UNIVERSIDADE D
COIMBRA

Bruno Miguel Sequeira dos Santos

MITOCHONDRIAL DYNAMICS AND MITOPHAGY IN CELLULAR MODELS OF PARKINSON'S DISEASE

Dissertação no âmbito do Mestrado em Bioquímica orientada pela Professora Doutora Ana Cristina Carvalho Rego e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Outubro de 2020

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Abstract

Alpha-synuclein (a-Syn) is a protein involved in both sporadic and some familial forms of Parkinson's disease (PD), a neurodegenerative movement disorder that largely affects dopaminergic neurons in the *substantia nigra pars compacta* and progressively the whole cortex. In PD-related synucleopathies, the protein forms oligomers that are toxic to the cell, disrupting intracellular calcium homeostasis, increasing the levels of reactive oxygen species (ROS) and causing the impairment of proteostase mechanisms, such as the ubiquitin-proteasome system (UPS) and autophagy-lysosome pathway (ALP). Mitochondrial dysfunction has been also largely described in PD, including complex I inhibition, energy depletion and increased mitochondrial ROS levels, which together exacerbate cytotoxic processes. Additionally, a-syn aggregates can abrogate mitophagy, a selective autophagy process that degrades damaged mitochondria, thus retaining damaged mitochondria in cells. Considering that PD features may arise from accumulated levels of WT a-syn, the main goal of this study was to define the cytotoxic events linked to deregulated proteostase, namely dysfunctional ALP, and mitophagy in human neuroblastoma catecholaminergic Tet-Off conditional SH-SY5Y cells. Here we show that WT a-syn overexpression causes increased cellular ROS levels and alterations in autophagic protein levels that may preclude WT a-syn from being degraded. Furthermore, when overexpressed, WT a-syn colocalizes with mitochondria leading to decreased mitochondrial membrane potential and altered morphology, which could endorse mitochondrial selective degradation through ALP/mitophagy pathways. Understanding the changes in autophagy/macroautophagy and mitophagy linked to increased levels of WT a-syn may help to identify selective neuroprotective strategies aimed to alleviate PD progression.

Keywords: Parkinson's disease; Alpha-synuclein; Mitochondrial function; Mitochondrial morphology; Autophagy

Resumo

Alfa-sinucleína (a-sin) é uma proteína envolvida em casos esporádicos e alguns casos familiares da doença de Parkinson (DP), uma doença neurodegenerativa que afeta a coordenação motora devido à degenerescência seletiva de neurónios dopaminérgicos da *substantia nigra pars compacta* e, progressivamente, do córtex cerebral. Nas sinucleínopatias relacionadas com a DP, é perturbada a homeostase do cálcio intracelular, aumentam os níveis de espécies reativas de oxigénio e os sistemas de degradação de proteínas, como o sistema ubiquitina-proteassoma e a via da autofagia-lisossoma, tornam-se disfuncionais. No contexto da DP foi anteriormente descrita a ocorrência de disfunção mitocondrial, incluindo a inibição do complexo I, depleção de energia e aumento dos níveis de espécies reativas de oxigénio produzidas pela mitocôndria, que, em conjunto, agravam os processos citotóxicos. Adicionalmente, os agregados de a-sin parecem inibir a mitofagia, um processo específico de autofagia que degrada mitocôndrias disfuncionais, fazendo com que estas sejam retidas na célula. Tendo em conta que as características da DP podem surgir da acumulação de a-sin WT o principal objetivo deste estudo foi definir a a desregulação da degradação de proteínas, nomeadamente a via da autofagia-lisossoma, e as alterações mitocondriais em células SH-SY5Y de neuroblastoma humano condicionadas por um sistema Tet-Off. Os resultados obtidos permitiram observarque a sobre-expressão de a-sin WT causa um aumento dos níveis de espécies reativas de oxigénio e alterações nos níveis de proteínas autofágicas associadas à degradação de a-sin WT. Além disso, após a sua sobre-expressão, aumenta a colocalização da a-sin WT com a mitocôndria, ocorrendo também uma diminuição do potencial da membrana mitocondrial e a alteração da morfologia mitocondrial, potencialmente conduzindo à degradação seletiva do organelo através da via autofágica-lisossomal/mitofagia. Desta forma, compreender as alterações na autofagia/macroautofagia e na mitofagia associadas a níveis aumentados de a-sin WT pode ajudar a identificar estratégias neuroprotetoras seletivas destinadas a atenuar a progressão da DP.

Palavras-chave: Doença de Parkinson; Alfa-sinucleína; Função mitocondrial; Morfologia mitocondrial; Autofagia

Abbreviations

a-syn – Alpha-Synuclein

ALP – Autophagy-Lysosome Pathway

AR – Aspect Ratio

ATF6 – Activated Transcription Factor 6

ATP – Adenosine Triphosphate

BBB – Blood Brain Barrier

BSA – Bovine Serum Albumine

DAT – Dopamine active transporter

DNA – Deoxyribonucleic Acid

DNMT1— DNA Methyltransferase 1

DOX – Doxycycline

Drp1 – Dynamin-Related Protein 1

DTT – Dithiothreitol

EIF2AK3 – Eukaryotic Translation Initiation Factor 2-Alpha Kinase 3

ESCRT – Endosomal Sorting Complexes Required for Transport

FBS – Fetal Bovine Serum

Fis1 – Mitochondrial Fission 1 Protein

GSH – Glutathione

H_2O_2 – Hydrogen Peroxide

HDAC6 – Histone Deacetylase 6

ICC – Immunocytochemistry

IRE1 – Inositol-Requiring Enzyme 1

KO – Knockout

LAMP2 – Lysosome-Associated Membrane Protein 2

LIR – LC3-interacting region

LRRK2 – Leucine-rich repeat kinase 2

MAM – Mitochondria-Associated Membranes

MCU – Mitochondrial Calcium Uniporter

MFN1 – Mitofusin-1

MFN2 – Mitofusin-2

MFNs – Mitofusins

MOM – Mitochondrial Outer Membrane

MPTP – 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MSA – Multiple System Atrophy

mtDNA – Mitochondrial DNA

Na_3VO_4 – Sodium Orthovanadate

NAC – Non-Amyloid Component

NaF – Sodium Fluoride

NMDA – *N*-methyl-D-aspartate

OPA1 – Mitochondrial Dynamin-like 120 kDa protein

PBS – Phosphate-Buffered Saline

PD – Parkinson's disease

PE – Phosphatidyletanolamine

PMSF – Phenylmethylsulfonyl Fluoride

PI3K – Phosphatidylinositol 3-kinase

PVDF – Polyvinylidene Fluoride

RIPA – Radioimmunoprecipitation Assay

ROS – Reactive species of oxygen

RPMI1640 – Roswell Park Memorial Institute's 1640 medium

SAM – S-adenosyl Methionine

SDS-PAGE – Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis

SEM – Standard Error of the Mean

SIRT2 – Sirtuin 2

SNARE – Soluble NSF Attachment Proteins Receptor

SOD1 – Superoxide Dismutase 1 [Cu-Zn]

SOD2 – Superoxide Dismutase 2

TBS-T – Tris Buffered Saline, with Tween 20

TIM – Translocase of the Inner Membrane

TMRM – Tetramethylrhodamine Methyl Ester

TOM – Translocase of the Outer Membrane

UPR – Unfolded Protein Response

UPS – Ubiquitin-Proteasome System

V-ATPase – Vacuolar-Type H+-ATPase

VMAT2 – Vesicular Monoamine Transporter 2

VPS – Vacuolar Protein Sorting

WT – Wild-Type

$\Delta\Psi m$ – Mitochondrial Membrane Potential

Contents

ABSTRACT III

RESUMO IV

ABBREVIATTIONS VI

CONTENTS X

LIST OF FIGURES XI

LIST OF TABLES XI

1. INTRODUCTION 1

Parkinson's disease 1
Alpha-synuclein structure and conformation 2
Alpha-synuclein function 3
Parkinson's disease pathological processes 5
Mutations 5
Epigenetics 8
Disease process 9
Degradation pathways 13
Mitochondrial dynamics 14
Objective 17

2. MATERIAL AND METHODS 18

Materials 18
Culture of stable SH-SY5Y cell lines conditionally expressing WT a-syn 18
Measurement of hydrogen peroxide (H_2O_2) levels 19
Mitochondrial membrane potential assay 19
Immunocytochemistry (ICC) 19
Western blotting 20
Statistical analysis 21

3. RESULTS 22

Wild-type alpha-synuclein overexpression in Tet-Off conditional SH-SY5Y cells is linked to increased ROS levels and differentially affected by autophagy inhibition 22

4. DISCUSSION 35

5. CONCLUSION 41

REFERENCES 42

List of figures

Figure 1: Sporadic PD initiation locals and a-syn aggregation steps. 10
Figure 2: A-syn aggregation effects in PD. 12
Figure 3: Mitochondrial Dynamics affected by a-syn. 16
Figure 4: A-syn and ROS levels in Tet-Off conditional SY-SY5Y cells. 24
Figure 5: Analysis of autophagic proteins Beclin-1, p62, LC3 and ubiquitin in Tet-Off conditional SH-SY5Y cells. 25
Figure 6: Influence of bafilomycin on p62 and ubiquitin levels in Tet-Off conditional SY-SY5Y cells. 28
Figure 7: Colocalization analysis between p62 or ubiquitin and a-syn a-syn in Tet-Off conditional SH-SY5Y cells. 29
Figure 8: A-syn overexpression influences mitochondrial membrane potential, colocalization between a-syn and mitochondria and mitochondrial morphology in Tet-Off conditional SH-SY5Y cells. 31
Figure 9: Analysis of colocalization between proteins involved in mitophagy and mitochondria in Tet-Off conditional SH-SY5Y cells. 34

List of tables

Table I: Known genes associated with PD with respective proteins, position in chromosome, and disease type. 7

1. Introduction

Parkinson's disease

Parkinson's disease (PD) is one of the most common neurodegenerative diseases in the world, first described in 1817 by James Parkinson. Over the age of 60, between 1% and 2% of the population is affected by this illness (Tysnes & Storstein, 2017), values that raise to 3% after 80 years old (Antony et al., 2013). Is more common in men than in woman (Tysnes & Storstein, 2017). Its symptoms are progressive and include motor symptoms like tremor while resting, bradykinesia, muscle rigidity, impaired posture and balance and loss of automatic movements, and non-motor symptoms in early stages like cognitive decline, depression, gastrointestinal symptoms, and olfactory and gustatory deficits, and in later stages mood alteration, sleep disturbances, and dementia are relatively common.

The main hallmarks of PD are the loss of dopaminergic neurons especially in substantia nigra pars compacta in the midbrain (Hirsch, 1994), loss of dopamine in striatum (Kish, Shannak, & Hornykiewicz, 1988), microgliosis (Croisier et al., 2005) and, normally, the formation of fibrous inclusion bodies on the surviving neurons (Gibb, Esiri, & Lees, 1987). These are named Lewy bodies, in the cell somata, and Lewy neurites, in axons and neurites, after its discoverer, Fritz Heirinch Lewy. Lewy bodies are an agglomerate of proteins, like ubiquitin (Kuzuhara et al., 1988), tyrosine hydroxylase (Nakashima & Ikuta, 1984), synaptophysin (Nishimura et al., 1994), and, for the most part, alpha-synuclein (a-syn) (Galvin et al., 1999; Spillantini et al, 1998; Tofaris & Spillantini, 2005), the last one promotes this aggregation when mutated or when is overexpressed. These inclusions are found in post-mortem studies of brains from patients with PD (Shashidharan et al., 2000).

PD have two main types, the familiar one, caused by hereditary mutations in the genome and could be dominant or recessive. The other type is idiopathic PD, known as sporadic PD, which is caused by a conjunction of environmental factors and genetic ones. Although, in both, Lewy bodies are almost every time verified. Familiar cases account for only 10% of PD cases, the other 90% corresponds to sporadic PD. This type of disease could be due environmental exposures like viral infections (Wetmur, Schwartz, & Elizan, 1979), toxins exposure, as 1-methyl-4-phenyl-1,2,3,6-tetrahydroxypyridine (MPTP)

(Langston & Ballard, 1983), industrials exposure (Rybicki et al., 1993), pesticides (Betarbet et al., 2000) and age is also a factor that could explain the disease (Mayeux et al., 1992), although not all the cases of idiopathic disease are known the cause or process that cause the pathology.

Even though Parkinson's disease is the most common disease that displays the presence of Lewy bodies, other diseases have this characteristic and are known as synucleinopathies. Some of these diseases are dementia with Lewy bodies (Grazia Spillantini et al., 1998), multiple system atrophy (MSA) (Gai et al., 1998), Parkinsonism-dementia complex of Guam (Yamazaki et al., 2000), pure autonomic failure (Arai et al., 2000), frontotemporal dementia (Bougea et al., 2017) and Krabbe disease (Smith et al., 2014).

Some genes mutations are identified to be related to PD. The SNCA gene codifies a-syn and is the most relevant gene in PD because expresses the protein that aggregates in Lewy bodies, which promotes the mechanism of toxicity. Leucine-rich repeat kinase 2 (LRRK2) is another gene that causes autosomal dominant PD (Marín, 2006) and most cases present the normal characteristics of PD including the Lewi bodies, although sometimes Lewi bodies are not found, and some additional features could be identified.

Genes like Parkin (West et al., 2002), DJ1 (Lucas & Marín, 2007), and PINK1 (Piccoli et al., 2008) are the ones that cause autosomal recessive disease. These genes encode proteins that are involved in some mechanisms of the disease caused by a-syn aggregation, like mitophagy, but the homozygous deletion of specific sites in these genes also cause parkinsonism symptoms including the formation of Lewy bodies, suggesting a relation between these deletions and the expression of a-syn.

Alpha-synuclein structure and conformation

A-syn is a protein of 14 kDa with 140 amino acids encoded by the *SNCA* gene, localized in the long arm of chromosome 4. The protein has three domains: (i) The N-terminus between amino acids (aa) 1 and 60, has a structure of α-helix (Bussell & Eliezer, 2003) and is capable of binding lipids (Perrin, Woods, Clayton, & George, 2000); (ii) The non-amyloid component (NAC), between aa 61 and 95, promotes the aggregation of a-syn to form fibrils; (iii) The last domain is the C-terminus, between aa 96 and 140, protects a-syn from aggregation and is responsible for ion and polyamine binding. All the

known mutations of *SNCA* identified to cause PD (A30P, E46K, H50Q, G51D, A53E and A53T) (Appel-cresswell et al., 2013; Kotzbauer et al., 2004; Krüger et al., 1998; Lesage et al., 2013; Pasanen et al., 2014; Polymeropoulos et al., 1997; Zarranz et al., 2003) are in the first domain. Besides these mutations, there are also cases of gene multiplication (Chartier-Harlin et al., 2004; Singleton et al., 2003). The two first domains, the N-terminus and NAC, could form an amphipathic alpha-helix that helps the bind to lipids from membranes, and especially the NAC domain has potential to be conformed in a β-sheet structure that facilitates the aggregation.

Is ubiquitously expressed in the brain (Jakes, Spillantini, & Goedert, 1994) but with higher incidence throughout the neocortex, hippocampus, olfactory bulb, striatum, thalamus, and cerebellum (Iwai et al., 1995). It was found to in red blood cells (Nakai et al., 2007), heart(Ueda et al., 1993; Uéda, Saitoh, & Mori, 1994), pancreas (Ueda et al., 1993; Uéda et al., 1994), and skeletal muscles (Askanas, Engel, Alvarez, McFerrin, & Broccolini, 2000; Uéda et al., 1994), although in much less concentration than in the brain (Ueda et al., 1993; Uéda et al., 1994).

A-syn is normally found as a soluble monomer in native conformation in the cytosol but may form multimers while binding to membranes, however, it is debated if a stable insoluble tetrameric form may occur in the cytosol (Bartels, Choi, & Selkoe, 2011). This binding to membranes favors the formation of multimers and consequently protects from the formation of a-syn fibrils (Davidson et al., 1998). a-syn was shown to be localized in the pre-synaptic terminal (Withers, George, Banker, & Clayton, 1997), nucleus (Kontopoulos, Parvin, & Feany, 2006), mitochondria (Li et al., 2007), endoplasmic reticulum (Calì et al., 2012) and in mitochondria-associated membranes (MAM) (Guardia-Laguarta et al., 2014).

Alpha-synuclein function

The protein was first isolated from the Torpedo electric organ (Maroteaux, Campanelli, & Scheller, 1988), where it was localized in the pre-synaptic terminal of nerves and it was predicted that it could be involved in synaptic function, which is coincident with further studies that demonstrated that a-syn interacts with synaptobrevin-2 (Burré et al., 2010), synapsin III (Zaltieri et al., 2015) and rab3A (R. H. C. Chen et al., 2013), which are vesicular proteins that are part of the Soluble NSF Attachment Proteins

Receptor (SNARE) complex, an essential complex in the fusion of vesicles with the pre-synaptic membrane (Südhof & Rothman, 2009).

In the presynaptic terminal of nerves, it is involved in some process of the synapsis, by interacting with synaptobrevin-2 (Burré et al., 2010), synapsin III (Zaltieri et al., 2015) and rab3A (R. H. C. Chen et al., 2013), that are vesicular proteins from the SNARE complex, an essential complex in the fusion of vesicles with the cellular membrane (Südhof & Rothman, 2009). Although this known interaction, there are others that could suggest various functions to a-syn in the cell. One of these interactions is with VMAT2, Knockout (KO) of a-syn raises the levels of VMAT2 in the membrane of vesicles causing more release of dopamine, serotonin transporters, and the dopamine active transporter (DAT), in absence of a-syn, dopamine and serotonin reuptake is decreased (Guo et al., 2008). One of the most controversial interaction is with phospholipases D1 and D2. a-Syn can inhibit the activity of these phospholipases (Gorbatyuk et al., 2010; Payton et al., 2004), suggesting that a-syn plays a role in the cleavage of membrane lipids. Another important protein that demonstrated interaction with a-syn is tyrosine hydroxylase, the key regulatory protein in dopamine synthesis, which is more active when phosphorylated. A-syn inhibits tyrosine hydroxylase activity (Li et al., 2011; Yu et al., 2004) by reducing phosphorylation and stabilizing dephosphorylated tyrosine hydroxylase (Peng et al., 2005). By interfering with tyrosine hydroxylase activity, a-syn is involved in the formation of dopamine. It is still discussed whether a-syn may have a role in lipid metabolism (Ellis et al., 2005), membrane curvature (Varkey et al., 2010) and/or a chaperone-like activity (Kim et al., 2000). Nevertheless, is hard to specify the normal function of a-syn in the cell. Whether by the compensatory effect of other proteins of the family, either by is overexpression being toxic or even because the unstructured structure of the protein that adopts different conformations depending on the situation, it is tough to test what is the a-syn specific function.

A-syn is a protein that is subject to diverse post-translational changes. Between them are the acetylation (Kang et al., 2012; Trexler & Rhoades, 2012), ubiquitination (Lee et al., 2008), truncation (Muntané, Ferrer, & Martinez-Vicente, 2012), sumoylation (Kunadt et al., 2015), glycation (Vicente Miranda et al., 2017), phosphorylation (Muntané et al., 2012), and nitration (Giasson et al., 2000). A-syn acetylation is a very important post-translational change in PD. A-syn is natively acetylated in lysine residues in N-

terminal. This acetylation increases the propensity to helical folding, which promotes binding to membranes (Dikiy & Eliezer, 2014). The binding to membranes will decrease the propensity to form the toxic oligomers (Bartels et al., 2014). Sirtuin 2 (SIRT2) is a deacetylase that could be involved in PD mechanism altering the native acetylated conformation of a-syn, removing the acetylation in the lysine residues (de Oliveira et al., 2017; Outeiro et al., 2007). These enzyme levels are higher in older people, supporting the possible effect in PD (Maxwell et al., 2011). HDAC6 is histone deacetylase that also deacetylases non-histone proteins. A-syn could be deacetylated by this protein in the lysine residues 6 and 10. The inhibition of HDAC6 with tubastatin A increases a-syn acetylation, and consequently reduce toxicity and up-regulates autophagy mediated by chaperones (Francelle, Outeiro, & Rappold, 2020). Phosphorylation is also an important modification of a-syn in PD. Phosphorylation in the serine 129 residue is found in the protein in Lewy bodies, which may imply a role of phosphorylation in a-syn aggregation (Anderson et al., 2006). This residue is phosphorylated by casein kinase 1 and 2 (Okochi et al., 2000), enzymes that demonstrate high activity in substantia nigra neurons (Desdouits et al., 1994).

Parkinson's disease pathological processes

Mutations

The first mutation found in this gene that cause PD was the A53T in an Italian-American family and in Greek families (Golbe et al., 1996; Polymeropoulos et al., 1997) and is the most common SNCA mutation with a mean onset at 46 years (Papapetropoulos et al., 2001) that often leads to cognitive decline and dementia (Polymeropoulos et al., 1997). A30P was discovered in a German family with a later onset in mean at 60 years with mild dementia (Krüger et al., 1998; Krüger et al., 2001), E46K in a Spanish family and onset between the ages of 50 and 65 years old with parkinsonism, dementia and visual hallucinations (Zarranz et al., 2004). The other three mutations were discovered more recently: A53E in a Finnish patient with an early onset at 36 years (Pasanen et al., 2014), G51D generally with early onset (Lesage et al., 2013) and H50Q with late onset (Kiely et al., 2015). Overexpression of wild-type a-syn, in duplicated or triplicated genes, also cause the aggregation (Chartier-Harlin et al., 2004; Kara et al., 2014; Singleton et al., 2003), with the triplicated phenotype bringing a more severe and earlier type of the disease than the duplicated (Ross et al., 2008).

LRRK2 is a 2527 amino acids protein also related with PD. Several missense mutations were identified but only eight are known to be associated with parkinsonism. Causing a late onset autosomal dominant (Funayama et al., 2002). The normal protein is a kinase that phosphorylates RAB GTPases, inhibiting these proteins function and localizes to vesicular structures. The disease resembles the normal PD symptoms with mild autonomic symptoms, DOPA-responsive disease, and age onset around the 50's being less rapid than idiopathic PD (Healy et al., 2008). Lewy bodies and loss of dopaminergic neurons in the substantia nigra are normal but sometimes are found abnormal characteristics like nigral degeneration without Lewy bodies, glial cytoplasmatic inclusions and fulminant plaque and tangle pathology (Zimprich et al., 2004).

Parkin is a E3 ubiquitin ligase protein with 465 amino acids and was the second gene found related to parkinsonism. Is a protein involved in mitophagy, an important process to clear damaged mitochondria from the cell (Narendra et al., 2010). Also have a role in ubiquitin-proteasome system (UPS), and mitochondrial maintenance, fusion and fission (Deng, Dodson, Huang, & Guo, 2008; Exner et al., 2007; Kamp et al., 2010). Mutations in this protein cause early onset juvenile PD and is autosomal recessive (Kitada et al., 1998). Foot dystonia, psychiatric symptoms and a dramatic response to treatment are typical (Khan et al., 2003). Disease caused by mutations in Parkin are characterized by heavy loss of neurons in substantia nigra pars compacta, lack of Lewy bodies in most cases and occasionally Tau pathology (Farrer et al., 2001; Van De Warrenburg et al., 2001). The lack of Lewy bodies are attributed to the young age of patients, because when found are in older patients (Doherty & Hardy, 2013; Farrer et al., 2001).

PINK1 is other protein related with PD. Is a protein of 581 amino acids and in cell is responsible for activate Parkin in mitophagy (Narendra et al., 2010), consequently have a role in clear damaged mitochondria from the cell. Consequently, parkin mutated non-functional could lead to an accumulate of damaged mitochondria and consequently cell apoptosis. In relation with PD there were already identified some mutations that cause slow progression early-onset PD (Marongiu et al., 2007) L-DOPA responsive. PD caused by mutations in this protein is autosomal recessive and resembles idiopathic PD symptoms without dementia. Loss in the substantia nigra pars compacta, Lewy bodies and aberrant neurites are characteristic but the locus coeruleus and amygdala were spared (Samaranch et al., 2010).

***Table I:* Known genes associated with PD with respective proteins, position in chromosome, and disease type.**

Gene	**Protein**	**Chromosome**	**Inheritance**
SNCA (PARK1/PARK4)	Alpha-synuclein	4q21	Autosomal Dominant
PARK2	Parkin	6q25·2–27	Autosomal Recessive
PARK3	Unknown	2p13	Autosomal Dominant
UCHL-1 (PARK5)	Ubiquitin c terminal hydrolase	4p14	Autosomal Dominant
PINK1 (PARK6)	Pten-induced putative kinase 1	1p35–36	Autosomal Recessive
PARK7	DJ-1	1p36	Autosomal Recessive
LRRK2 (PARK8)	Leucine-rich repeat kinase 2	12p11·2–q13·1	Autosomal Dominant
ATP13A2 (PARK9)	Lysosomal type 5 ATPase	1p36.13	Autosomal Recessive
PARK10	Unknown	1p32	Unknown
GIGYF2 (PARK11)	GRB interacting GYF protein 2	2q37.1	Autosomal Dominant
PARK12	Unknown	Xq21-q25	X-linked
HTRA2 (PARK13)	HTRA serine	2p13.1	Autosomal Dominant
PLA2G6 (PARK14)	Phospholipase A2	22q13.1	Autosomal Recessive
FBXO7 (PARK15)	F-box only protein 7	22q12.3	Autosomal Recessive
PARK16	Unknown	1q32	Unknown
VPS35 (PARK17)	Vacuolar protein sorting 35	16q11.2	Autosomal Dominant
EIF4G1 (PARK18)	Eukaryotic translation initiation factor 4	3q27.1	Autosomal Dominant
DNAJC16 (PARK19)	DNAJ/HSP40 homolog subfamily C member 6	1p31.3	Autosomal Recessive

DJ-1 protein is codified by the gene PARK7 is a protein of 189 amino acids. Is involved in combating oxidative stress acting as a stress sensor and antioxidant, and have a role in maintaining the normal dopaminergic function (Lev et al., 2009; Menzies, Yenisetti, & Min, 2005; Mitsumoto & Nakagawa, 2001). Normal protein could also protect against PD because inhibits a-syn aggregation (Shendelman et al., 2004) and be

involved in PINK1 transcription (Requejo-Aguilar et al., 2015). PD caused by DJ-1 is autosomal recessive early-onset caused by deletions in the gene (Bonifati et al., 2003).

Epigenetics

Deoxyribonucleic Acid (DNA) methylation is a process where a methyl group is transferred from S-adenosyl methionine (SAM) to a cytosine residue from the DNA by action of methyltransferases and occurs normally in CpG pairs. This modification is from great importance in some genes, because the presence of methylated cytosine (5-methylcytosine) changes chromosome conformation, histones interaction with DNA and gene expression (Cao, Zhang, & DU, 2013). Methylation could interfere with binding sites of transcription factors inhibiting expression and is also recognized by Methyl-CpG binding proteins that activates a co-repressor protein contributing to gene silence (Boyes & Bird, 1991). So, aberrant methylation sites could be cause of disease by altering the expression of certain genes (Masliah et al., 2013). Age is a factor that promotes aberrant methylation, causing in different genes hypermethylation and hypomethylation. With more advanced age, more prone to epigenetic changes people are, and consequently it is more likely to suffer from a disease caused by these changes (Maegawa et al., 2010).

With the relation between age and epigenetic changes made, and being PD a disease prone to occur in more elderly population, it was supposed that some cases of idiopathic PD could be explained by those alterations. Hence, it was studied the epigenetics patterns of SNCA gene, that expresses a-syn. It was found that SNCA own two CpG islands, one in the first exon, and the second in the first intron (Matsumoto et al., 2010). *In vitro* methylation of the CpG in the first intron demonstrate that promoter activity in those cases was reduced concluding that methylation in the first intron should reduce a-syn expression (Jowaed et al., 2010; Matsumoto et al., 2010). This was corroborated by studies from PD patients' samples where was verified hypomethylation in the first intron from the SNCA gene in substantia nigra and in blood samples (Jowaed et al., 2010; Pihlstrøm et al., 2015). DNA methyltransferase 1 (DNMT1), is a methyltransferase involved in DNA methylation by binding to hemimethylated DNA maintaining methylation patterns after DNA replication (Long, He, Huang, & Li, 2014). a-syn could interact with these methyltransferase sequestering Dmnt1 outside the nucleus, and a-syn aggregation inclusively enhance this sequestration. With Dmnt1 outside nucleus, methylation will be impaired (Desplats et al., 2011). This hypomethylation

would cause a-syn overexpression leading to protein aggregation and consequently to the next PD mechanisms which will lead to loss of dopaminergic neurons and PD symptoms. Apart to these results, contrary results were also found, with no difference in methylation patterns in SNCA gene between normal samples and PD ones (Guhathakurta et al., 2017; J. Richter et al., 2012).

Another methylation influence in PD could be found in hyperhomocysteinemia condition, caused by a deficient folate diet. Homocysteine is a precursor of methionine that gives origin to SAM and homocysteine in high levels is considered dangerous to dopaminergic neurons (Duan et al., 2002). Hyperhomocysteine will lead to oxidative stress by N-methyl-D-aspartate (NMDA) receptors activation which will activate nitric oxide synthase leading to radicals formation and mitochondria dysfunction (Jara-Prado et al., 2003; W. K. Kim & Pae, 1996; Lipton et al., 1997). Exposure to pesticides and endocrine disruptors are also possible that can alter methylation patterns and contribute to PD by inducing reactive oxygen species (ROS) formation (Kajta & Wójtowicz, 2013; Kumar & Thakur, 2017; Preciados, Yoo, & Roy, 2016).

Disease process

The process of aggregation starts with monomers aggregating into dimers, trimers, oligomers, protofibrils, and then fibrils, forming the insoluble Lewy bodies (Figure 1). Even though during several years it was thought that the most toxic form were the Lewy bodies, various studies demonstrated that oligomers are the most toxic forms (Pountney, Voelcker, & Gai, 2005); moreover, oligomers may propagate from cell to cell through exosomes or nanotubes (Danzer et al., 2012; Rostami et al., 2017; Vekrellis et al., 2009).

The aggregation in sporadic cases could start at the olfactory bulb or the enteric nervous system (Figure 1) because are locals more exposed to possible environmental factors and consequently prone to higher levels of oxidative stress therefore high chances to the formation of a misfolded protein that easily aggregate (Del Tredici & Braak, 2016). A-syn aggregates can propagate from nerve cell to nerve cell. This transfer of a-syn aggregates from cell to cell was first verified after an autopsy of a PD patient, that had received a graft of fetal dopaminergic neurons as an experiment treatment several years before. This patient had Lewy bodies in the host surviving dopaminergic cells, which is normal, but the cells that were grafted possessed similar protein aggregates, suggesting,

for the first time, that these could be transferred to other cells. . After this discovery is said that PD could be a prion-like disease because these movements of misfolded proteins propagating for other cells are the mechanism of action of prion diseases (Goedert, Clavaguera, & Tolnay, 2010; Olanow & Brundin, 2013).

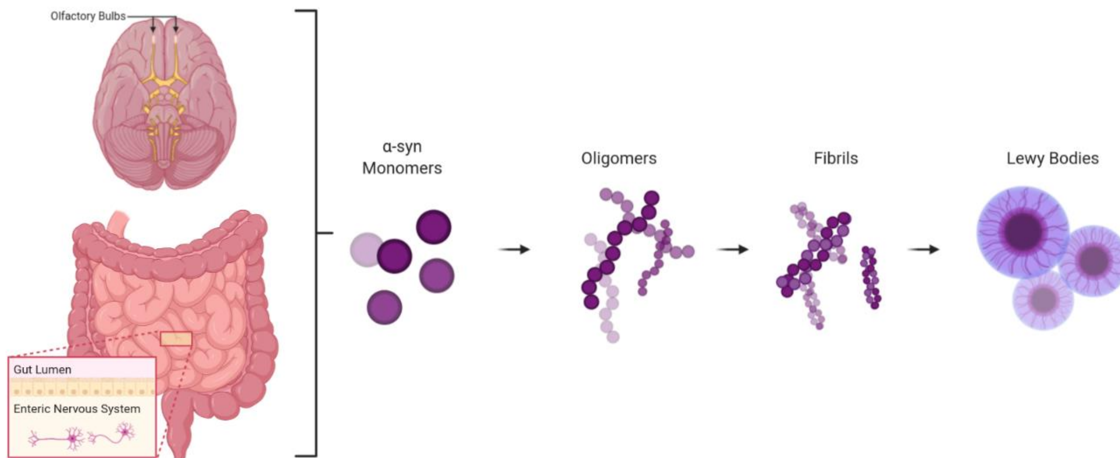

Figure 1: Sporadic PD initiation locals and a-syn aggregation steps. Olfactory bulb and enteric nervous system are suggested to be places that could initiate the process of a-syn aggregation because are susceptible to environmental factors. This aggregation from monomers that can be transported from cell to cell, until the brain where will form Lewy bodies, characteristic from PD. (Original illustration created with BioRender.com)

Inside the cells, these oligomers are responsible for several events that will in the final result in the apoptosis of the neurons, which justifies the loss of neurons in PD. Normally these toxic aggregates would be degraded by the cell, but the oligomers disrupt the ubiquitin-proteasome machinery and the autophagy-lysosomal pathway (ALP) (Nogalska et al., 2010; Pan et al., 2008). As a result, a-syn oligomers accumulate in the cell and further increase protein aggregation.

a-syn oligomers can create pores in the membrane disrupting the calcium homeostasis because permits the entry of ions from extracellular space (Luth et al., 2014). The oligomers can also cause damage in mitochondria and endoplasmic reticulum promoting the stress of these organelles (Luth et al., 2014). The influx of calcium improves the a-syn oligomerization rate (Nath et al., 2011). Increased intracellular levels of calcium raises the entry of calcium to the mitochondria through the mitochondrial calcium uniporter (MCU) (Figure 2). Within the mitochondrial matrix, calcium controls the production of energy because it activates enzymes that play a key role in metabolism (Denton, 2009; Nichols & Denton, 1995). However, when in excess, mitochondrial calcium causes disruption of the mitochondrial membrane potential ($\Delta\Psi m$), which is

normally maintained high by the 'pump' of protons from the mitochondrial matrix to the intermembrane space through complexes I, III and IV. Mitochondrial inhibition of complex I has been long recognized in *postmortem* brain tissue, human cells and PD animal samples; MPTP, which causes PD symptoms (Dauer et al., 2002; Langston & Ballard, 1983; Tretter, Sipos, & Adam-Vizi, 2004) after crossing the blood brain barrier (BBB) and is converted to MPP+, being taken up by the DAT in dopaminergic neurons, also inhibits complex I (Mizuno et al., 1988). Selective mitochondrial disruption is observed in the presence of rotenone or paraquat, which were previously used as pesticides. Under these conditions, mitochondrial ROS levels augment, despite the presence of antioxidants in the organelle matrix, such as superoxide dismutase 2 (SOD2), reduced glutathione and glutathione peroxidase and reductase, and cytochrome C is released, accelerating ROS production (Luth et al., 2014) (Figure 2).

An important characteristic of nigral dopaminergic neurons is the autonomous activity that they demonstrate even without synaptic input (Albin, Young, & Penney, 1989; Gonon & Bloch, 1997). This property could be influenced by an L-type channel that could be open at a hyperpolarized potential, which permits a larger influx of Ca^{2+} than in normal neurons (Nedergaard, Flatman, & Engberg, 1993; Puopolo, Raviola, & Bean, 2007). This influx had to be buffered by cellular mechanisms like buffering proteins and storage at mitochondria and endoplasmic reticulum. However, there are fewer buffer proteins like calbindin (Yamada et al., 1990), and the pores formed by a-syn oligomers enhance the influx of calcium ion. The influx of calcium improves the a-syn oligomerization rate (Goodwin et al., 2013; Nath et al., 2011). More calcium inside cells leads to an increase in the calcium that enters to mitochondria. Levels of oxidative damage and nitric oxide augments, cytochrome C is released and $\Delta\Psi m$ is dysregulated (Green & Kroemer, 2004; Perier et al., 2005; Vila & Przedborski, 2003). The higher levels of ROS and nitric oxide promote a high number of mutations in mitochondrial DNA (mtDNA), which will impair the expressed mitochondrial complexes and consequently energy production (Kurihara et al., 2012; C. Richter, 1992; Swerdlow et al., 1996; Tieu, Ischiropoulos, & Przedborski, 2003).

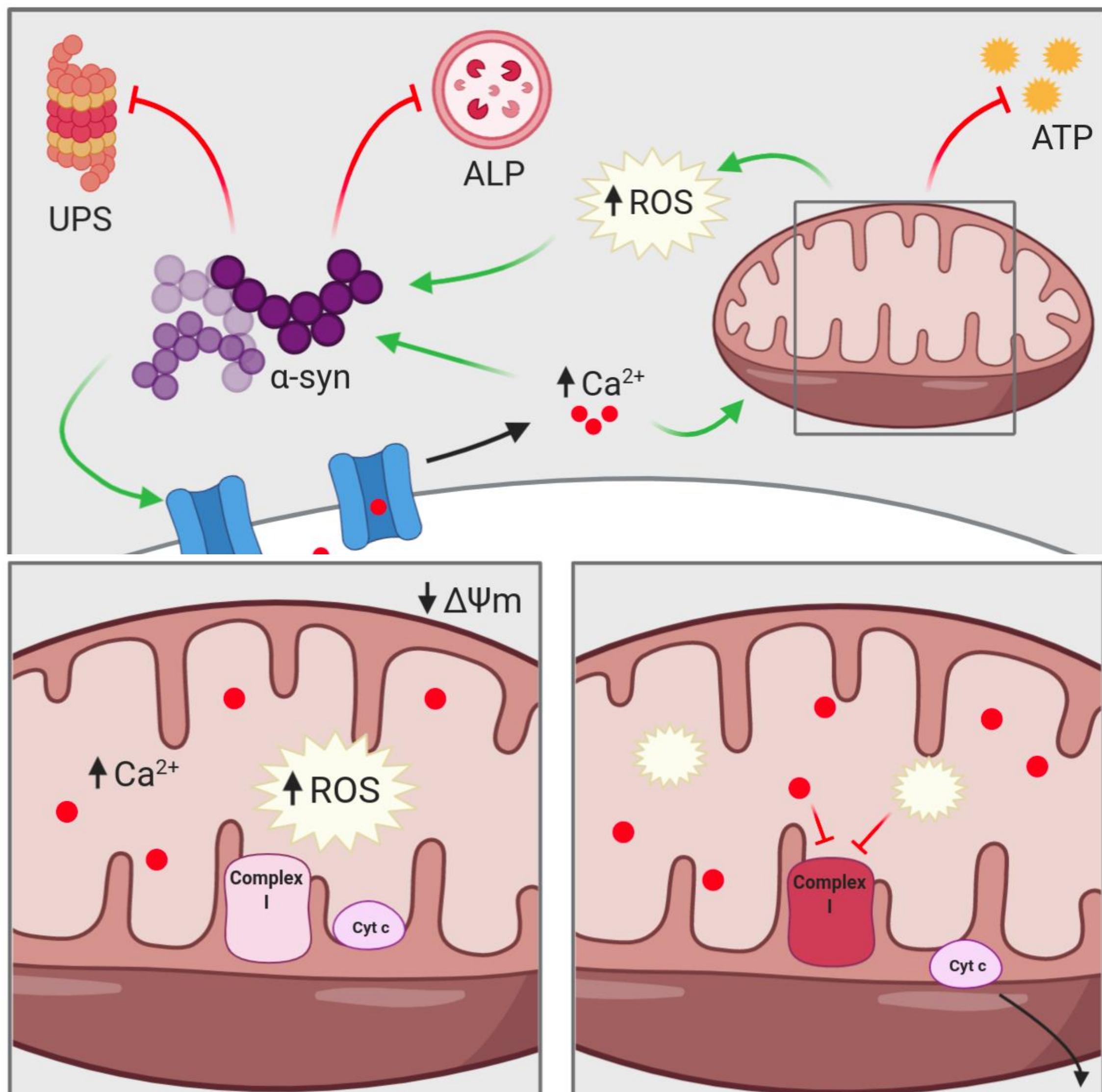

Figure 2: A-syn aggregation effects in PD. A-syn aggregation leads to disruption in calcium homeostasis and autophagy inhibition. The excess of calcium in mitochondria will lead to ROS production and decrease the mitochondrial membrane potential. This will impair complex I causing energy depletion. The damage in mitochondria will lead to cytochrome C release that ultimately leads to neuron apoptosis. (Original illustration created with BioRender.com)

UPS and ALP are inhibited by a-syn oligomers (Crews et al., 2010; McNaught & Jenner, 2001; Winslow et al., 2010; Winslow & Rubinsztein, 2011). Thus, misfolded a-syn stays in the cell, and mitochondria are marked to mitophagy, remaining dysfunctional in the cell without being degraded (Geisler et al., 2010). Autophagosomes that are relevant for mitophagy have in their surface the LC3 proteins (LC3-II) that bind with p62 on the surface of mitochondria, allowing the mitophagy process (Youle & Narendra, 2011), but when oligomers of a-syn are present in PD condition, the autophagosomes have much less LC3 in the membrane, inhibiting mitophagy (Winslow et al., 2010). The accumulation of these proteins and dysfunctional mitochondria will finally lead to the

activation of apoptosis-related proteins, like caspases, leading to neuronal degeneration (Tatton, 2000).

Degradation pathways

Cell mechanisms to degrade proteins and organelles are crucial to maintain the normal cell mechanism, allowing cells to degrade cellular components like misfolded proteins and protein aggregates or impaired organelles, avoiding these components from causing cell damage.

Autophagy is one of the processes of degradation used by the cell. It mainly degrades insoluble protein aggregates and long lived proteins or impaired organelles, like mitochondria (a selective process named mitophagy) (Anding & Baehrecke, 2017; Ravikumar, Duden, & Rubinsztein, 2002; Roberts & Deretic, 2008). Autophagy pathways include microautophagy, chaperone-mediated autophagy and macroautophagy. The later process has been mostly described to be dysfunctional in several neurodegenerative diseases. Macroautophagy (here refered as autophagy) results in the formation of an autophagosome that fuses with lysosomes, degrading the content of the autophagosome. The process starts with ULK1 activation that phosphoryl ATG13 and FIP200 (Hosokawa et al., 2009). These three proteins form a complex that promotes the initiation of phagophore membrane formation (Park et al., 2016). This membrane is then formed by the action of the phosphatidylinositol 3-kinase (PI3K) complex, composed by proteins like Beclin-1, ATG14 and Vacuolar protein sorting (VPS) (VPS15 and VPS34), that creates Phosphatidylinositol-3-Phosphate, and other ATG proteins (ATG5, ATG12 and ATG16) that act as elongation factors (Funderburk, Wang, & Yue, 2010; Merrill et al., 2017; Romanov et al., 2012). During this process LC3 is added to the membrane (forming LC3-II, the lipidated form of LC3-1), an important autophagosome membrane receptor in autophagy (Pankiv et al., 2007). Ubiquitin is involved in macroautophagy by forming polyubiquitinated chains linked to proteins. When ubiquitin chains are formed by monomers linked at Lys-63, these chains are targeted to autophagy (Tan et al., 2008). These polyubiquitin chains are recognized by adaptors proteins like p62 that interacts with LC3 in the autophagosome. When the components to be degraded are inside the autophagosome, this can fuse with lysosomes by the action of SNAREs, Rab GTPases and lysosome-associated membrane protein 2 (LAMP2) (Fortunato et al., 2009; Hubert et al., 2016; Kern, Dikic, & Behl, 2015; Moreau, Renna, & Rubinsztein, 2013).

Ubiquitin is also involved in other form of protein degradation, like the UPS. This system is responsible for degrading soluble misfolded proteins or short-lived proteins (Finley, 2012; Fuertes, Villarroya, & Knecht, 2003). These proteins are ubiquitinated through a mechanism that involves three type of enzymes. E1 enzymes, or ubiquitin-activating enzymes, activate ubiquitin when it attaches to E1 in an ATP-dependent manner. Activated ubiquitin then binds to E2, which are ubiquitin-conjugating enzymes. Finally, E3 enzymes (ubiquitin ligase) bind to the protein that ought be degraded and transfer activated ubiquitin from E2 to the protein (Mulder et al., 2016; Scheffner, Nuber, & Huibregtse, 1995). The process is repeated to form a polyubiquitylated chain, with ubiquitin monomers linked to each other in Lys-48 (Chau et al., 1989). The polyubiquitylated protein will then be identified by the 19S regulatory complex of the 26S proteasome, being degraded at the 20S catalytic core complex. Deubiquitinated enzymes or ubiquitin proteases cleave the peptide bond between ubiquitin and its substrate protein before the protein gets inside the proteasome, thus releasing polyubiquitin or ubiquitin monomers that can be re-used to regulate the degradation of proteins via the proteasome and the lysosome.

Mitochondrial dynamics

Mitophagy is an important cellular process of autophagy by which mitochondria are selectively degraded. Two important proteins for this procedure are Parkin, an E3 ubiquitin ligase, and PINK1, a serine/threonine kinase (Eiyama & Okamoto, 2015), which when mutated cause familial PD forms. Pioneer experiments in flies showed that KO of both proteins causes motor deficits and disintegration of mitochondrial cristae. Parkin rescues PINK1 KO but not the inverse, therefore it was concluded that Parkin is downstream of PINK1, in the same cellular pathway (Eiyama & Okamoto, 2015). In healthy mitochondria, PINK1 is imported by translocase of the outer membrane (TOM) and translocase of the inner membrane (TIM). PINK1 N-terminal is cleaved by proteases in the mitochondria matrix and after that, it is transported back to the cytosol where it is degraded by the proteasome (Lazarou et al., 2012; Yamano & Youle, 2013). However, depolarized mitochondria have the import machinery impaired, so the import of PINK1 fails. The protein stays in the membrane and autophosphorylates (Okatsu et al., 2012) and dimerizes (Okatsu et al., 2013), keeping the kinase domain in the cytosol, leading to ubiquitin phosphorylation (Zhou et al., 2008). Phosphorylated ubiquitin binds to Parkin,

altering the conformation of this protein (Wauer et al., 2016), and facilitating its phosphorylation and activation by PINK1 (Sauvé et al., 2015; Wauer et al., 2016). Active Parkin causes the ubiquitination of mitochondrial outer membrane (MOM) proteins (Shiba-Fukushima et al., 2012). After the ubiquitination of MOM proteins, ubiquitin-binding adaptors (e.g. p62) bind to ubiquitin, enabling autophagy by forming an autophagosome through linking with LC3-II (Padman et al., 2019; Park et al., 2014). Mitochondria proteins marked with ubiquitin can also be recognized by the endosomal sorting complexes required for transport (ESCRT) in the membrane of endosomes (Hammerling et al., 2017). After engulfing the mitochondria, the endosomes or autophagosomes can fuse with lysosomes that enables mitochondrial degradation.

In PD, when calcium is highly concentrated all over the cell, mitochondrial trafficking stops, and depolarized mitochondria stop producing adenosine trisphosphate (ATP) (Miller & Sheetz, 2004). The disruption of trafficking also inhibits the mechanism of fusion (Figure 3). Indeed, mitochondria are very dynamic organelles that are transported along the neurons and form a network throughout the cell that continuously undergo fusion and fission (Hollenbeck & Saxton, 2005; Okamoto & Shaw, 2005; Shaw & Nunnari, 2002). These processes regulate the number of mitochondria in the cell, the transport of the organelle to cellular locations where energy is required, modulate mitochondrial metabolism and maintain mtDNA integrity (Westermann, 2002). Dynamin-like GTPases mitofusin-1 (Mfn1) and mitofusin-2 (Mfn2), both located at MAM, and Mitochondrial Dynamin-like 120 kDa protein (OPA1), a protein from the inner membrane, participate in mitochondrial fusion (Cervenyet al., 2007; Hoppins et al., 2007; Song et al, 2007). Damaged mitochondria undergo fission from the network. Ubiquitination of MFNs certifies that impaired mitochondria do not fuse again, assuring that they are degraded (Gegg & Schapira, 2011). Fission is promoted by the action of dynamin-related protein 1 (DRP1) that translocates from the cytosol to the MOM, where it interacts with Mitochondrial Fission 1 Protein (Fis1) (Hoppins et al., 2007). The main proteins involved in mitochondrial transport are Miro1 and Milton (Stowers, Megeath, Górska-Andrzejak, Meinertzhagen, & Schwarz, 2002). Miro1 is an adaptor protein to dynein and kinesin, motor proteins involved in retrograde and anterograde neurite movement, respectively, along the microtubules. Miro1 is also calcium-sensitive, so when calcium is in excess, Miro1 dissociates from motor proteins (Cai & Sheng, 2009). This mechanism is essential to anchor mitochondria where ATP is needed. When mitochondria

are damaged, Miro1 has to be removed from mitochondria so that the process can continue, but when a-syn is overexpressed Miro1 is not removed and is accumulated in mitochondria, inhibiting mitophagy (Hsieh et al., 2016).

Considering that mitochondria transport is highly important in neurons, damage of this transport along the microtubules may result in cell death. Mitochondrial trafficking is disrupted in oxidative stress, and the latter process is highly predominant in sporadic forms of PD (Trushina & McMurray, 2007). A-syn is related to microtubule stabilization (Cartelli et al., 2016). In PD, a-syn oligomers are colocalized with free tubulin, suggesting that in PD conditions a-syn inhibits the formation of new microtubules by binding free tubulin. Consequently, mitochondrial trafficking through microtubules is impaired (Prots et al., 2013).

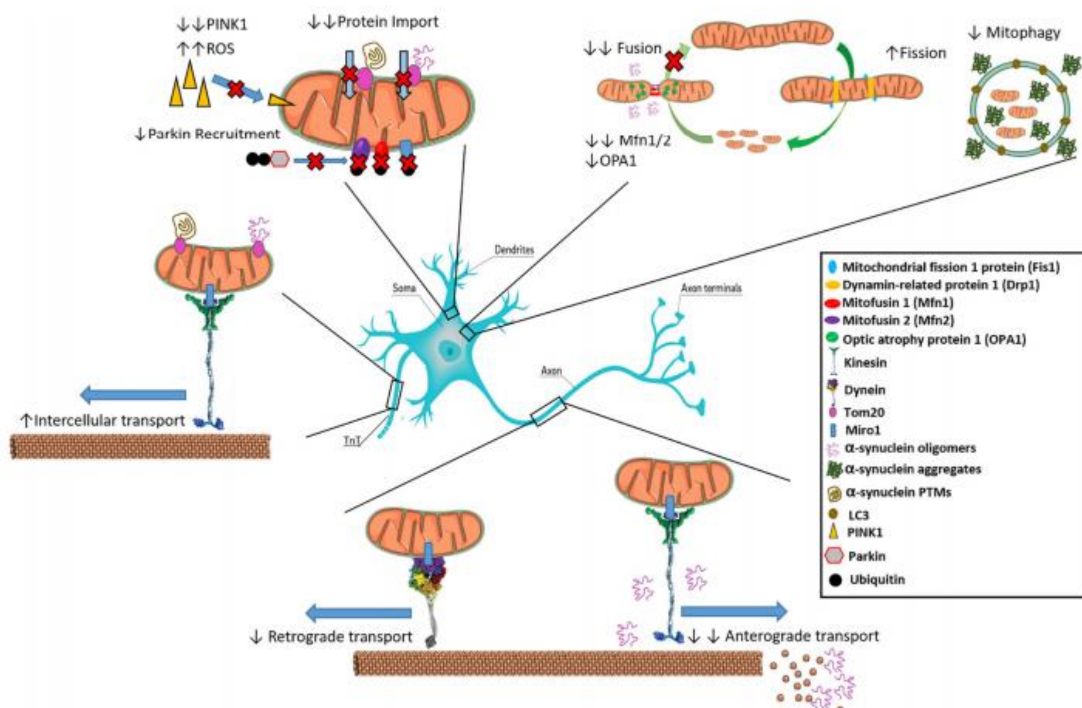

Figure 3: Mitochondrial Dynamics affected by a-syn. A-syn overexpression promotes mitochondria fission, resulting in fragmentated smaller mitochondria. Fusion is impaired, with low levels of two proteins needed in that process (Mitofusins and OPA1). Transport along the cell is also impaired, as it is mitophagy *(Valdinocci et al., 2019)*

Objective

The objective of this study was to define the impact of wild-type (WT) a-syn overexpression, a protein accumulated in both sporadic forms of PD and in familial forms of *SNCA* gene triplication, on cell toxicity and in the levels of autophagy proteins and a-syn degradation, and further define the intricate relationship between these processes and mitochondrial function, morphology and mitophagy regulation.

For this purpose, we used human SH-SY5Y cell line conditionally expressing WT-a-syn by a Tet-OFF system (Vekrellis et al., 2009). Two cellular phenotypes have been previously identified, the neuroblast-like cells and the epithelial-like cells (Ross, Spengler, & Biedler, 1983); moreover, this line can be differentiated into neuron-like phenotype with retinoic acid (Encinas et al., 2002). Neuroblast-like cells demonstrated activity of tyrosine hydroxylase and dopamine-β-hydroxylase, which converts dopamine into noradrenaline (Ross et al., 1983). Since these cells can synthetize both neurotransmitters, they are described as catecholaminergic (Ross et al., 1983). Non polarized cell bodies and few truncated processes are typical in undifferentiated cells (Kovalevich & Langford, 2013), resembling immature catecholaminergic neurons (Lopes et al., 2010; H. R. Xie, Hu, & Li, 2010).

2. Material and methods

Materials

Secondary antibodies for Western blotting were obtained from Thermo Scientific Pierce (Waltham,MA ,USA). Roswell Park Memorial Institute's 1640 medium (RPMI 1640), Fetal bovine serum (FBS) and Geneticin were acquired from GIBCO (Paisley, UK). Protease inhibitor cocktail (chymostatin, pepstatin A, leupeptin and antipain), Horseradish Peroxidase, Bovine serum albumin (BSA), hygromycin B, doxycycline (DOX), L-Glutamine, Triton X-100, Tween-20, Bafilomycin A1 and FCCP were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). ECF was acquired from GE Healthcare (Uppsala, Sweden). Amplex Red and Hoechst 33342 were obtained from Invitrogen/Molecular Probes (Life Technologies Corporation, Carlsbad, CA, USA). BioRad Protein Assay were acquired from BioRad Laboratories, Inc. (Munich, Germany). Sodium bicarbonate was obtained from Fisher Scientific (Hampton, NH, EUA). All other reagents were of analytical grade.

Culture of stable SH-SY5Y cell lines conditionally expressing WT a-syn

Stable SH-SY5Y cell lines conditionally expressing WT a-syn (Vekrellis et al., 2009) (SH-SY5Y WT a-syn cells, kindly donated by Prof. Leonidas Stefanis (Division of Basic Neurosciences, Biomedical, Research Foundation of the Academy of Athens, Soranou Efesiou, Athens, Greece)) were cultured in RPMI 1640 medium, supplemented with 10% FBS, 50 μg/mL hygromycin, 250 μg/mL geneticin (G418), 2 μg/mL DOX, 1 g/L sodium bicarbonate and 2 mM L-glutamine. SH-SY5Y cells were maintained in RPMI 1640 with DOX. Cells were plated at a density of 3.125×10^4 cells/cm^2 for western blotting, at 5.714×10^4 cells/cm^2 for immunocytochemistry, or at 9.375×10^4 cells/cm^2 for Amplex red and mitochondrial membrane potential assays. When plated, cells were maintained in medium in the presence (+ DOX) or in the absence (- DOX) of DOX (2 μg/mL) for one to four days, at a temperature of 37ºC, in an atmosphere of 95% air and 5% CO_2. Briefly, this Tet-Off system supports the expression of the gene of interest, a-

syn, in the absence of DOX. When present, bafilomycin (50 nM) was added 24h before experiments.

Measurement of hydrogen peroxide (H_2O_2) levels

After a wash with phosphate-buffered saline (PBS), 100 µL/well of Krebs medium (135 mM NaCl, 5 mM KCl, 0.4 mM KH_2PO_4, 5.5 mM Glucose, 1.8 mM $CaCl_2$, 1 mM, 20 mM HEPES, pH 7,4), 0.5 U/mL horseradish peroxidase and 10 mM Amplex red were added. Amplex red is a probe to H_2O_2, highly sensitive and a colorless reagent that in the presence of extracellular H_2O_2 uses the peroxidase to react with the hydrogen peroxide, producing resorufin, a fluorescent red substrate. The fluorescence was measured in a fluorimeter SpectraMax Gemini EM (Molecular Devices, USA) at 540 nm excitation and 590 nm emission wavelengths for 12 minutes, at 37ºC.

Mitochondrial membrane potential assay

Cells were incubated with 300 nM tetramethylrhodamine methyl ester ($TMRM^+$) in Krebs medium for 30 minutes, at 37ºC. After this period the fluorescence was measured in a fluorimeter SpectraMax Gemini EM (Molecular Devices, USA) at 540 nm excitation and 580 nm emission for 5 minutes, at 37ºC. After this read 2 µM FCCP was added per well for another 5 minutes at the same temperature.

TMRM+ is a fluorescent compound that is sequestered by healthy mitochondria, with high negative potential. Contrariwise, mitochondria with low membrane potential will sequester less $TMRM^+$. After adding the uncoupling agent FCCP, $TMRM^+$ that have been retained inside mitochondria will leave the organelle, allowing to evaluate how healthy mitochondria are, relatively to the fluorescence intensity. Thus, the healthier the mitochondria are, the higher is the mitochondrial transmembrane potential and more $TMRM^+$ enters the organelle due to its charge.

Immunocytochemistry (ICC)

Cells are fixed with 4% paraformaldehyde for 15 minutes, washed three times in PBS, permeabilized with 0.2% Triton X100 in PBS for 2 minutes, washed again in PBS and blocked with 3% BSA in PBS, for 30 minutes. Then each lamella was incubated

overnight with the primary antibody (in PBS containing 3% BSA). The next day, lamellas were washed with PBS and incubated with the secondary antibody (in PBS containing 3% BSA) during an hour. After this time, lamellas were washed again and incubated for 5 minutes in the dark with 2μg/mL Hoechst (in PBS) to label the cell's nucleus. Lamellas were then mounted in laminas, previous clean with ethanol, after putting a drop of mounting medium.

The primary antibodies used were mouse anti-a-syn antibody (BD Bioscience #610786), rabbit anti-p62 antibody (Sigma P0067) and rabbit anti-ubiquitin antibody (DakoCytomation Z0458) all at a final dilution of 1:200, and rabbit anti-parkin antibody (Abcam ab15954) at 1:350. The secondary antibodies used were Alexa Fluor^{TM} 488 donkey anti-mouse IgG, Alexa Fluor^{TM} 568 donkey anti-mouse IgG and Alexa Fluor^{TM} 647 goat anti-rabbit IgG, all at a dilution of 1:300. The mitochondria were labelled with Mito-DsRed (1 μg/mL) transfected with lipofectamine 3000 in the day before cell fixation.

The samples were observed in a confocal microscope LSM 710 (Zeiss Microscopy, Germany) with a Pln Apo 63x/1.4 Oil DICII objective, and images obtained with ZEN software.

Western blotting

To obtain extracts for western blotting analysis, cells plated are washed three times in PBS. Then radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris HCl, 5 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, pH 7.5) containing 100 nM okadaic acid, 25 nM sodium fluoride (NaF), 1 mM sodium orthovanadate (Na_3VO_4), 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 μg/ml protease inhibitor cocktail (chymostatin, pepstatin A, leupeptin and antipain) was added to the cells and let RIPA buffer act for 15 minutes, to then, scrape the cells to an Eppendorf for protein quantification by the BioRad protein assay in a microplate reader Spectra Max Plus 384 (Molecular Devices, USA). Values obtained in this quantification were used to prepare the samples using a sample buffer 6x concentrated (300 mM Tris-HCl pH 6.8, 12% SDS, 30% glycerol, 600 mM DTT, 0.06% bromophenol blue) and sample buffer 1x. The samples were then denaturated at 95°C, for 5 min.

Protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, at 15%) and passed to a polyvinylidene difluoride (PVDF) membrane activated in 100% methanol. Membranes were then washed three times in tris-buffered saline with 0.1% tween 20 (TBS-T) (25 mM Tris-HCl and 150 mM NaCl, at pH 7.6). The membranes were then left overnight incubating in 5% BSA in PBS with the primary antibody. The next day, membranes were washed three times with TBS-T and incubated with TBS-T, 5% BSA in PBS and the secondary antibody, for an hour. After this hour, membranes were washed again three times in TBS-T. The membranes were then incubated with ECF for bands visualization using the BioRad ChemiDoc Touch Imaging System (BioRad, Munich, Germany).

The primary antibodies used were rabbit anti-p62 antibody (Sigma P0067), rabbit anti-ubiquitin antibody (DakoCytomation Z0458), rabbit anti-LC3 antibody (Cell signaling #12741), mouse anti-actin antibody (Abcam ab205), and rabbit anti-Beclin-1 antibody (Cell signaling #3738S), all at 1:1000, and mouse anti-a-syn antibody (BD Bioscience #610786) at a dilution of 1:500. The secondary antibodies used were Goat Anti-Rabbit (H+L), Alkaline Phosphatase Conjugated Thermo Scientific Pierce #31340 and Goat Anti-Mouse (H+L) , Alkaline Phosphatase Conjugated Thermo Scientific Pierce #31320, both at a dilution of 1:10000.

Statistical analysis

Results were obtained through the mean ± standard error of the mean (SEM) of the data acquired with the number of experiments specified in each figure legend. Comparative analysis was obtained with the non-parametric test Kruskal-Wallis, Dunn´s multiple comparisons test and Mann-Whitney test with significance at $p<0.05$. Results were analysed with the software GraphPad Prism 8.0.2 (GraphPad Software, San Diego, CA, USA). Western blotting images were analysed with BioRad Image Lab 6.0.1 software (BioRad, Munich, Germany), and ICC images were analyzed by Fiji ImageJ 2.1.0. with macros used to measure integrated density of proteins, colocalization between two proteins, colocalization between a protein and mitochondria, and mitochondria parameters [perimeter, roundness, aspect ratio (AR, defined by the ratio between major and minor axes of an ellipse equivalent to the mitochondrion), and area occupied by mitochondria in the cell].

3. Results

Wild-type alpha-synuclein overexpression in Tet-Off conditional SH-SY5Y cells is linked to increased ROS levels and differentially affected by autophagy inhibition

To investigate WT a-syn overexpression, Tet-Off conditional human neuroblastoma SH-SY5Y cells were exposed to culture medium without DOX for 1 and up to 4 days. Analysis by western blotting to a-syn revealed that three and four days without DOX caused a significant increase in a-syn levels (normalized to beta-actin) by 1.5-fold and 1.4-fold, respectively, when compared with cells incubated with DOX (control, 0 days without DOX) (Figure 4A). Analysis by ICC also revealed a significant increase in a-syn levels by 1.85-fold in cells incubated for four days without DOX, confirming a-syn overexpression under these conditions (Figure 4C,E).

High levels of ROS are generally a characteristic of a-syn overexpression (Junn & Mouradian, 2002). To characterize altered redox profile in these cells the levels of extracellular hydrogen peroxide (H_2O_2) were measured after 1-4 days without DOX, corresponding to changes in a-syn levels. The fluorescence of resorufin was proportional to H_2O_2 levels, which were significantly higher on the first day without DOX (with an increase by 1.3-fold), when compared with cells incubated with DOX (control). Also, after three and four days without DOX significant differences in H_2O_2 levels were found (Figure 4B). Interestingly, higher ROS levels (after three and four days without DOX) were coincident with enhanced a-syn levels, as defined by WB and ICC (Figure 4A-C).

To evaluate the role of autophagy in SH-SY5Y cells expressing a-syn basal levels (maintained with DOX) and following a-syn overexpression, cells were exposed to bafilomycin (Baf, 50 nM), 24h before cell fixation. Bafilomycin is an inhibitor of autophagic lysosomal pathway. This drug inhibits the function of the vacuolar-type H^+-ATPase (V-ATPase) (Bowman, Siebers, & Altendorf, 1988). V-ATPase is responsible for the acidification of lysosomes and endosomes (Yoshimori et al., 1991). Bafilomycin was also reported to prevent the fusion between autophagosomes and lysosomes (Boya et al., 2005). Thus, bafilomycin is expected to increase the levels of proteins in cells if they are being degraded through autophagy/macroautophagy. Data indicate increased levels of

a-syn following Baf exposure under basal conditions and after two days without DOX, but not after four days without DOX (Figure 4D,E).

Data indicate that a-syn is overexpressed in Tet-Off conditional SH-SY5Y cells and that this overexpression is toxic to cells, as evaluated by formation of ROS. The results with bafilomycin suggest dysfunctional autophagy after four days of a-syn expression in SH-SY5Y cells.

Modified levels of autophagic proteins in Tet-Off conditional SH-SY5Y cells

Based on data obtained in the presence of Baf that suggested the involvement of modified autophagy in Tet-Off conditional SH-SY5Y cells, the levels of autophagy protein markers, p62, LC3, Beclin-1 and ubiquitin, were analysed by western blotting.

Beclin-1 is a subunit of PI3KC3 that promotes the maturation of autophagosomes when binding to positive cofactors like ATG14, PINK1 and parkin (Liang et al., 2010; Y. Xie, Kang, & Tang, 2016).(Liang et al., 2010)(Liang et al., 2010)(Liang et al., 2010)(Liang et al., 2010) Despite its relevance in the initial steps of macroautophagy, no differences were found by western blotting in cells incubated in the presence or absence of DOX (Figure 5A).

P62 is an autophagic receptor that interacts with ubiquitin and LC3. When activated/lipidated LC3 (due to its interaction with membrane phosphatidyletanolamine (PE)), LC3II is involved in autophagosome formation. Analysis of p62 revealed higher levels of this protein after three and four days cells without DOX (Figure 5B), while activated LC3 (LC3II/LC3I ratio) levels were significantly higher following four days without DOX (Figure 5C). Ubiquitin is a protein involved in various processes in cells. This protein can bind to other proteins forming polyubiquitinated chains, marking proteins for the UPS or autophagy. Ubiquitin western blotting results demonstrated higher levels of ubiquitinated proteins at four days cells without DOX (Figure 5D). Data show that three of the four autophagy related proteins analysed (being Ub also involved in UPS) have higher levels at four days in cells treated without DOX, suggesting that these proteins are being accumulated possibly due to autophagy impairment.

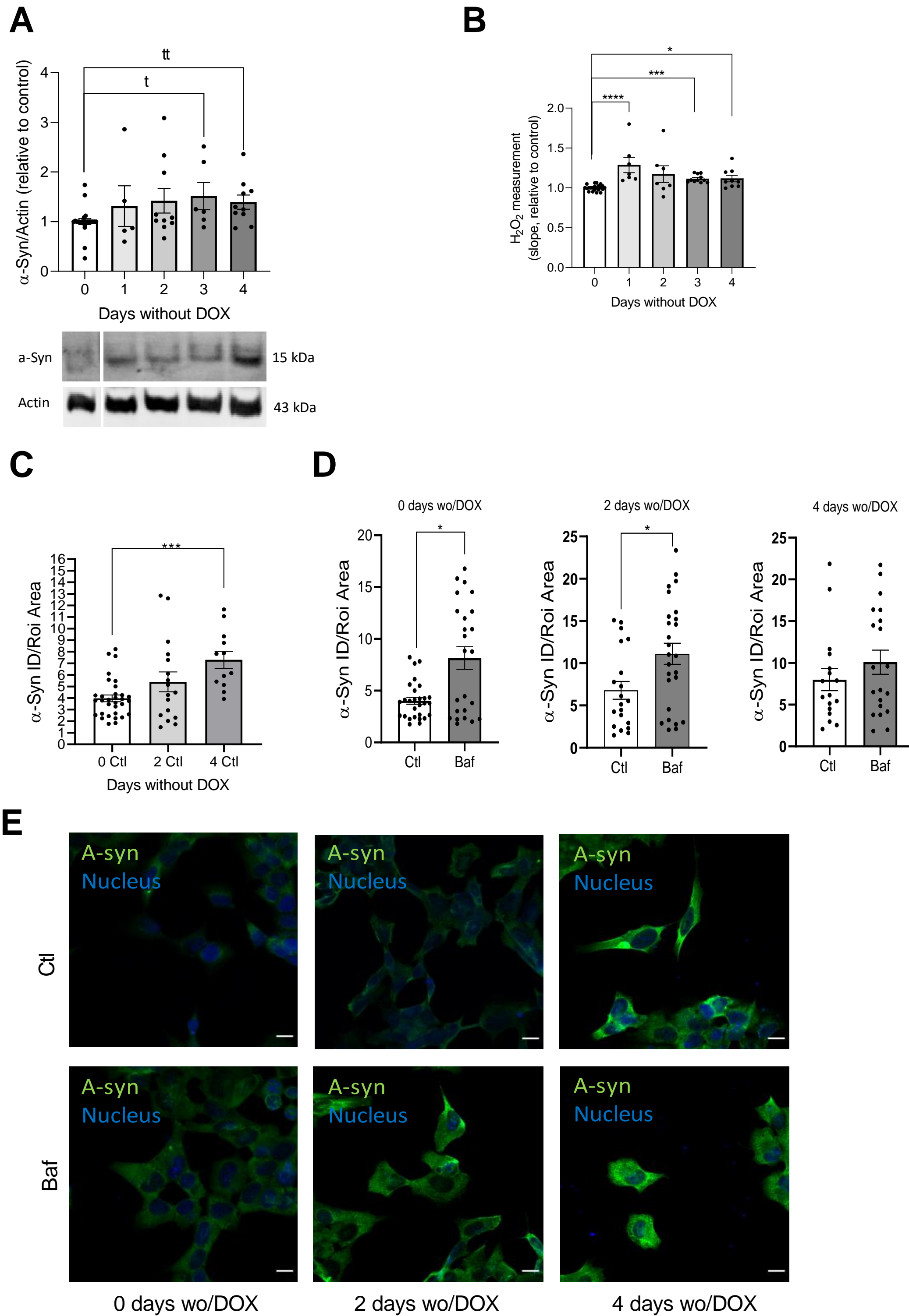

Figure 4: A-syn and ROS levels in Tet-Off conditional SY-SY5Y cells. A-syn levels were determined by Western Blotting in cells incubated in the presence of 2 µg/mL DOX (control; 0 days -DOX) or absence of DOX for one, two, three and four days (**A**). Hydrogen peroxide levels were measured, for 12 minutes by the Amplex-red method in cells in medium with DOX and after one, two, three and four days without DOX (**B**). A-syn levels were also determined by immunocytochemistry (ICC) with DOX and two and four days without DOX (**C, E**). ICC data comparing a-syn expression levels in control cells (with DOX) or in the absence of DOX for 2-4 days and cells incubated with bafilomycin are shown in (**D, E**). ICC representative images obtained at the confocal microscope after nuclei labelling with Hoechst (in blue) and anti-a-syn (in green); scale bar = 10 µm (**E**). Data are the mean ± SEM of 4 to 13 independent experiments, performed in triplicates with 5-9 cells analyzed per lamella. Statistical analysis: t $p<0.05$, tt $p<0.005$ by the Mann-Whitney test, *$p<0.05$, ***$p<0.0005$, ****$p<0.0001$, by the Kruskal-Wallis test.

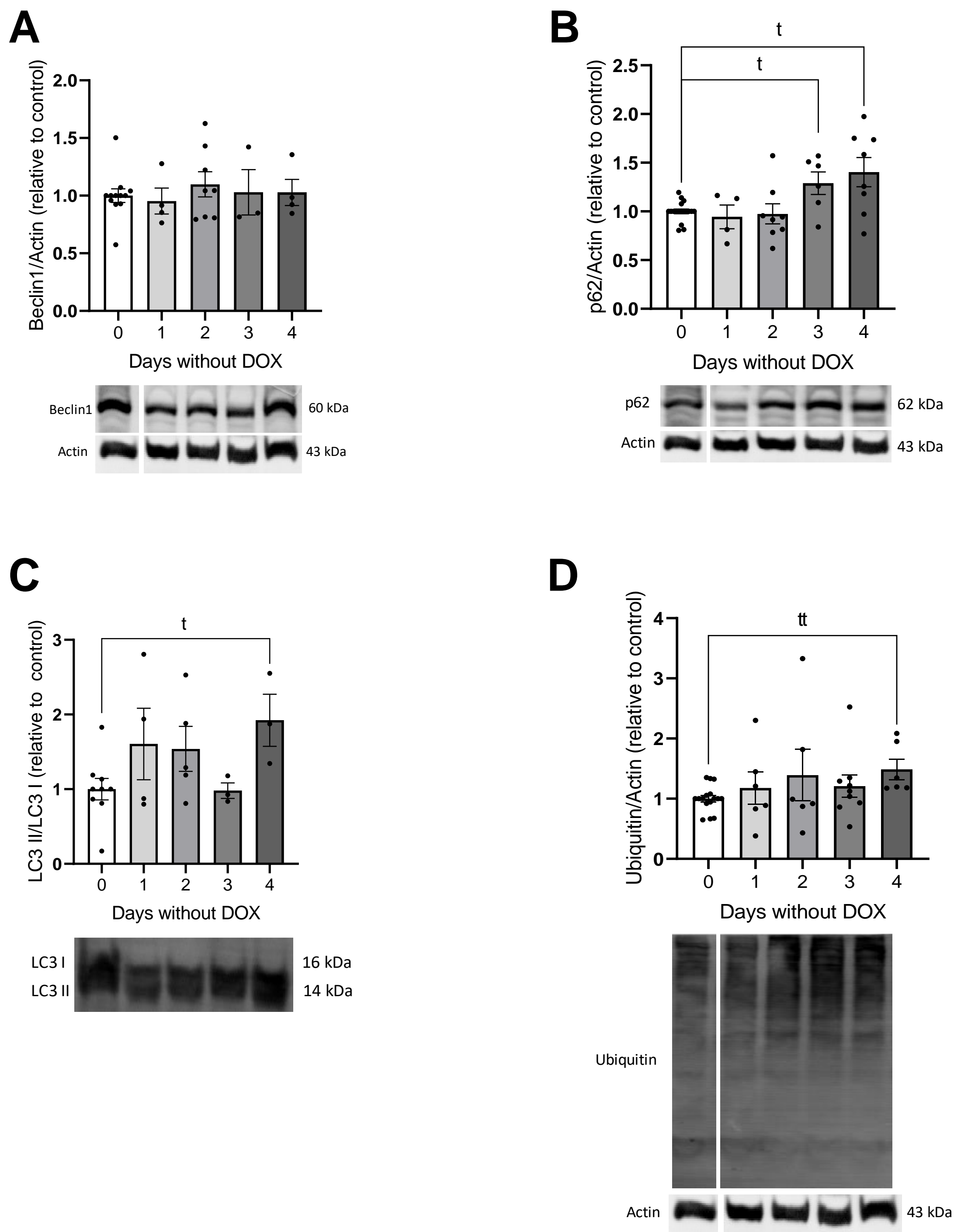

Figure 5: Analysis of autophagic proteins Beclin-1, p62, LC3 and ubiquitin in Tet-Off conditional SH-SY5Y cells. The cells were incubated in the presence of DOX (control; 0 days -DOX) and after one, two, three and four days without DOX. Data represented in graphs are the mean ± SEM of 3-8 independent experiments and representative western blots were labelled against: Beclin-1 (**A**), p62 (**B**), LC3 II/LC3 I ratio (**C**), and ubiquitin (**D**). Statistical analysis: t $p<0.05$, tt $p<0.005$ by nonparametric Mann-Whitney test.

Bafilomycin modulates p62 and ubiquitin protein levels in SH-SY5Y cells overexpressing a-synuclein

Coincident with the results obtained in the western blot for p62 after four days without DOX, this protein was shown to accumulate at the same time relatively to zero (+ DOX) and two days without DOX when assessed by ICC (Figure 6A). In the presence of bafilomycin, p62 levels augmented when compared to control conditions in cells exposed to DOX. However, after two and four days without DOX no significant differences were found in cells treated vs non-treated with bafilomycin (Figure 6B).

Ubiquitin levels were significantly higher in cells subjected to two and four days without DOX (Figure 6D). However, bafilomycin did not affect ubiquitin total levels in none of the days without DOX tested (Figure 6E).

The data suggest that autophagy processes could have been initiated in cells overexpressing a-syn, since p62 and ubiquitin are accumulated under those conditions..

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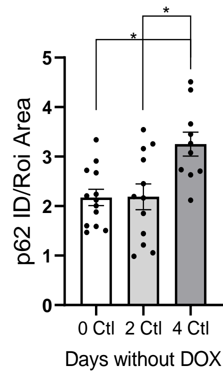

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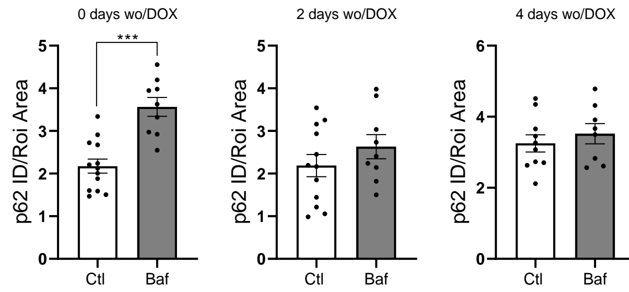

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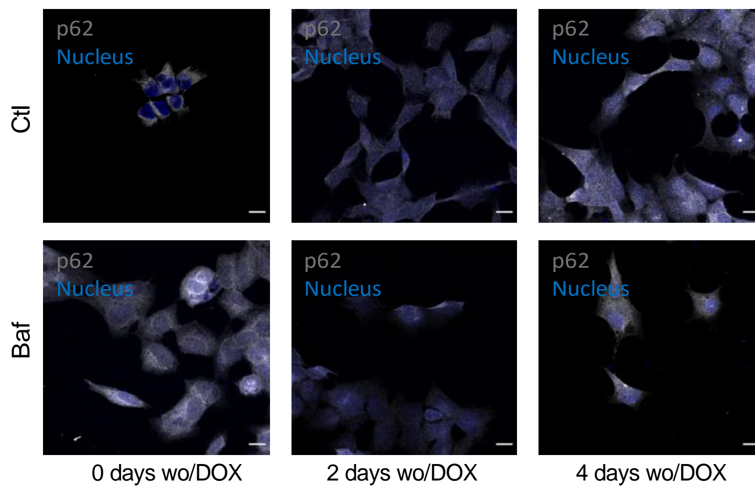

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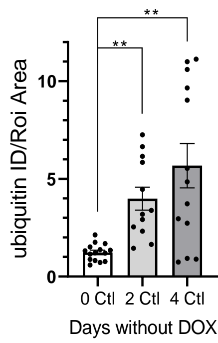

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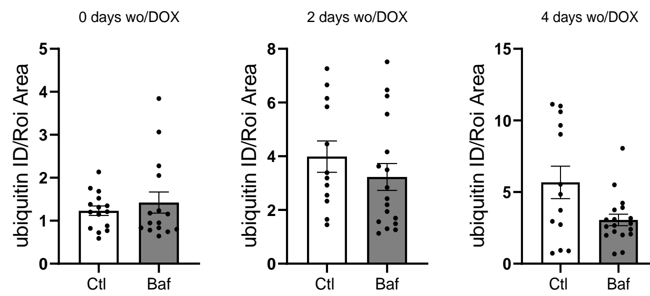

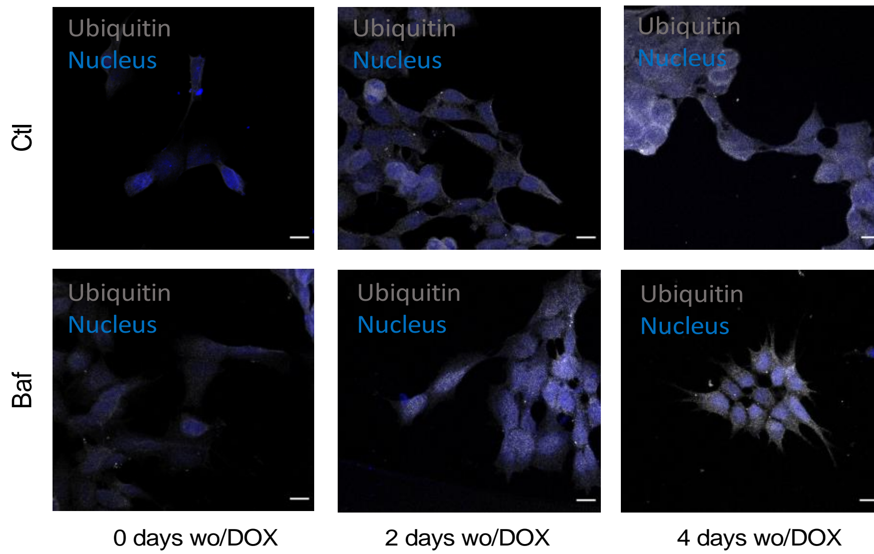

Figure 6: Influence of bafilomycin on p62 and ubiquitin levels in Tet-Off conditional SY-SY5Y cells. p62 (**A**,**C**) and ubiquitin (**D**,**F**) cellular levels were analyzed by immunocytochemistry (ICC) in cells exposed to DOX (0 days without DOX) or after two and four days without DOX. p62 (**B**) and ubiquitin (**E**) relative levels were also determined before and after 50 nM bafilomycin treatment. Representative images from ICC obtained from a confocal microscope, with nuclei labelled with Hoechst (in blue) and p62 (**C**) or ubiquitin (**F**) (in grey); scale bar= 10 µm. Bars in graphs are the mean ± SEM of 2 independent experiments in duplicates with 5-9 cells analyzed per lamella. Statistical analysis: *p<0.05, **p<0.005, ***p<0.0005 by the Kruskal-Wallis test.

Alpha-synuclein colocalization with autophagic proteins in Tet-Off conditional SH-SY5Y cells

Colocalization between a-syn and p62 and between a-syn and ubiquitin in Tet-Off SH-SY5Y cells, as determined by analysis of Pearson's coefficient, was analysed after ICC. Colocalization between p62 and a-syn was lower at two and four days without DOX, when compared to cells in medium with DOX (Figure 7A,B). No significant differences were observed when assessing the colocalization between a-syn and ubiquitin (Figure 7C,D). These data suggest that, in contrast with ubiquitin, a-syn interaction with p62 may constitute a limiting factor for a-syn degradation.

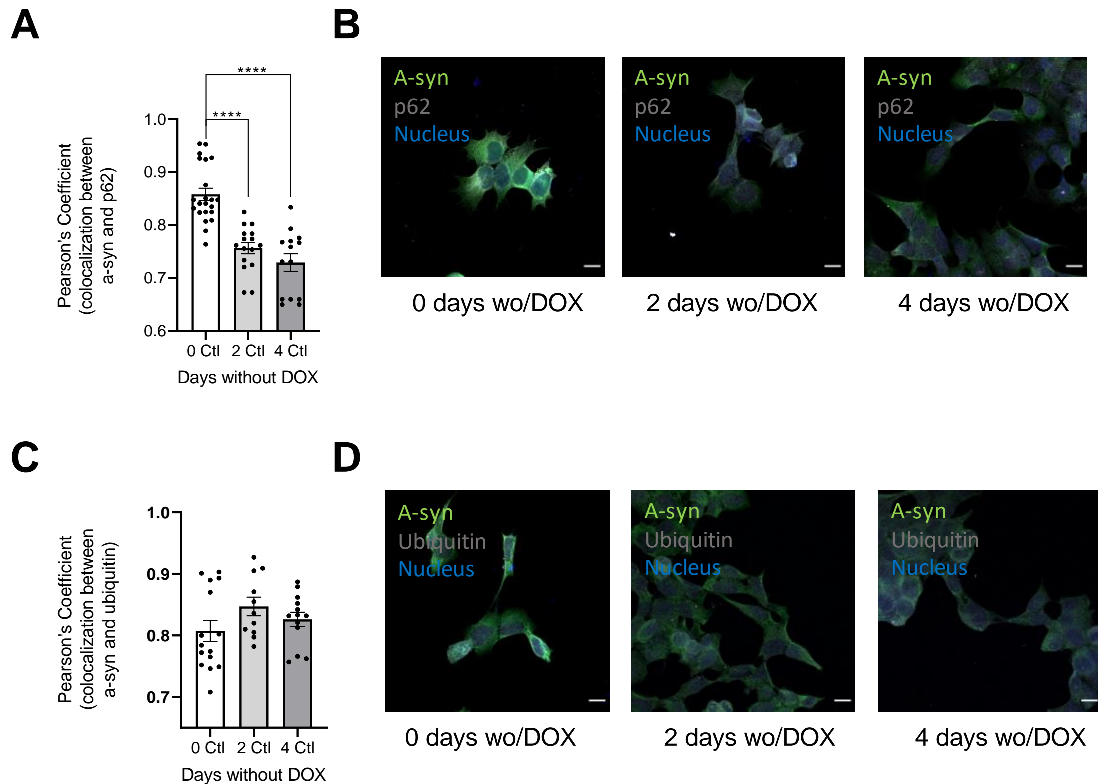

Figure 7: Colocalization analysis between p62 or ubiquitin and a-syn a-syn in Tet-Off conditional SH-SY5Y cells. Colocalization between a-syn and p62 (**A,B**) or between a-syn and ubiquitin (**C,D**) was defined by determining the Pearson's coefficient after ICC in cells in medium with DOX (control), and incubated without DOX for two and four days. **B** and **D** panels show representative images obtained by confocal microscopy with nucleus labelled in blue, a-syn in green and p62 or ubiquitin in grey; scale bar = 10 µm Bars in graphs represent the mean ± SEM of 2 independent experiments, run in duplicates, with 5-9 cells analyzed per lamella. Statistical analysis: ****p<0.0001, with Kruskal-Wallis test.

Mitochondria dysfunction and colocalization with alpha-synuclein in Tet-Off conditional SHSY-5Y cells

Mitochondria are organelles especially susceptible to damage by ROS production (Musatov & Robinson, 2012). In PD, mitochondria dysfunction has been largely documented (e.g. Wang et al., 2012), leading to impairment in mitochondrial trafficking, or ATP production, which further exacerbates ROS production by the organelle. In particular, WT a-syn overexpression was previously shown to reduce complex I activity and enhance mitochondrial ROS levels (Perfeito et al., 2017). Therefore, in this study we assessed whether mitochondria membrane potential could be affected by a-syn overexpression in Tet-Off conditional SH-SY5Y cells.

Mitochondria overexpressing a-syn for two days (-DOX) showed significant low ΔΨm (lower TMRM+ fluorescence after addition of FCCP) when compared to +DOX (Figure 8A). Although $TMRM^+$ fluorescence values were also apparently reduced after three and four days without DOX, the differences were not significant.

Because a-syn oligomers have been shown to interact with mitochondria (Di Maio et al., 2016; Parihar et al., 2008), colocalization between mitochondria and a-syn is also an important measure in the context of PD. Induction of a-syn overexpression after two and four days without DOX caused an increased in the levels of a-syn colocalizing with mitochondria, when compared to conditions in which cells expressed normal levels of a-syn (+DOX) (Figure 8B).

Mitochondrial morphology is also affected in PD (Trimmer et al., 2000; Walter et al., 2019). Therefore, four parameters of mitochondrial morphology were defined, namely perimeter, roundness, AR (defined by the ratio between major and minor axes of an ellipse equivalent to the mitochondrion), and the area occupied by mitochondria in the cell. No changes in AR were found between control (+DOX) and a-syn overexpression (-DOX). However, mitochondrial perimeter, roundness and area occupied by mitochondria in the cell showed significant differences. Both mitochondrial perimeter and roundness were reduced after two days without DOX, comparing with cells in medium with DOX. Concordantly with reduced roundness and perimeter, after two days overexpressing a-syn (-DOX) mitochondria area in cells was higher (Figure 8C), suggesting increased mitochondrial fusion.

Results suggest decreased ΔΨm and enhanced mitochondrial fusion when a-syn is overexpressed, which can be facilitated by its direct interaction with the organelle.

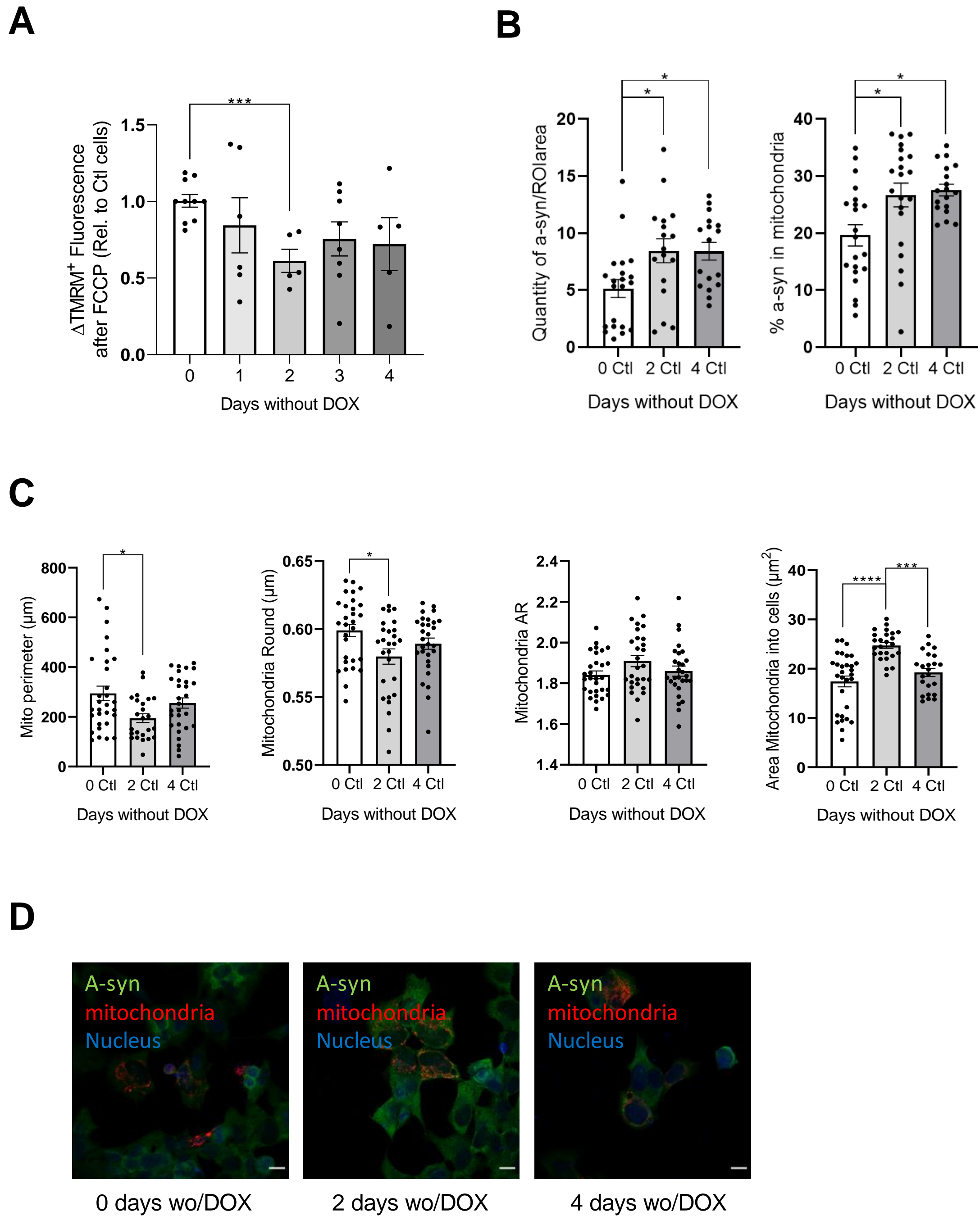

Figure 8: A-syn overexpression influences mitochondrial membrane potential, colocalization between a-syn and mitochondria and mitochondrial morphology in Tet-Off conditional SH-SY5Y cells. Mitochondrial transmembrane potential was measured by TMRM+ assay. TMRM+ (300 nM) was incubated for 30 minutes and basal fluorescence was assessed for five minutes. Then, FCCP (2 µM) was added and fluorescence was measured again for five minutes (**A**). Quantity and percentage of a-syn colocalized with mitochondria in cells that express basal levels of a-syn (control, +DOX) and cells that overexpress a-syn for two and four days (maintained in the absence of DOX) (**B**). Four mitochondrial parameters (perimeter, roundness, aspect ratio (AR) and mitochondrial area in cells) measured in cells in medium with DOX and cells in medium without DOX for two and four days (**C**). Representative images obtained from a confocal microscope with nucleus labelled with Hoechst (in blue), a-syn in green and mitochondria labelled (in red) after transfection with mito-DsRed; scale bar = 10 µm (**D**). Bars in graphics represent the mean ± SEM of 5 independent experiments, with 5 to 9 cells analyzed per lamella. Statistical analysis: *p<0.05, ***p<0.0005, ****p<0.0001 by Kruskal-Wallis test.

Modified markers of mitophagy following WT alpha-synuclein overexpression

To evaluate modified mitophagy due to overexpression of WT a-syn, in this part of the study we determined colocalization between mitochondria and markers of autophagy, namely parkin, p62 and ubiquitin.

Parkin is a E3 ubiquitin-protein ligase involved in mitophagy. This protein ubiquitinates several mitochondrial proteins, marking impaired mitochondria for mitophagy. Therefore, we determined, by Pearson's coefficient, the colocalization between parkin and mitochondria following a-syn overexpression. Despite the slight increase in colocalization, no significant differences were found (Figure 9A,B).

Colocalization between p62 and mitochondria was also measured. Data showed inconsistent results, with a decrease observed after two days without DOX, and an increase detected following four days without DOX (Figure 9C,D). The colocalization between ubiquitin and mitochondria was shown to be reduced along the days without DOX (Figure 9E). Nevertheless, in cells with bafilomycin the effect was contrary to what was expected, with lower values of colocalization in cells expressing basal levels of a-syn (+DOX), but a significant enhanced colocalization after four days without DOX (Figure 9F).

These data suggest that mitochondria are apparently being marked to mitophagy by parkin, however its activity does not seem to sufficient to label mitochondrial proteins with ubiquitin, as less ubiquitin is detected in mitochondria along the days; consequently, p62 is also less present in mitochondria at two days without DOX. However, after four days without DOX, not only p62 interaction with mitochondria is increased, but ubiquitin levels rise after inhibiting ALP with bafilomycin, suggesting that mitophagy could be activated at this stage. However, more studies will be required to define the changes in mitophagy exerted by WT a-syn overexpression.

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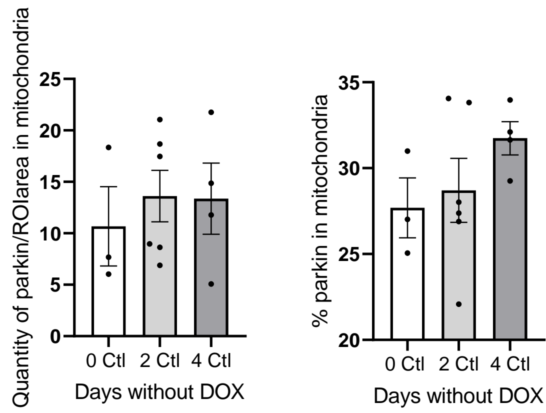

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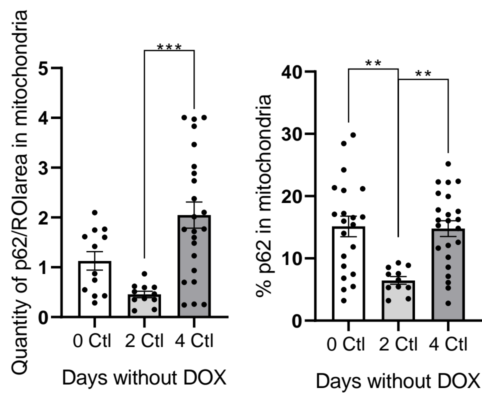

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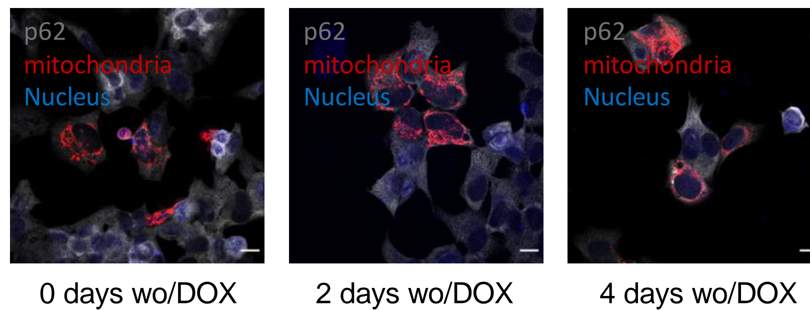

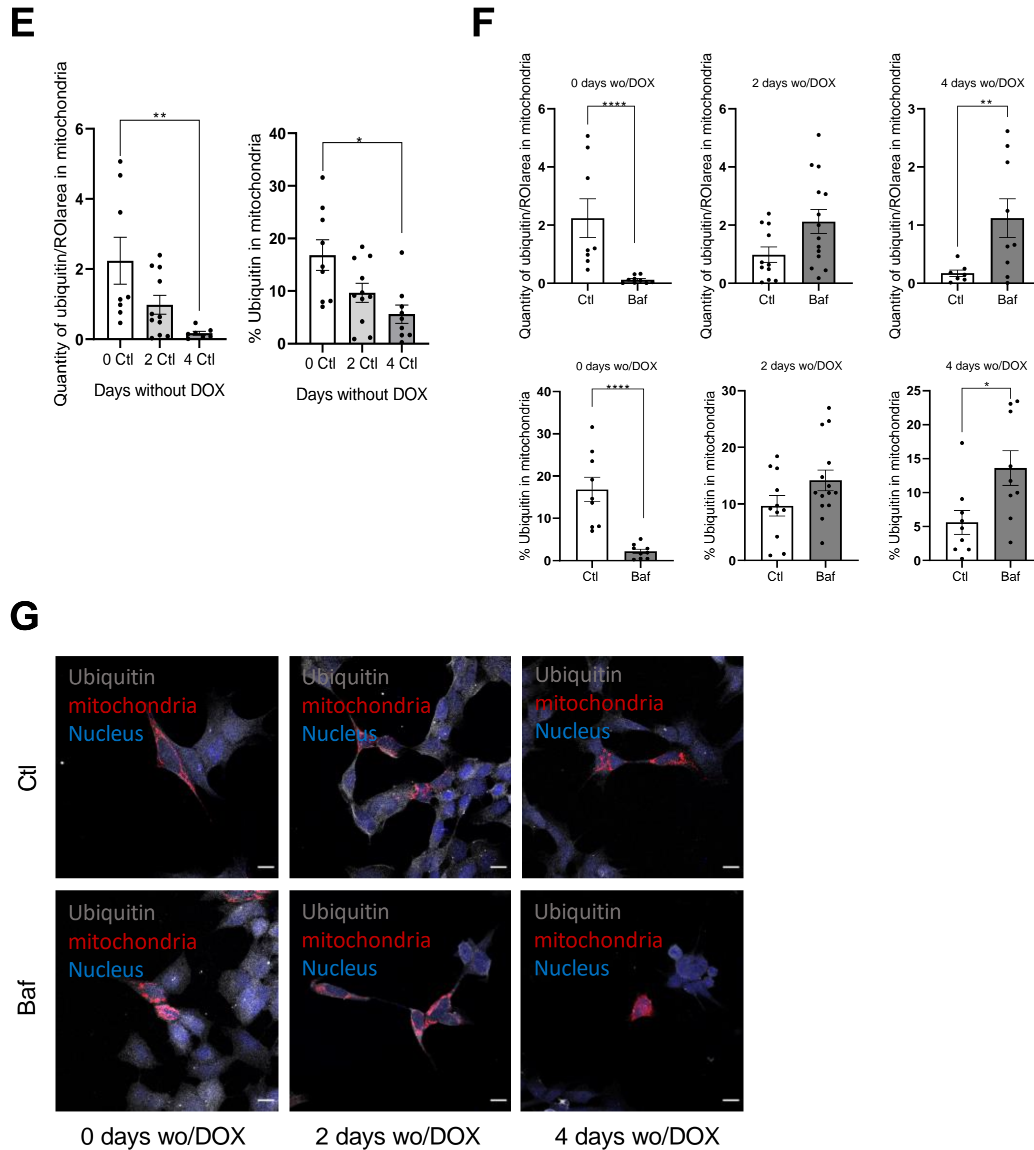

Figure 9: Analysis of colocalization between proteins involved in mitophagy and mitochondria in Tet-Off conditional SH-SY5Y cells. Quantity and percentage of parkin (A), p62 (C) and ubiquitin (E,F) colocalized with mitochondria in cells expressing basal levels of a-syn (+DOX; 0 days without DOX) and cells overexpressing a-syn (-DOX) for two and four days. Representative images obtained by confocal microscopy with nucleus labelled with Hoechst (in blue), parkin, p62 and ubiquitin in grey and mitochondria (in red) after transfection with mito-DsRed; scale bar = 10 µm (B,D,G). Ubiquitin quantity and percentage in mitochondria was also determined in the absence or presence of 50 nM bafilomycin for 24h in cells exposed to +DOX (0 days without DOX) or incubated without DOX for two and four days (F). Data are the mean ± SEM of 2 independent experiments, performed in duplicates, with 3-9 cells analyzed per lamella. Statistical analysis: *p<0.05, *p<0.005 ***p<0.0005, ****p<0.0001 by Kruskal-Wallis test.

4. Discussion

The results show that overexpression of WT a-syn in Tet-Off inducible SH-SY5Y cells is associated with increased ROS and enhanced autophagic protein levels, which may result from inefficiency of the ALP (as evaluated by the effect of bafilomycin). Decreased interaction between a-syn and p62 might also implicate reduced protein degradation. Furthermore, overexpressed WT a-syn was also highly colocalized with mitochondria, which could be accounted for decreased $\Delta\Psi m$, altered organelle morphology and its possible altered degradation through ALP/mitophagy.

The SH-SY5Y cell line exhibits various dopaminergic markers. SH-SY5Y cells express tyrosine hydroxylase, and consequently can produce dopamine, adrenaline and noradrenaline, and also express dopamine transporters and dopamine receptors (Oyarce & Fleming, 1991; Ross et al., 1983). When differentiated, normally with retinoic acid, expression of these catecholaminergic markers tend to increase (Presgraves et al., 2003), while cellular proliferative rate is decreased (Encinas et al., 2002).In the present study, SH-SY5Y cells were modified to overexpress a-syn by a Tet-off system controlled by DOX, such that following DOX absence WT a-syn is overexpressed. In the context of PD familial cases of related with gene multiplication, overexpression of this protein causes the formation of aggregates (Melnikova et al., 2020; Oliveras-Salvá et al., 2013); oligomers formation, in particular, are hazardous to neurons, impairing processes like autophagy (Winslow et al., 2010; Winslow & Rubinsztein, 2011), mitophagy (Shaltouki et al., 2018) and other mitochondrial processes related with organelle dynamics (Gui et al., 2012; Plotegher, Gratton, & Bubacco, 2014). Thus, a-syn overexpression in cell lines are expected to mimic pathological effects of PD patient-derived cells (El-Agnaf et al., 1998).

Of relevance, we observed an increase in relative levels of WT a-syn overexpression after four days in the absence of DOX (about 1.4 fold increase by WB and about 2-fold increase by ICC) when compared to cells maintained in media with DOX (Figure 4A,C,E). Confirmation of a-syn overexpression is concordant with previous studies using these cells (Perfeito et al., 2017; Pantazopoulou et al., 2020). Also, concordantly with previous studies in the group (Perfeito et al., 2017), a-syn showed

higher colocalization with mitochondria after two and four days without DOX (Figure 8B,D).

Indeed, excess a-syn can translocate to mitochondria and associate to the mitochondrial membrane (Robotta et al., 2014; Shavali et al., 2008; Wang et al., 2019). a-Syn N-terminal was described to physically interact with mitochondrial membranes, altering membrane properties like permeability (Shen et al., 2014). a-Syn is also internally present in mitochondria, but in much lower levels (Devi et al., 2008; Martin et al., 2006). This interaction between a-syn and mitochondria causes defects in mitochondria, leading to inhibition of complex I activity and increased ROS production, further promoting mitochondrial dysfunction and energy depletion (Brian J. Tabner et al., 2005; Janetzky et al., 1994; Parker, Parks, & Swerdlow, 2008). As a consequence of energy depletion, high ROS levels and the inability to perform mitophagy of damage mitochondria, apoptotic cell death can be triggered, as seen in PD patient's neurons (Liu, Wang, & Wang, 2020; Merad-Boudia et al., 1998). Indeed, ROS levels and mitochondrial dysfunction are important pathological markers in PD. Our results show significant differences in cells in culture after one, three and four days without DOX, with the higher levels occurring in the first day (Figure 4B). The results are partially concordant with a-syn overexpression; apparently a slight increase in a-syn levels at day one without DOX are enough to trigger increased ROS levels. High mitochondrial ROS production were previously observed in SH-SY5Y cells subjected to 5 days without DOX (Perfeito et al., 2017). These cells also showed decreased mitochondrial protein levels and activity of superoxide dismutase 1 [Cu-Zn] (SOD1), an enzyme that dismutates the superoxide radical, and reduced levels of the antioxidant GSH, supporting the higher ROS levels in cells overexpressing a-syn. These findings also support altered mitochondrial function, which was analyzed in the present study through changes in mitochondrial transmembrane potential. As defined by using the $TMRM^+$ fluorescent probe, SH-SY5Y cells overexpressing WT a-syn for two days showed significant decrease in $\Delta\Psi m$ (Figure 8A), which occurred concomitantly with enhanced interaction of a-syn with the organelle. Abnormal $\Delta\Psi m$ was previously observed in human PD cells due to high calcium accumulation and ROS formation (Hettiarachchi et al., 2009; Parihar et al., 2009).

Autophagy and mitophagy are processes essential for normal cellular function, and in PD cells both processes are impaired (Hsieh et al., 2016; Krebiehl et al., 2010; Winslow et al., 2010; Winslow & Rubinsztein, 2011). In PD, after a-syn oligomerization,

lysosomal function is impaired, preventing a-syn oligomers from being degraded (Meredith et al., 2002); moreover, the relative number of lysosomes is decreased, and lysosomal membranes get more permeable (Chu et al., 2009; Dehay et al., 2010). This lysosomal permeabilization facilitates the exit of the proteolytic enzymes from the lysosome, contributing to cell death (Dehay et al., 2010). Autophagosomes are another component essential to autophagy. In this process autophagosomes merge with lysosomes to form autophagolysosomes. Due to lysosomal impairment, fusion with autophagosomes does not occur and these vesicles begin to accumulate in the cell (Manzoni & Lewis, 2013).

Considering that bafilomycin is an inhibitor of autophagic lysosomal pathway, this compound was tested in SH-SY5Y cells inducibly expressing a-syn to evaluate the influence of both autophagy and mitophagy pathways. Interestingly, when present at low/basal levels or after overexpression for two days, a-syn was shown to be largely degraded by autophagy, as determined by the increase in protein levels after treatment with bafilomycin (Figure 4D). Apparently, prolonged a-syn overexpression (after four days without DOX) affected autophagic degradation of a-syn (Figure 4D). However, other inhibitors and activators of (macro)autophagy should be tested to define the involvement of autophagy in a-syn accumulation in SH-SY5Y cells.

Diverse proteins are involved in autophagy regulation, including p62, Beclin-1, LC3 and ubiquitin. Beclin-1 is a protein involved in autophagy, namely in the maturation of autophagosomes. Although in PC12 cells a-syn overexpression caused reduced Beclin-1 levels (Wang et al., 2016), in the present study Beclin-1 levels were similar in cells subjected, or not, to DOX (Figure 5A). Unchanged levels of this protein may indicate that the maturation of autophagosomes is preserved despite enhanced cellular levels of a-syn.

P62, or sequestosome-1, is an autophagy receptor that acts like a bridge between proteins marked for autophagy and autophagosomes, where it binds to lipidated LC3 or LC3II (Moscat & Diaz-Meco, 2009; Pankiv et al., 2007). Increased levels of p62 in SH-SY5Y cells (Figure 5B, 3A) were observed concomitantly with enhanced a-syn levels, particularly after four days without DOX that accounted for apparent decreased lysosomal degradation of a-syn. These data highly suggest that the autophagic machinery is impaired in PD, with p62 being accumulated and thus not degraded by the lysosomes. Indeed, accumulation of p62 is expected if autophagy is impaired (Korolchuk et al., 2009), and in PD p62 was shown to accumulate in Lewy bodies (Kuusisto, Parkkinen, & Alafuzoff,

2003; Zatloukal et al., 2002). Indeed, p62 levels rose in cells expressing basal levels of a-syn after exposure to bafilomycin (to inhibit ALP); however, after two and four days without DOX, this scenario was not verified (Figure 6B).

LC3 is also related to autophagy, but more important than the total level of this protein is the LC3-II/LC3-I ratio, i.e. between the activated/lipidated form that participates in autophagy and locates in the surface of autophagosomes, and soluble LC3-I. In PD, autophagic vesicles were shown to have less LC3-II associated with the membrane (Wills et al., 2012; Winslow & Rubinsztein, 2011), however other studies report high LC3-II/LC3-1 ratio (Liou et al., 2016; B. Liu et al., 2015). Concordantly, we verified the second scenario, with a rise in LC3-II/LC3-I ratio in cells overexpressing a-syn (Figure 5C). Normal levels of Beclin-1 suggest normal maturation of autophagosomes, while higher LC3 levels associated with autophagosomal membranes suggest that the autophagy process could be impaired after LC3 membrane interaction.

Ubiquitin is a protein that exists free or bound to other proteins as monomers or in chains. Binding to proteins in chains may have several cellular functions, being one of them to mark proteins for degradation (Jin et al., 2008; Thrower et al., 2000). These proteins are marked to be degraded by the UPS. UPS impairment in PD leads to accumulation of ubiquitin and ubiquitinated proteins (Höglinger et al., 2003), including polyubiquitinated a-syn in Lewy bodies (Hasegawa et al., 2002). However, macroautophagy also involves the ubiquitin system; as an example, ubiquitinated cargos are recruited to the phagophore (autophagosome initiation) via an autophagy receptor with a ubiquitin-binding domain and a LC3-interacting region (LIR) (Gómez-Díaz & Ikeda, 2019). In SH-SY5Y cells overexpressing a-syn during two or four days we were able to observe an increase in ubiquitin levels (Figure 5D, 3D), which was not affected by bafilomycin (Figure 6E), suggesting that ubiquitin or ubiquitinated proteins were not degraded by autophagy. As p62 and ubiquitin are both autophagic proteins they should aid in prevention of a-syn accumulation, leading to degradation of this protein. Therefore, a high colocalization between each one of these two proteins and a-syn should be expected in the context of PD (e.g. Kuusisto et al., 2003). Nevertheless, in this study p62 colocalized with a-syn was lower with higher a-syn overexpression (Figure 7A,B), in contrast with ubiquitin (Figure 7C,D), suggesting that a-syn interaction with p62 was a limiting factor for a-syn degradation.

Mitochondrial morphology is regulated by dynamic processes of fusion and fission. Fusion is an important process to maintain mtDNA stability (Chen et al., 2010). Fission reduces mitochondria size, facilitating transport and mitophagy (Burman et al., 2017). Mitochondria fission can be promoted by PD conditions (Nakamura et al., 2011; Wang et al., 2011; Xie & Chung, 2012), which should reduce the mitochondrial perimeter, as verified after two days without DOX, but contrarily after four days without DOX this reduction was not seen (Figure 8C). In the context of PD, enlarged and swollen mitochondria were previously found (Trimmer et al., 2000). Also, PD conditions favour more circular mitochondria (Dagda et al., 2009; Xie & Chung, 2012). In contrast to what has been described, mitochondrial roundness was reduced after a-syn overexpression for two days, suggesting enlarged mitochondria; however, this was not confirmed by the analysis of the aspect ratio (Figure 8C). Mitochondrial AR is the ratio between the major mitochondrial axis and the minor mitochondrial axis. Despite this, we observed increased area occupied by mitochondria after two days of a-syn overexpression, which could be related with mitochondrial biogenesis. In this perspective, both measurement of mtDNA copy number and the levels of mitochondrial transcription factors (e.g. PGC-1a in the nucleus, and the resulting TFAM in mitochondria) could give additional information regarding this process.

Mitophagy is a specialized autophagy process that is normally triggered under conditions of mitochondrial dysfunction and/or enhanced mitochondrial fission. Parkin is a protein recruited from the cytosol to begin mitophagy in damaged mitochondria, due to mitochondrial undegraded Pink1, which further phosphorylates/activates Parkin. So, if damaged mitochondria are not cleared up from the cell, more Parkin will be recruited, leading to the retention of this protein in the organelle (Kawahara et al., 2008). However, no significant changes in parkin levels were observed in mitochondria in cells overexpressing a-syn (Figure 9A,B). One of the reasons for mitophagy impairment is the upregulation of Miro by a-syn overexpression (Hsieh et al., 2016; Shaltouki et al., 2018). Miro is a protein involved in mitochondrial motility, anchoring mitochondria to microtubules (Cai & Sheng, 2009). But when mitochondria are marked to mitophagy, Miro is phosphorylated and removed from mitochondria stopping mitochondrial movement and initiating mitophagy (Shlevkov, 2016). However, when a-syn is overexpressed removal of Miro from mitochondria does not occur due to a possible direct interaction of a-syn (Shaltouki et al., 2018). Under conditions linked to A53T a-syn

mutation, this mutant protein promotes the activation of p38 MAPK that phosphorylates parkin at serine 131, affecting the role of parkin in mitophagy (Chen et al., 2018). Being a E3 ligase, parkin requires ubiquitin binding of mitochondrial proteins for mitophagy (Chan et al., 2011; Geisler et al., 2010).

P62 may serve as an adaptor for ubiquitinated mitochondrial proteins (Ding et al., 2010). In some PD cases, ubiquitination of damaged mitochondria is impaired (Lee et al., 2010). The results show that mitochondria ubiquitination is impaired in cells overexpressing a-syn (low colocalization between ubiquitin and mitochondria, Figure 9E), despite similar mitochondrial levels of Parkin. P62 colocalization with mitochondria is more inconsistent; at two days without DOX the levels of p62 are reduced when compared to cells in the presence of DOX (Figure 9C). If p62 serves as an adaptor to ubiquitin, this make sense because mitochondria are not highly ubiquitinated, so p62 will not be used as an adaptor; however, at four days without DOX the values of colocalization returned to levels similar to cells not overexpressing a-syn. Interestingly, inhibiting ALP in cells overexpressing a-syn caused increased levels of ubiquitinated mitochondria, suggesting activated mitophagy, aimed to degrade dysfunctional mitochondria. Analysis of additional mitophagy markers will be required to determine the influence of WT a-syn overexpression on mitochondrial degradation.

5. Conclusion

Data show that WT a-syn overexpression by SH-SY5Y cells causes increased levels of ROS and mitochondrial dysfunction linked to altered organelle morphology, concomitantly with increased levels of a-syn colocalized with mitochondria. A-syn overexpression and related increased levels of different autophagic proteins (particularly after lysosomal inhibition), but not Beclin-1, implicate dysfunctional ALP. As a consequence, accumulation of a-syn itself was observed, which could be accounted for by decreased a-syn interaction with p62. Nevertheless, under these conditions, ubiquitinated mitochondria is apparently being degraded through the ALP/mitophagy. Overall, these are features of cytotoxic events that may occur in the context of PD, and thus understanding the control of each one of these intricate pathways involving protein quality control and selective mitophagy may be helpful to identify selective therapeutic strategies in this debilitating movement disorder.

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