% CO2.

2.1.1.3 Human neuroblastoma cell line SH-SY5Y

SH-SY5Y human neuroblastoma cells were purchased from ATCC. Cells were grown in 75 cm2 tissue culture flasks maintained in a humidified incubator at 37ºC and 5% CO2.

2.1.1.4 SH-SY5Y cell line inducible expressing WT α-synuclein

The generation of a stable cell line inducible expressing WT ASYN was described previously (Vekrellis et al., 2009). Briefly, naïve SHSY-5Y cells were transfected with the Tet-Off vector and selected with 500 μg/mL G418. Determination of the inducibility of the resistant clones was determined by transient transfection of a pTRE-LUC vector, in the presence or absence of dox (2μg/mL) in TET-Off-approved medium, and one clone (2-22) was further used for generation of stable pTRE-ASYN expression.

2.1.1.5 Human HEK293T cell line

Human HEK293T cells were obtained from ATCC. Cells were grown in 75 cm2 tissue culture flasks maintained in a humidified incubator at 37ºC and 5% CO2.

2.2 Chemicals and Cell media

MPP^+ was purchased from Sigma. DMEM medium was obtained from Gibco-Invitrogen. Optimem medium was obtained from Gibco-Invitrogen. Non-dialyzed and

dialyzed fetal bovine serum was obtained from Gibco-Invitrogen. NT2 ρ0 cell growth medium consisted of Optimem supplemented with 10% heat inactivated fetal bovine serum, 200 µg/ml sodium pyruvate, 150 µg/ml uridine, and 100 IU/ml penicillin and 50 µg/ml streptomycin. NT2 cybrid selection medium consisted of Optimem supplemented with 10% non-dialyzed heat inactivated fetal bovine serum and penicillin–streptomycin. Cybrid expansion medium consisted of Optimem supplemented with 10% heat inactivated fetal bovine serum and penicillin–streptomycin. MPP^+ medium consisted of cybrid expansion medium with 1 mM MPP^+. SHSY-5Y cell growth medium consisted in DMEM and Ham´s F12 medium with 10% supplemental fetal bovine serum and 100 IU/ml penicillin and 50 µg/ml streptomycin. HEK283 cells growth consisted in DMEM supplemented with 10% fetal bovine serum and penicillin–streptomycin. SHSY-5Y cells were cultured in RPMI 1640, 10% fetal bovine serum. Stable cells inducible over-expressing WT ASYN were maintained in 250 lg/mL G418 and 50 lg/mL Hygromycin B. ASYN expression was switched off with dox (2 µg/mL). Stock cultures were kept in the presence of dox. Analysis in stable cells inducible over-expressing WT ASYN was performed 4 days after removal of dox from cell's medium.

2.3 Subcellular proteome extraction in cultured cells

In order to obtain pure mitochondrial and cytosolic protein extracts, protein subcellular extraction was performed in cell culture using ProteoExtract® Subcellular Proteome Extraction Kit from Calbiochem®, according to the manufacturer's protocol. Briefly, each extraction buffer is sequentially incubated with cell pellet taking advantage of the differential solubility of certain subcellular compartments in the specific buffer. After incubation, the subcellular compartment solubilized in the

respective extraction buffer is separated from cell pellet by appropriate centrifugation force.

2.4 Lymphocytes isolation

Venous blood was collected by venipuncture in K_2EDTA-containing tubes. Lymphomonocytes were isolated by Ficoll-Histopaque 1077 density gradient centrifugation. Briefly, blood samples were carefully layered on Ficoll-Histopaque 1077 and centrifuged for 20 min at 2500rpm at room temperature, in a swing-out rotor, without brake. Lymphomonocytes were collected from the interface between serum and Ficoll-Histopaque 1077 gradients and washed once in PBS. The solution was then centrifuged to pellet the cells for 10 min at 1500rpm, at room temperature. Cells were the lysated with in buffer containing 25 mM HEPES, 1 mM EDTA, 1 mM EGTA, 2 mM $MgCl_2$, protease inhibitors (commercial protease inhibitor cocktail from Sigma), 0.1 M PMSF (Sigma), 0.2 M DTT (Sigma), 1% Triton X-100 , 2mM ortovanadate and 50 mM sodium fluoride.

2.5. Immunoblotting

For the analysis of total protein levels, individual cybrid cell lines were scraped in buffer containing 25 mM HEPES, 1 mM EDTA, 1 mM EGTA, 2 mM $MgCl_2$, protease inhibitors (commercial protease inhibitor cocktail from Sigma), 0.1 M PMSF (Sigma), 0.2 M DTT (Sigma), 1% Triton X-100 , 2mM ortovanadate and 50 mM sodium fluoride. Cell suspensions were frozen three times in liquid nitrogen and

centrifuged at 20,000 g for 10 min at 4 ºC. The resulting supernatants were removed and stored at −80°C. Protein concentrations were determined by the Bradford method and equal amounts of protein (50 µg) were used for immunoblotting. For the SDS-PAGE experiments samples were resolved by electrophoresis in SDS polyacrylamide gels and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Non-specific binding was blocked by gently agitating the membranes in 5% non-fat milk or 5% BSA, for phosphorilated proteins, and 0.1% Tween in TBS for 1h at room temperature. The blots were subsequently incubated with the respective primary antibodies overnight at 4°C with gentle agitation (1:1000 Polyclonal Rabbit anti-phospho-Drp1 (Ser616) antibody from Cell Signaling Technology; 1:750 Polyclonal Rabbit anti-Fis1 antibody from Imgenex; 1:520 Rabbit Polyclonal anti-Opa1 antibody from Abcam; 1:10,000 monoclonal anti-alpha-tubulin antibody from SIGMA; 1:200 Polyclonal Rabbit anti-Drp1 antibody from Santa Cruz Biotechnology, Inc.; 1:200 Polyclonal Rabbit anti-Mfn1 antibody from Santa Cruz Biotechnology, Inc.; 1:200 Polyclonal Rabbit anti-Tom20 antibody from Santa Cruz Biotechnology, Inc.; 1:500 Monoclonal Mouse anti-Mfn2 antibody from Abnova; 1:1000 mouse; 1:500 Monoclonal Mouse anti-glyceraldehyde-3-phosphate dehydrogenase from Millipore). Blots were washed with TBS containing 0.1% non fat milk and 0.1% Tween three times (each time for 10 min), and then incubated with the appropriate horseradish peroxidase- conjugated secondary antibody for 2 h at room temperature with gentle agitation. After three washes specific bands of interest were detected by developing with an alkaline phosphatase enhanced chemical fluorescence reagent (ECF from GE Healthcare). Fluorescence signals were detected using a Biorad Versa-Doc Imager, and band densities were determined using Quantity One Software.

2.6 Immunocytochemistry

Cells were all plated at density of $0.05x10^6$ and after incubation periods cells were washed twice with PBS and fixed for 30 min at room temperature using 4% paraformaldehyde. The fixed cells were washed again with PBS, permeabilized with 0.2% Triton X-100, and blocked with 3% BSA. The permeabilized cells were incubated with primary antibody (1:100 polyclonal rabbit anti-Tom20 from Santa Cruz Biotechnology; and 1:400 mouse monoclonal anti-α-sunuclein from Santa Cruz Biotechnology). Afterward cells were incubated 1h with appropriate secondary antibody (1:200 alexa fluor 594 and 488 from Molecular probes, Eugene, OR, USA). Finally cells were washed in PBS, incubated for 5 minutes with Hoechst 33342 (15mg/L in PBS, pH 7.4) in the dark. Cells were visualized by confocal microscopy.

2.6 HEK293T transfection procedure

HEK293T cells were transfected using FuGENE® 6 Transfection Reagent according to manufacturer's protocol. The Reagent:DNA ratio used was 6:1 (µl, for transfection reagent, and µg for DNA, respectively). The Opa1 (WT) construct was a kind gift from Xiongwei Zhu, Case Western Reserve University, Cleveland, OH 44106, USA. The expression vector for Myc-tagged wild-type OPA1 was based on pCMV-Tag vector (Stratagene).

2.5 Fluorometric reactive oxygen species production

ROS production was measured using dihydroethidium (DHE), a dye that in cytoplasm exhibits blue fluorescence, however, once this probe is oxidized to ethidium, it intercalates within DNA, staining the cell nucleus a bright fluorescent red. HEK293 cell were plated at the density of $0.05x10^6$ in 48-well plates and transfected the next day for 48h. After the 24h treatment for the last 24h of transfection, cells were washed once with PBS (at 37ºC), and the incubated with 7.5 µM DHE (from a stock solution of 1 mM in DMSO) in Krebs medium, at 37ºC, and put in the incubator in light-protected conditions, for 1h at 37ºC.

Fluorescence was monitored every 5 min for 1h, at 37ºC, by using a Spectramax GEMINI EM fluorocytometer (Molecular Devices), with excitation and emission wavelengths corresponding to 518 and 605 nm, respectively. After the measure, Krebs medium was removed and the cells were cells were scraped in buffer containing 25 mM HEPES, 1 mM EDTA, 1 mM EGTA, 2 mM MgCl2, and protease inhibitors (0.1 M PMSF, 0.2 M DTT, 1% Triton X-100 and 1:1,000 dilution of a protease-inhibitor cocktail) for posterior protein quantification by the Bradford method.

Chapter 3
Results

3.1. Mitochondrial fragmentation and altered distribution in different PD models

In order to investigate the morphology and distribution of the mitochondrial network in the cybrids it was preformed an immunostaining against the mitochondrial protein Tom20. PD cybrids exhibited a more perinuclear distribution of the mitochondrial network when compared to control cybrids, with and apparent loss of mitochondrial interconnectivity (Fig. 6). This inference is based on the appearance of fragmented mitochondria nearby nucleus with disorganized mitochondrial network that no longer connect with each other. It was also tested the effect of acute MPP^+ exposure, a CXI inhibitor. Incubation with 1mM of MPP^+ for 24 hours fragmented mitochondrial network in a great extent, resulting in the appearance of round-shaped disconnected perinuclear mitochondria. Taken together, PD cybrids exhibit an intermediary mitochondrial network between CT cybrids and MPP^+ treated CT cybrids.

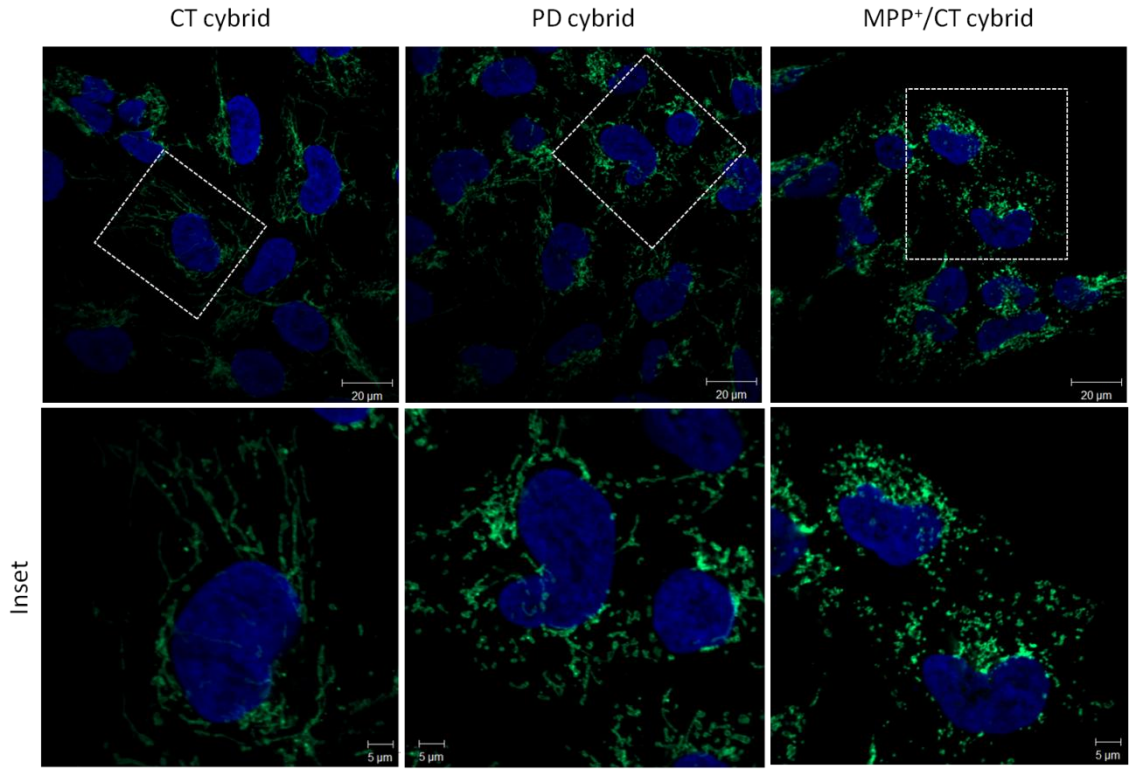


Figure 6 – Mitochondrial fragmentation and altered distribution in PD cellular models. Representative immunofluorescence pictures evidencing mitochondrial network in CT and PD Cybrids, under basal conditions, and in MPP^+-treated CT cybrids (1mM, 24 hours). Immunostaining was performed against the mitochondrial protein Tom20. PD cybrids mitochondrial network morphology is placed between the pronounced mitochondrial fragmentation and perinuclear distribution in MPP^+-treated CT cybrids and the interconnected well distributed CT cybrids mitochondrial network. Green: Tom20, Blue: Hoechst. (Magnification x63)

3.2. MPP^+ modulates fission and fusion proteins levels in CT cybrids in a distinct manner from the observed in basal PD cybrids

Observed the abnormal mitochondrial network in PD cybrids and MPP+ treated CT cybrids we next asked whether there was a disturbance of the proteins involved in the mitochondrial fission and fusion process. The immunoblot performed from total cellular lysates revealed a balance of the protein levels between CT and PD cybrids except in levels of the pro-fusion protein Mfn2 that were increased in PD cybrids (Fig. 7). Treatment of CT cybrids with the CXI inhibitor MPP^+ resulted in a different

modulation of protein levels, significantly decreasing the fusion-stimulatory phosphorylation of Drp1 at Ser616 and Mfn2 levels, when compared to basal CT Cybrids. Strikingly, MPP+ treatment modulates the conversion of the isoforms of the mitochondrial protein Opa1 involved in the fusion process. Indeed, it is observed a conversion of the denominated long isoforms (LI) into the short isoforms (SI), 24h after treatment with MPP^+. Summarizing, the acute CXI inhibition appear to regulate the levels of the proteins involved in mitochondrial fission fusion process differently from a chronic CXI inhibition.

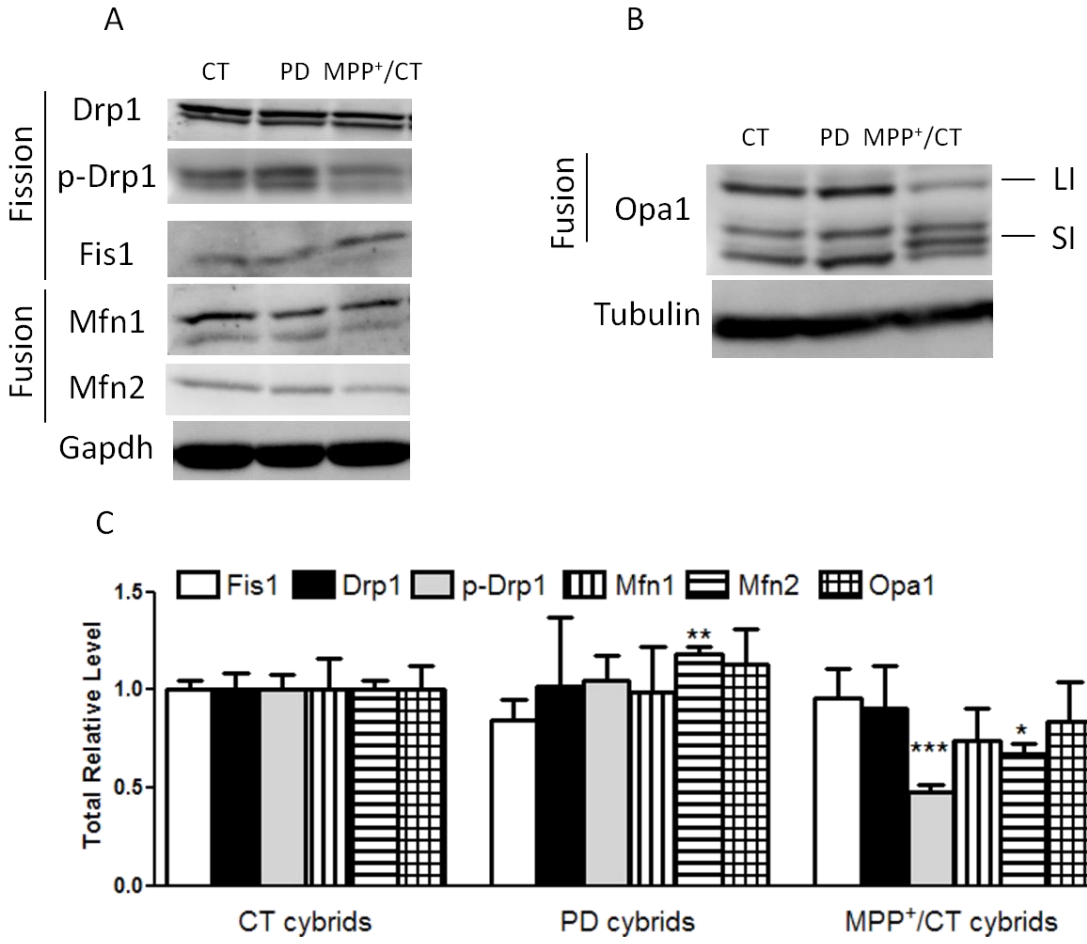


Figure 7 – Accute MPP^+ treatment modulates mitochondrial fission/fusion proteins levels unlike the chronic pathological state, the PD cybrid. Representative immunoblot (*A*,*C*) and quantification analysis (*B*,*D*) revealed that Mfn2 levels are increased in PD cybrids when compared to CT cybrids. MPP^+ treatment of CT cybrids reduces the levels of the fusion protein Mfn2 and the phosphorylation of the fission protein Drp1 (Ser616) (B). MPP^+ also modulates the conversion of Opa1 long isoforms (LI) into short isoforms (SI) (C). Data represent mean ± SEM values derived from, at least, three independent determinations. (*$p < 0.05$; **$p < 0.01$; ***p <0.001, significantly different when compared to CT cybrid group; Student's t-test).

3.3. PD cybrids exhibit decreased mitochondrial levels of the pro-fusion protein Opa1

Given that slight differences in the protein levels were seen between CT and PD cybrids we next asked whether the subcelullar localization of the proteins was similar. For this purpose CT and PD cybrid cells were fractionated in mitochondrial and cytosolic extracts. The results from the fractionation process revealed that PD cybrids have a significant reduction in mitochondrial levels of the pro-fusion protein Opa1, when compared to CT cybrids (Fig. 8). However, no significant differences were seen in the levels of the other proteins in both fractions, inclusive in Mfn2 mitochondrial levels.

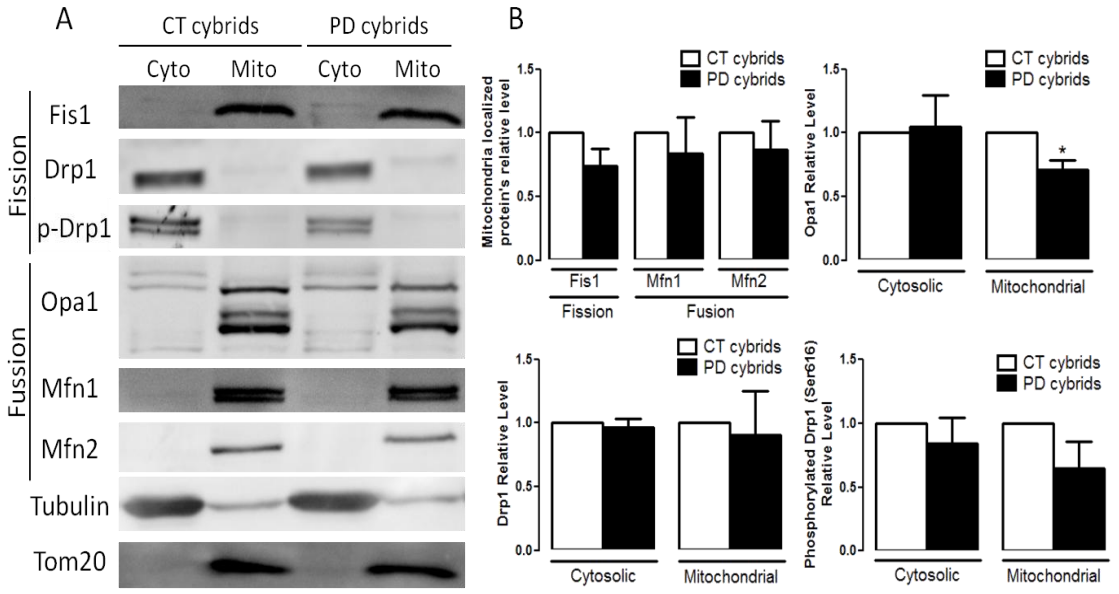


Figure 8 – Decreased Opa1 mitochondrial levels in PD cybrids. CT and PD cybrids cells were harvested and fractionated into mitochondrial and cytosolic fractions. Representative immunoblot (A) and quantification analysis (B) of the proteins levels involved in mitochondrial fission and fusion revealed a decrease in the mitochondrial levels of the pro-fusion Opa1 in PD cybrids. Equal protein amounts (50 µg) were loaded and confirmed with Tubulin and Tom20 for the cytosolic and mitochondrial fractions, respectively. The purity of the mitochondrial and cytosolic fractions is confirmed by the negligible contamination each fraction with the marker of the other fraction. Data represent mean ± SEM values derived from three independent determinations. (* $P < 0.05$, when compared to CT cybrid group; Student's *t*-test).

3.4. Protein expression in PD patients' lymphocytes

In order to get a better insight from how the mitochondrial network morphology is regulated *in vivo*, mitochondrial fission/fusion proteins were analyzed in PD patients, comparing to age-matched healthy subjects. Total lysates from lymphocytes, isolated from venous blood, were immunobloted and no significant statistical variations were seen between PD and CT subjects (Fig. 9), although we could see an increase in pDRP1 levels and a decrease in OPA1 levels, which point towards an increase in mitochondrial fragmentation.

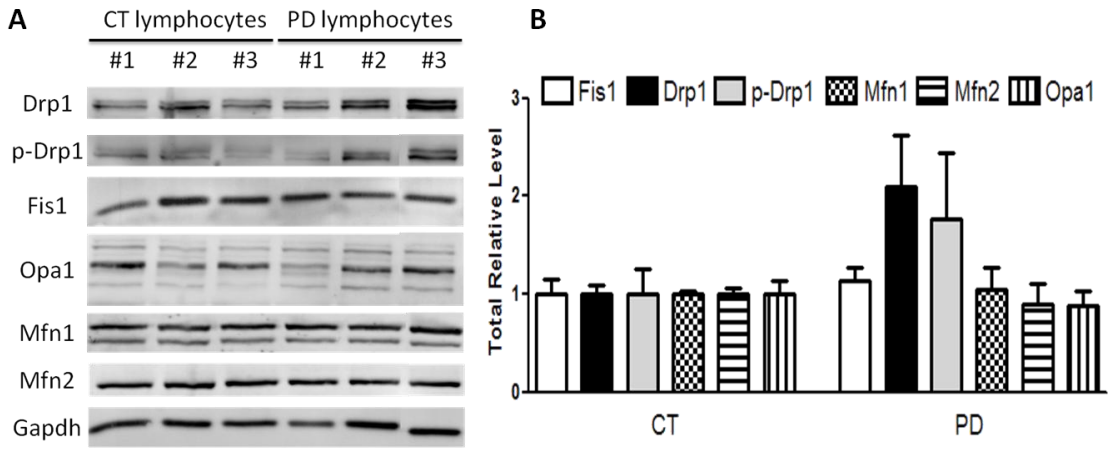


Figure 9 – Basal expression of mitochondrial fission and fusion proteins in PD lymphocytes. Lymphocytes were isolated form PD and age-matched healthy subjects and total lysates were immnunobloted. Representative immunoblot (A) and quantification analysis (B) of the proteins levels involved in mitochondrial fission and fusion revealed no statistical significant differences in the total protein levels of PD subjects, relative to controls. Equal protein amounts (50 µg) were loaded and confirmed with Gapdh. Data represent mean ± SEM values derived from three independent determinations.

3.5. Mitochondrial fragmentation in mitochondrial DNA depleted cells, associated with aberrant protein expression

To gain a better understand of the contribution of mtDNA to the mitochondrial network, immunocytochemistry against the mitochondrial protein Tom20 was performed in mtDNA depleted NT2 cell line. Upon basal condition NT2 ρ0 cell display a pronounced fragmented mitochondrial network with very small round mitochondria instead of a reticulated network seen in NT2 CT cybrids (Fig. 10, 6).

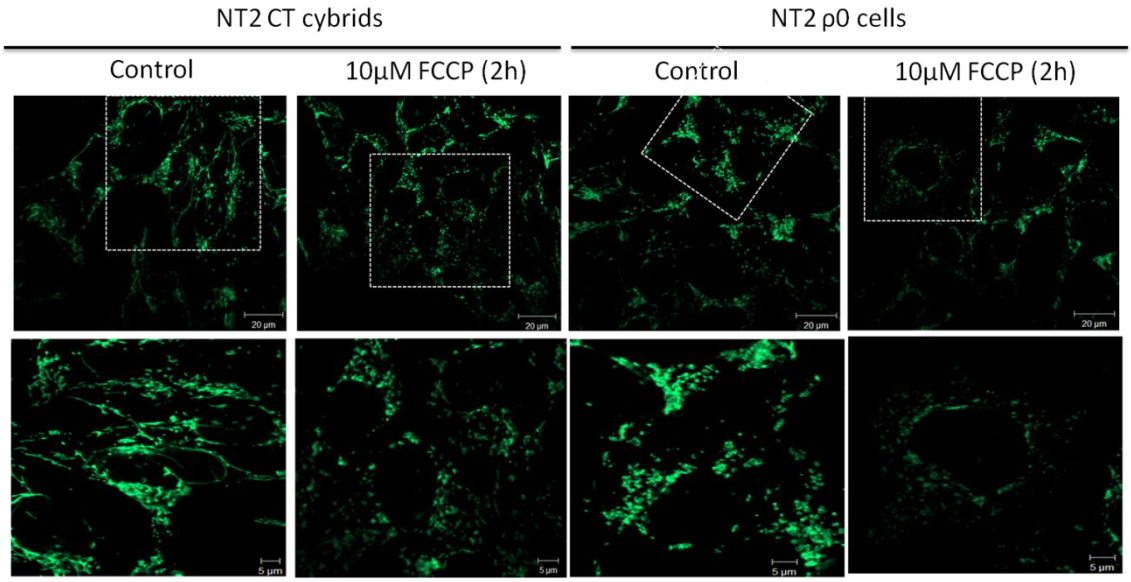
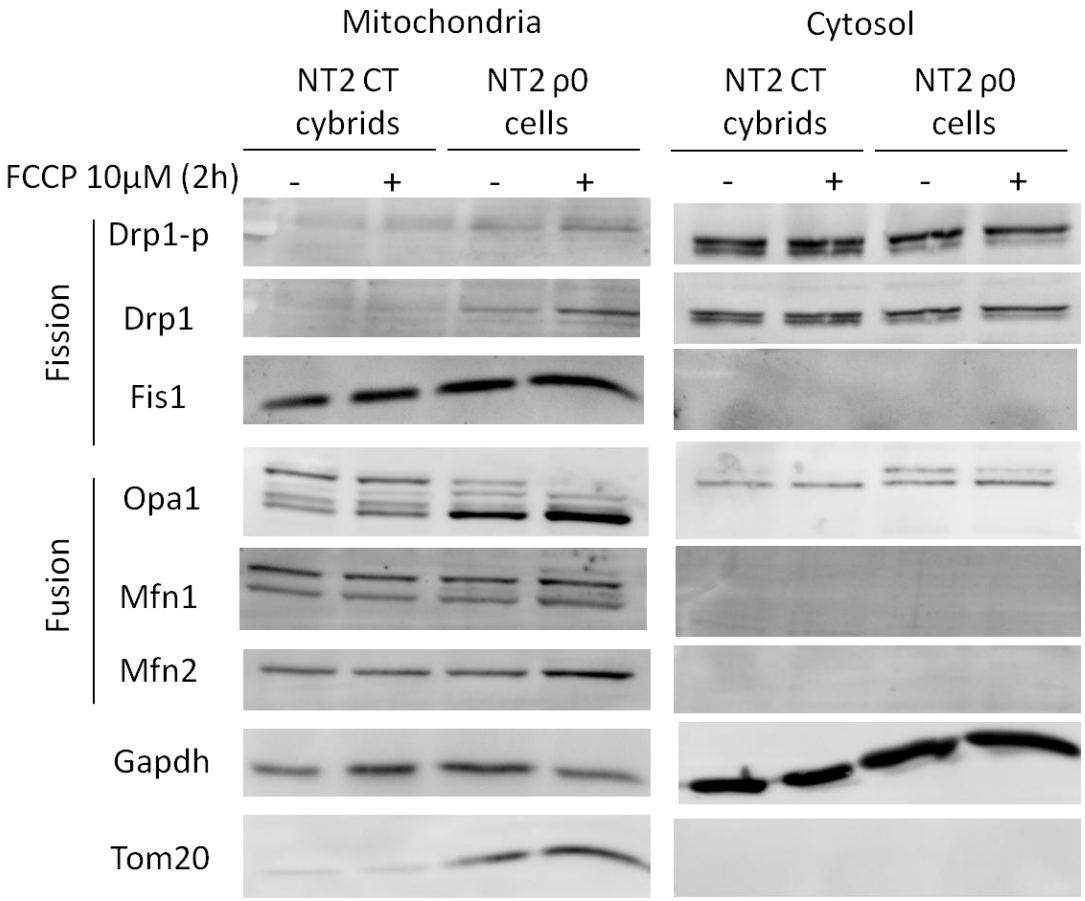


Figure 10 – Mitochondrial fragmentation in mitochondrial DNA depleted cells. Representative immunofluorescence pictures evidencing mitochondrial network in NT2 cells, containing or not mtDNA, under basal conditions, or treated with FCCP (10µM, 2 hours). Immunostaining was performed against the mitochondrial protein Tom20. NT2 ρ0 cells exhibit mitochondrial fragmentation when compared to NT2 CT cybrids. Green: Tom20.

Indeed, an impaired fusion or increased fission may cause fragmentation of the mitochondrial network in the absence of mtDNA. To distinguish between these possibilities, we evaluated in NT2 ρ0 cells the proteomics of the proteins involved in the mitochondrial fission and fusion process. In agreement with previous observations in

PD cybrids (Fig.8), cells lacking mtDNA revealed a significant reduction in mitochondrial levels of the pro-fusion protein Opa1 long isoforms (LI) (Fig. 11). Moreover, it was observed a decrease in Mfns in the mitochondrial fration (Fig.11). Our results strongly suggest that the organelles are unable to fuse.

A



B

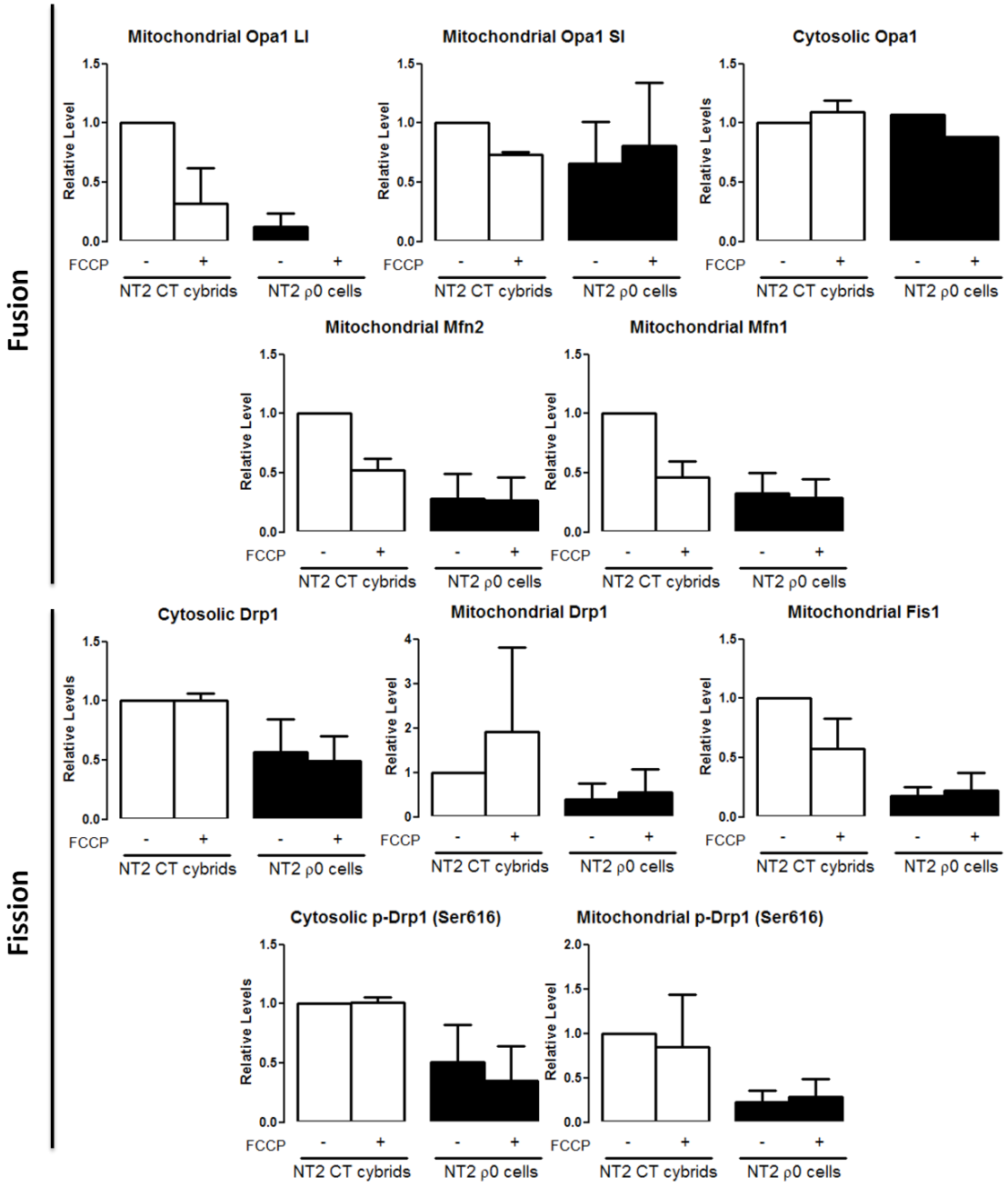


Figure 11 – Fusion and Fission protein expression levels in mitochondrial DNA depleted cells. **C**ells were harvested and fractionated into mitochondrial and cytosolic fractions. Representative immunoblot (A) and quantification analysis (B) of the proteins levels involved in mitochondrial fission and fusion revealed a decrease in the mitochondrial levels of the pro-fusion Opa1 and Mfn1 in NT2 □0 cells. Equal protein amounts (50 µg) were loaded and confirmed with Tubulin and Tom20 for the cytosolic and mitochondrial fractions, respectively. The purity of the mitochondrial and cytosolic fractions is confirmed by the negligible contamination each fraction with the marker of the other fraction. Data represent mean ± SEM values derived from two independent determinations.

3.6 Abnormal fusion and fission in ASYN SH-SY5Y overexpressing cells

To further verify the physiological relevance of the involvement of ASYN in modulating mitochondrial morphology, mitochondrial morphology were monitored by imaging of cells, incubated with Tom20. When wild-type ASYN was overexpressed in SH-SY5Y cells, a decreased mitochondrial fragmentation was observed (Figure 12). In order to validate our results and experimental conditions (4 days overexpressing ASYN) we treated both cells with FCCP, and observed an increase in the mitochondrial fragmentation pattern.

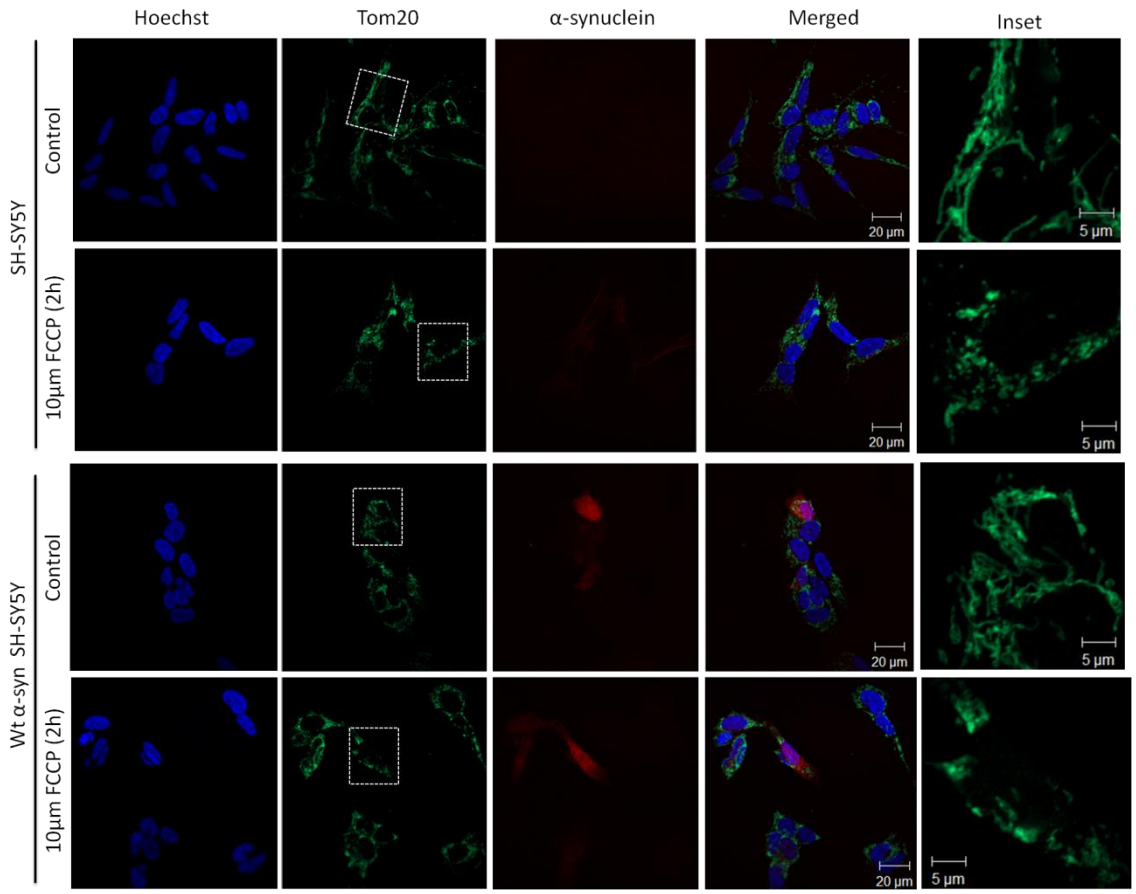
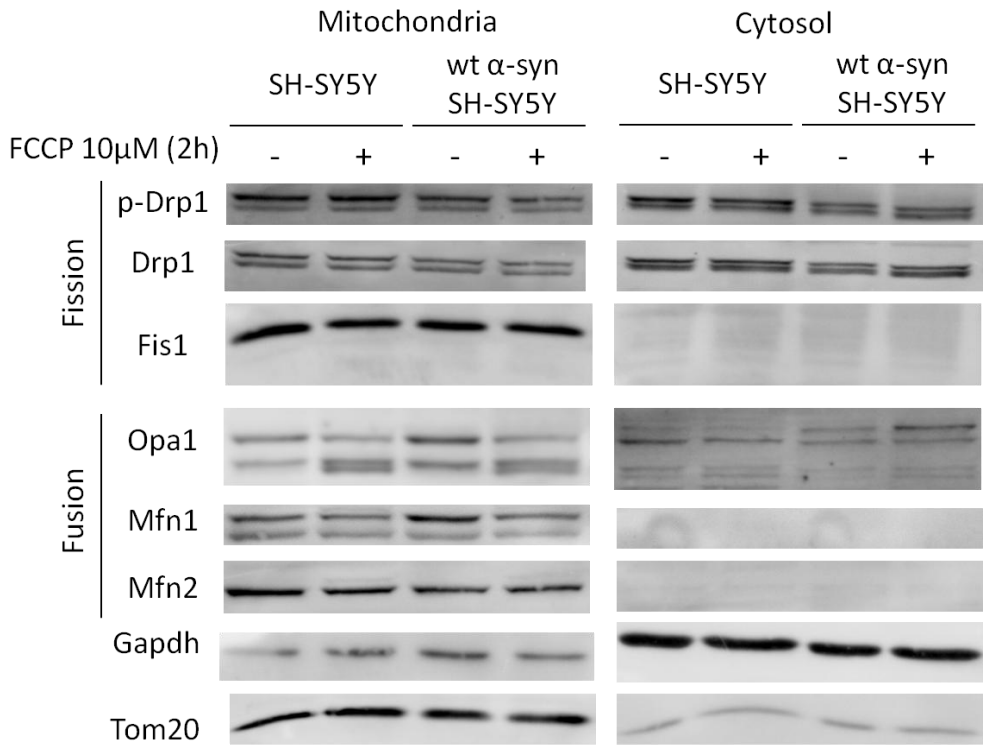


Figure 12 –Mitochondrial fragmentation in wt ASYN overexpressing cells. Representative immunofluorescence pictures evidencing mitochondrial network in SH-SY5Y cells, overexpressing or not wt α-synuclein, under basal conditions, or treated with FCCP (10μM, 2 hours). Immunostaining was performed against the mitochondrial protein Tom20 and α-synuclein. Green: Tom20, Red: α-synuclein, Blue: Hoechst.

Overexpression of ASYN affected the expression levels and subcellular localization of Mfn1, Mfn2, Opa1 and pDrp1 (Fig. 13). Accordingly to our immunocytochemistry data, we observed a decrease in pDRP1 and increased in mitochondrial OPA1 and Mfn1 expression and localization. Our results clearly indicate that ASYN induced a decrease in mitochondrial fission or stimulates mitochondrial fusion.

A


Mitochondria
Cytosol
SH-SY5Y
wt α-syn SH-SY5Y
SH-SY5Y
wt α-syn SH-SY5Y
FCCP 10µM (2h)
- + - + - + - +
Fission
p-Drp1
Drp1
Fis1
Fusion
Opa1
Mfn1
Mfn2
Gapdh
Tom20

B

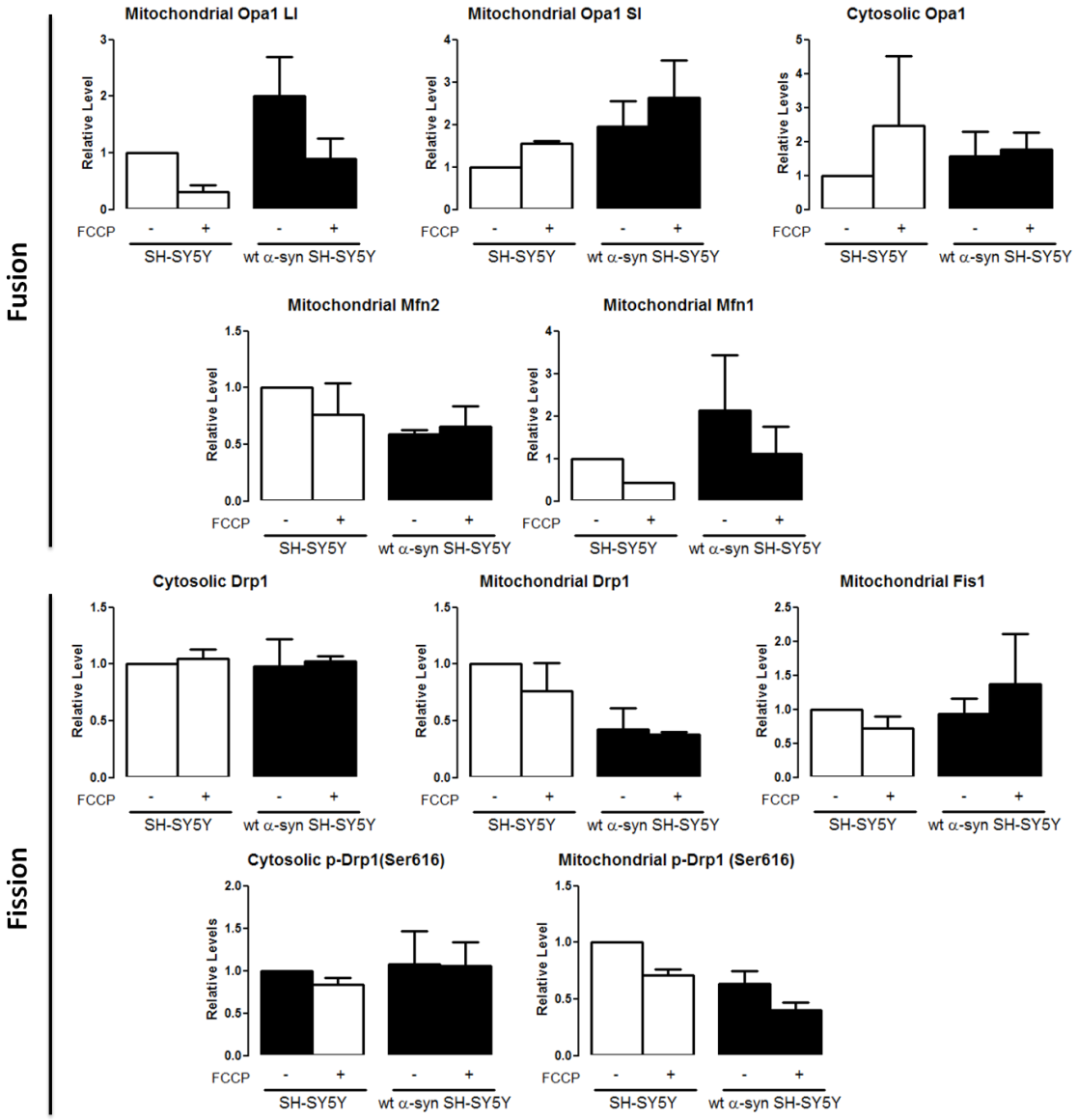


Figure 13 – Fusion and Fission protein expression levels in wt ASYN overexpressing cells. **C**ells were harvested and fractionated into mitochondrial and cytosolic fractions. Representative immunoblot (A) and quantification analysis (B) of the proteins levels involved in mitochondrial fission and fusion revealed a decrease in the mitochondrial levels of the pro-fusion Opa1 and Mfn1 in NT2 □0 cells. Equal protein amounts (50 µg) were loaded and confirmed with Tubulin and Tom20 for the cytosolic and mitochondrial fractions, respectively. The purity of the mitochondrial and cytosolic fractions is confirmed by the negligible contamination each fraction with the marker of the other fraction. Data represent mean ± SEM values derived from two independent determinations.

3.7 MPP^+-induced ROS production is independent of OPA1 mitochondrial levels

Mitochondria are the primary source for endogenous ROS, and a significant increase in ROS levels could be detected in HEK293T cells after 24 h of treatment with MPP^+ (Fig. 14). MPP^+-treated HEK293T cells show an increase in superoxide radical production. When challenged with rotenone, a complex I inhibitor, HEK293T cells increase significantly superoxide radical production. Interestingly, MPP^+ and rotenone co-treatments decreased the detection of ROS levels, probably due to the loss of cell numbers after this trigger.

To explore the relationship between MPP^+-induced mitochondrial fragmentation and ROS production, we also measured ROS levels in OPA1 overexpressing HEK293T cells treated with MPP^+ for 24h. Basal level of ROS was similar in nontreated OPA1 overexpressing cells compared with control cells. Unexpectedly, MPP^+-induced ROS production was not dependent on mitochondrial OPA1 levels. Cells treated with MPP^+ that overexpressed OPA1 showed an increase in ROS production (Fig. 14). These findings indicate Opa1 overexpression doesn't rescue MPP^+ induced ROS production in HEK293T cells.

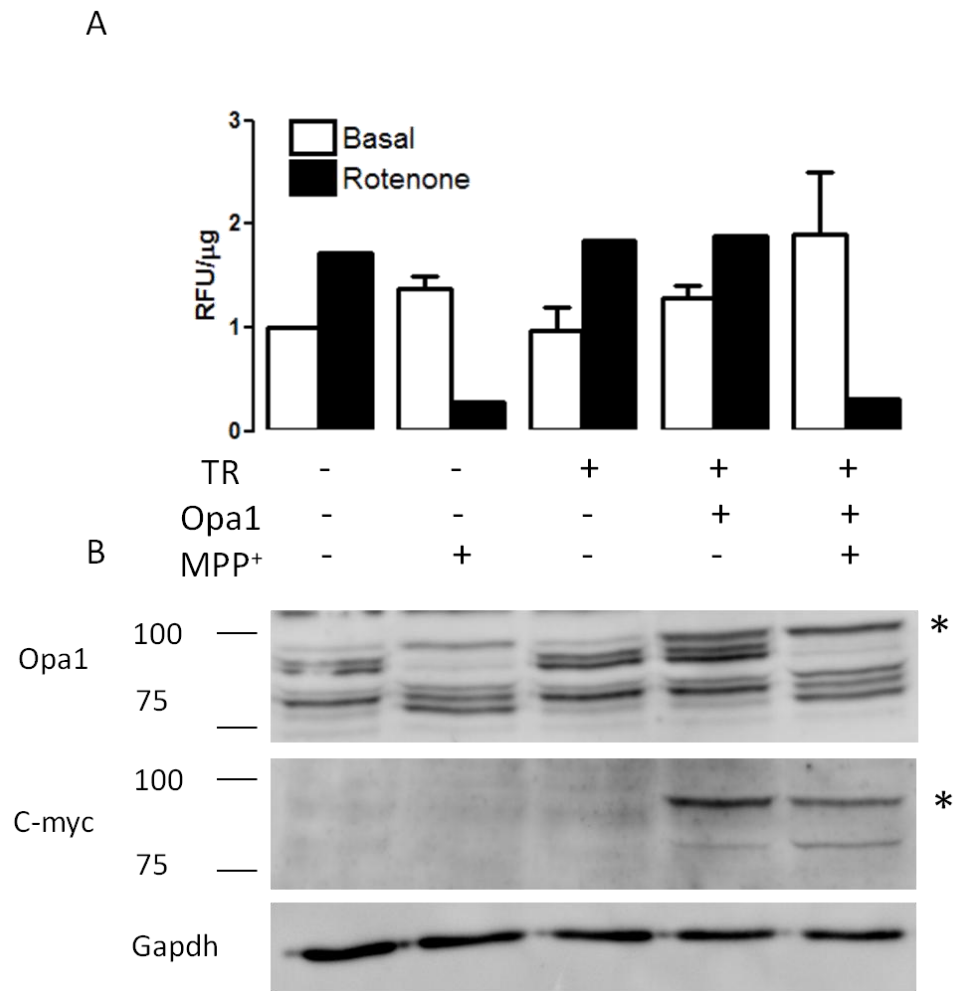


Figure 14 – Opa1 overexpression does not protect against MPP^+ induced ROS production. Quantification of ROS production (A) revealed that MPP^+ treatment increases ROS production which cannot be rescued by previous Opa1 overexpression. Transfection efficiency was confirmed by western blot analysis (B). Asterisks point for the appearance of an additional staining of Opa1 after transfection, which is also reacts to C-myc immunostaining. Equal protein amounts (50 µg) were loaded and confirmed with Gapdh. Data represent mean ± SEM values derived from two independent determinations. TR: Transfection reagent.

Chapter 4
Discussion

The data presented in this work supports the idea that an abnormal mitochondrial morphology and distribution is a consequence of OXPHOS deficiency. Mitochondria in our PD cellular model exhibit an intermediary phenotype between healthy state and acute CXI inhibition-induced severe mitochondrial fragmentation (Fig. 6). Although MPP^+ treatment of our CT cybrids (1mM, 24h) resembles CXI activity deficiency of our PD cybrids (Esteves et al., 2008), fission/fusion proteins' levels modulation is very different. Indeed, our PD cybrids exhibits a reduction of mitochondrial Opa1 levels and accumulation of the Mfn2 levels, both proteins involved in the mitochondrial fusion process (Fig. 7, 8). Treatment of CT cybrids with MPP+ also modulates Opa1 levels, however it significantly reduces Mfn2 levels and the fission-stimulatory phosphorylation of Drp1 at Ser616.

Several CXI inhibitors were described to induce mitochondrial fragmentation in a Drp1- dependent manner (Benard et al., 2007, Gomez-Lazaro et al., 2008, Wang et al., 2011c). Indeed, Wang and colleagues have recently shown that MPP+ mediates Drp1-dependent mitochondrial fragmentation in SH-SY5Y cells as the attenuation of mitochondrial fragmentation by Drp1 knockdown almost prevents the decrease of mitochondrial membrane potential, calcium handling disruption, increased ROS generation and deficient mitophagy, placing mitochondria fragmentation as an upstream event mediating MPP^+-induced toxicity (Wang et al., 2011c). Our results with MPP^+ treatment of CT cybrids are, in some extent, in agreement with the previous study. They had described that at 24h after MPP^+ treatment, during which they had seen a biphasic increase in Drp1 levels, Drp1 levels are similar to control, which is also seen in our MPP^+ treated CT cybrids (Fig. 7). Although it was shown that Drp1 plays an important role in cell death, it might not be the only player, and therefore Wang and co-workers

study lacks information regarding mitochondrial proteins involved in mitochondrial fusion.

Increasing studies have been highlighting a tight regulation of the proteins involved in the mitochondrial fusion process in neurodegeneration, namely Opa1 and Mfn1 & 2. Indeed, decreased expression of these proteins have been described in neurodegenerative diseases, namely in AD and HD (Wang et al., 2008, Wang et al., 2009, Manczak et al., 2011, Shirendeb et al., 2011).

Between our chronic and acute mitochondrial model of PD it appears to be a nexus at Opa1 and Mfn's levels modulation. Indeed, our PD cybrids exhibits reduced mitochondrial Opa1 isoforms levels (Fig. 7, 8) and MPP+ treatment modulates Opa1 levels by converting Opa1 long isoforms into the short ones (SI) (Fig. 7). Along with the fragmented mitochondrial phenotype observed here (Fig. 6), disorganization of mitochondrial cristae structure, mitochondrial membrane potential reduction and mitochondrial cytochrome *c* release were also described in our PD cybrids (Esteves et al., 2008), in agreement with results observed upon Opa1 siRNA-mediated knockdown in HeLa cells (Olichon et al., 2003, Griparic et al., 2004). Indeed, it was suggested that loss of mitochondrial Opa1 accelerates cytochrome *c* release and causes a block in mitochondrial fusion, placing apoptotic mitochondrial fragmentation downstream of these events (Arnoult et al., 2005).

On the other side, whereas in our acute MPP^+-model there is a propensity for a reduction in Mfns levels (Fig. 7), Mfns levels in our PD cybrids remain identical, or higher (Fig. 7, 8). In the field of Mfns' levels regulation it was been extensively described that upon mitochondrial damage Mfn1 & 2 are ubiquitinated by Parkin and degraded in a proteasome dependent manner (Tanaka et al., 2010, Chan et al., 2011). Therefore, it is possible that upon MPP^+ induced damage in CT cybrids mainly Mfn2 is

ubiquitin tagged and degraded by proteasome leading to the decreased Mfn2 levels. However, in our PD cybrids impairment in the UPS (Martins-Branco unpublished work) may either prevent ubiquitination of Mfns or inhibit their proteasome mediated degradation, culminating in the accumulation of defective fragmented mitochondria. Future experiments accessing Mfns ubiquitination can quickly elucidate this hypothesis.

Moreover, our results in PD patients lymphocytes, show a trendline towards an increase in pDRP1 and a decrease in OPA1 levels (Fig. 9), although no statistical significance was obtained with such small sample. These results are in accordance with what we observed in PD cybrids, obtained after fusion of PD patients platelets with mtDNA depleted cells.

Interestingly, Opa1 was shown to be crucial for mtDNA replication and distribution as silencing of Opa1 variants led to mtDNA depletion and to altered mtDNA distribution throughout the mitochondrial network (Elachouri et al., 2011). As the already described cellular deficits of our PD cybrid cell lines are consequences of the inheritance of PD patient mtDNA (Esteves et al., 2008), targeting Opa1 function seems appropriate to maintenance of mitochondrial genome integrity in a cellular model of a disease. In order to address this issue we performed experiments in cells without mtDNA, and observed a similar pattern of mitochondrial morphology and intracellular localization as in PD cybrids (Fig. 10 and 6). Although, we observed a comparable decrease in mitochondrial OPA1 levels, these cells have more dramatic proteomic alterations, like a decrease in mitochondrial Mfn 2 and 1 (Fig. 11). Indeed, NT2 ρ0 cells FCCP, what could be explained by a dramatic alteration towards mitochondrial fragmentation under basal conditions (Fig. 10).

Studies using dominant optic atrophy (DOA) patients fibroblasts with inhibited mitochondrial fusion, caused by mutation in the OPA1 gene, showed disturbed

oxidative metabolism with impaired mitochondrial ATP synthesis after treatment with CXI inhibitor (Zanna et al., 2008).

Since familial PD can also be caused ASYN gene duplication/triplication, which leads to enhanced protein levels of ASYN (Singleton et al, 2003) and patients with sporadic PD show an increase of ASYN mRNA and oligomers as was observed in our sporadic PD model, PD cybrids (Esteves et al., 2011) we wanted to access the effect of wt ASYN in mitochondrial fragmentation/elongation. It was previously described that ASYN mediated mitochondrial fragmentation independently of mitochondrial fusion and fission proteins (Kamp et al 2010). Our results, using the same cell line, but with a stable tranfection of wt ASYN, show a decrease of mitochondrial fragmentation (Fig. 12) with a decreased expression of the fission protein pDRP1 and increases in mitochondrial fusion proteins, OPA1, Mfn1. Our findings suggest that ASYN gene duplications or triplications may lead to increased amounts of ASYN inside the mitochondria (Esteves et al., unpublished work), which may promote mitochondrial fusion in order to dilute toxic ASYN oligomers. More studies with cells overexpressing ASYN need to be done in order to fully understand the contradictory results obtained by Kamp and co-workers (2010) showing that increased amounts of ASYN bind to mitochondria inhibiting mitochondrial fusion and therefore trigger disease pathology.

While it remains to be determined whether and how mtDNA-induced changes in bioenergetic function cause mitochondrial fragmentation, we demonstrated that a compromised OXPHOS causes an excessive mitochondrial fragmentation triggered by a cleavage of the OPA1 LI (Fig. 7 and 8). However, the involvement of mitochondrial fragmentation and perinuclear distribution in OXPHOS-mediated impairments in mitochondrial membrane potential, calcium handling, ROS generation and mitophagy as well as eventual cell death are still undisclosured. Indeed, the attenuation of

mitochondrial fragmentation by OPA1overexpression did not prevent MPP+-induced ROS production (Fig. 14). It was previously described that DRP1 knockdown prevented MPP+-mediated ROS production (Wang et al., 2011). Indeed, Opa1 up-regulation already shown to restore mitochondrial morphology and to protect neurons against excitotoxicity (Jahani-Asl et al., 2011). Although no changes in Opa1 levels and distribution were seen upon exposure to NMDA, this protein was required for calpastatin-mediated neuroprotection, placing Opa1 downstream of calpain activation following an excitotoxic stimulus. Our cybrid cellular model also presents show an exacerbate calpain activation, which may be mediating OPA1 clevadge (Esteves et al., 2011).

Although we still need to address OPA1/mitochondrial fusion involvement in OXPHOS-mediated mitochondrial membrane potential depletion, mitochondrial calcium buffering, and mitophagy, we strongly believe that changes in mitochondrial dynamics and turnover might render neurons susceptible to degeneration in PD.

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