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Universidade de Coimbra

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Interplay between Alpha-Synuclein and Oxidative Stress in Parkinson´s Disease Cell Models

Interrelação entre Alfa-Sinucleína e Stresse Oxidativo em Modelos Celulares da Doença de Parkinson

Rita Catarina Gonçalves Perfeito

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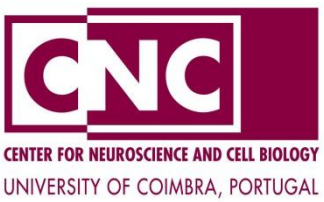

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Act as if what you do makes a difference. It does.

William James
1842-1910

Believe in yourself! Have faith in your abilities! Without a humble but reasonable confidence in your own powers you cannot be successful or happy.

Norman Vincent Peale
1898-1993

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Abbreviations

$^{\cdot}OH$	Hydroxyl radical
3-MA	3-methyladenine
3-NT	3-nitrotyrosine
6-OHDA	6-hydroxydopamine
8OHG	8-hydroxyguanosine
Ac-DEVD-AFC	N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin
AD	Alzheimer's disease
AIF	Apoptosis inducing factor
Alpha-syn	Alpha-synuclein
ALS	Amyotrophic lateral sclerosis
ANK	Ankyrin
ANOVA	Analysis of variance
ARE	Antioxidant response element
ARM	Armadillo
ASK1	Apoptosis signalling-regulating kinase 1
ATP	Adenosine triphosphate
ATP13A2	ATPase type 13A2
Bak	Bcl-2 homologous antagonist-killer protein
Bax	Bcl-2-associated X protein
BBB	Blood brain barrier
Bcl-2	B-cell leukaemia/lymphoma 2
BSA	Bovine serum albumin
BSO	L-buthionine-S-sulfoxamine
bZip	Basic leucine-zipper
CBP	CREB binding protein

CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate
CK	Casein kinase
CMA	Chaperone mediated autophagy
CNC	cap'n'collar
CNS	Central nervous system
COR	C-terminal of Roc
CREB	c-AMP response element binding protein
CS	Citrate synthase
Cu/ZnSOD (SOD1)	Copper-zinc superoxide dismutase
Cyt c	Cytochrome c
DA	Dopamine
DAT	Dopamine transporter
DCF	Dichlorodihydrofluorescein
DHE	Dihydroethidium
DIC	Dicarboxylate carrier
DLB	Dementia with Lewy bodies
DMPO	5,5-dimethyl pyrroline *N*-oxide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOPAC	3,4-dihydroxyphenylacetic acid
Dox	Doxycycline
DTT	1,4-ditiotreitol
EAAC1	Excitatory amino acid carrier 1
ECF	Enhanced chemifluorescence
ECSOD (SOD3)	Extracellular superoxide dismutase
EDTA	Ethylene diamine tetraacetic acid
EGCG	Epigallocatechin gallate
EGTA	Ethylene glycol tetraacetic acid

EHT	Eicosanoyl-5-hydroxytryptamide
EPR	Electric paramagnetic resonance
ERRα	Estrogen-related receptor α
et al.	*et alii* (from Latin), and others
ETC	Electron transport chain
FBS	Fetal bovine serum
FBXO7	F-box protein 7
G418	Geneticin
GCL	Glutamate-cysteine ligase
GCLc	Glutamate-cysteine ligase catalytic subunit
GCLm	Glutamate-cysteine ligase modulatory subunit
GFP	Green fluorescent protein
GIGYF2	Grb10-interacting GYF protein 2
GPx	Glutathione peroxidase
GRed	Glutathione reductase
GRK	G-protein receptor coupled kinase
GS	Glutathione synthase
GSH	Glutathione
GSSG	Oxidized glutathione
GST	Glutathione-S-transferase
GWASs	Genomewide association studies
h	hour
H_2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
H_2O_2	hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNE	4-hydroxynonenal
HO-1	Heme oxygenase-1
hONS	Human olfactory neurosphere-derived

HSP60	60 kDa Heat shock protein
IBR	in-between-ring
iPS	Induced pluripotent stem
Keap 1	Kelch-like ECH-associated protein 1
LAMP-2A	Lysosome-associated membrane protein 2A
LBs	Lewy bodies
L-DOPA	Levo-3,4-dihydroxyphenylalanine
LNs	Lewy neurites
LRR	Leucine-rich repeat domain
LRRK2	Leucine-rich repeat kinase 2
MAF	Musculoaponeurotic fibrosarcoma oncogene
MAO	Monoamine oxidase
MAP	Mitogen-activated protein
MAP2	Microtubule associated protein
MAPKKK	Protein kinase kinase kinase
MARE	MAF recognition element
MBP	Myelin basic protein
MDA	Malondialdehyde
min	minute
MMP^+	1-methyl-4-phenylpyridinium
MnSOD (SOD2)	Manganese superoxide dismutase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrapyridine
MRC	Mitochondrial respiratory chain
MSA	Multiple system atrophy
mtDNA	Mitochondrial DNA
mUs	Milliunits
NAC	Non-amyloid beta-component
NADH	Nicotinamide adenine dinucleotide

NADPH	Nicotinamide adenine dinucleotide phosphate
ND1	Mitochondrial encoded NADH dehydrogenase 1
ND6	NADH-ubiquinone oxidoreductase chain 6
NDA	2,3-naphthalenedicarboxaldehyde
NDUFA4	NADH dehydrogenase (ubiquinone) 1 α-subcomplex 4
NEM	*N*-ethylmaleimide
NFkB	Nuclear factor kappa B
NO	Nitric oxide
NQO1	NADPH:quinone oxidoreductase 1
NRF	Nuclear respiratory factor
Nrf2	Nuclear transcription factor NF-E2-related factor 2
NRs	Nuclear receptors
O_2	Molecular oxygen
$O_2^{\cdot -}$	Superoxide anion /radical
$ONOO^-$	Peroxynitrite
OPA	ortho-phethaldialdehyde
PBS	Phosphate buffered saline
PD	Parkinson's Disease
PGC-1alpha	Peroxisome proliferator activated receptor γ co-activator 1 alpha
PI	Propidium iodide
PINK1	PTEN-induced putative kinase 1
PKC	Protein Kinase C
PLA2G6	Phospholipase A2 group VI
PLK	Polo-like kinase
PMSF	Phenylmethylsulfonyl fluoride
PP2A	Protein phosphatase 2 A
PPAR	Peroxisome proliferator activated receptor

Prx	Peroxiredoxin
PVDF	Polyvinylidene difluoride
RA	Retinoic acid
rAAV	Recombinant adeno-associated virus
RFU	Relative fluorescence unit
RNA	Ribonucleic acid
RNAi	RNA interference
RNS	Reactive nitrogen species
Roc	Ras of complex protein
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Ser	Serine
siRNA	Short-interfering RNA
SN	Substantia nigra pars compacta
SNCA	Alpha-synuclein
SOD	Superoxide dismutase
tBHQ	Tert-butylhydroquinone
TBP	TATA box-binding protein
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TFAM	Mitochondria transcription factor A
TH	Tyrosine hydroxylase
TKL	Tyrosine kinase-like
Trx	Thioredoxin
Tyr	Tyrosine
Ub	Ubiquitin

UBL	Ubiquitin-like
UCHL1	Ubiquitin C-terminal hydrolase 1
UPS	Ubiquitin proteasome system
VMAT	Vesicular monoamine transporter
WT	Wild-type
XO	Xanthine oxidase

Abstract

Parkinson's disease (PD) is the most common motor and age-related neurodegenerative disorder affecting more than 1% of the population above age 65. Neurologically, PD patients present symptoms of progressive deterioration of tremor, rigidity of movements, bradykinesia and postural instability. The major neuropathological features of the disease are characterized by a progressive degeneration of dopaminergic neurons in the *substantia nigra pars compacta* (SN) and the formation of intracytoplasmic inclusions, called Lewy bodies (LBs), which are mainly composed by alpha-synuclein (alpha-syn). Despite the efforts over the years, the etiopathogenesis of PD is still not fully understood. 95% of cases are sporadic and result from a combination of environmental factors and genetic susceptibilities. The remaining 5% are the result of genetic mutations, including in the alpha-syn gene, which is involved in autosomal dominant inherited forms of PD. Nevertheless, sporadic and genetic forms of PD share common processes, namely oxidative stress and mitochondrial dysfunction, which have been of great interest to understand the mechanisms involved in dopaminergic neurodegeneration in PD.

In this study, we modelled mitochondrial dysfunction and oxidative stress in PD, respectively, by using rotenone, a complex I inhibitor of the mitochondrial respiratory chain and iron ($FeSO_4$), a classical oxidative stress inducer, in the human neuroblastoma cell line SH-SY5Y, expressing wild-type (WT) or mutant A53T alpha-syn, using two different systems of protein overexpression.

In the first part of the work, we focused on the effect of mutant A53T *versus* WT alpha-syn modifications in transiently transfected SH-SY5Y cells and analysed the susceptibility of these cells to prolonged exposure (4 days) to iron ($FeSO_4$) and rotenone on the generation of reactive oxygen species (ROS) and its correlation with cell death, formation of alpha-syn inclusions and alpha-syn phosphorylation at serine (Ser) 129. Our data showed that mutant A53T alpha-syn increased ROS levels, ubiquitin (Ub)-labelled alpha-syn aggregates,

phosphorylation at Ser129 and a decrease in protein phosphatase 2A (PP2A) activity, when compared to WT alpha-syn. Furthermore, prolonged exposure to iron or rotenone, enhanced the production of ROS in cells expressing WT or mutant A53T alpha-syn, which correlated with alpha-syn inclusion formation, Ser129 phosphorylation and mitochondrial depolarization. Our data suggest that enhanced ROS formation is associated with alpha-syn aggregation and phosphorylation at Ser129, in particular mutant A53T alpha-syn, which may precede degeneration of PD affected cells.

Next, we used the neuroblastoma cell line SH-SY5Y overexpressing WT alpha-syn in a doxycycline (Dox) regulated manner, in a Tet Off system. Here, we correlated the occurrence of oxidative stress in cells inducibly overexpressing WT alpha-syn (-Dox) before and after short exposure (2 h) to iron. We gave evidence for raised ROS generation under basal conditions in cells overexpressing WT alpha-syn, compared to cells expressing the endogenous protein (+Dox), without changes in cell reducing capacity or alterations in the levels of apoptotic proteins. Furthermore, increased ROS levels were closely related with a decrease in the activities and levels of proteins involved in the antioxidant defense pathways, namely superoxide dismutase 1 (SOD1), SOD2, reduced glutathione (GSH) and oxidized glutathione (GSSG), while enzymes of the glutathione redox cycle were unchanged. Accordingly, the levels of the catalytic subunit of glutamate-cysteine ligase (GCLc) were also diminished. Further analysis of nuclear fractions demonstrated that nuclear factor erythroid 2-related factor 2 (Nrf2), which activates the gene transcription of these antioxidant enzymes following mild intracellular ROS production, showed decreased levels in –Dox cells. Short treatment with iron, although largely augmenting ROS production in both +Dox and –Dox cells, triggered a significant increase in the levels and activities of these antioxidant proteins, apparently independently of alpha-syn overexpression, suggesting a compensatory intrinsic defense mechanism against short oxidizing stimulus.

Finally, we investigated the role of WT alpha-syn expression in mitochondrial dysfunction, strongly associated with PD pathology. Using the same cellular model regulated by Dox, we showed that cells overexpressing alpha-syn and retaining high amount of alpha-syn in

mitochondria, displayed increased levels of mitochondrial superoxide anion, which were not triggered by treatment with iron. Accordingly, mitochondria from –Dox cells showed a significant decrease in the activity of complex I of the respiratory chain, with no changes in citrate synthase activity, corroborating previous studies concerning mitochondrial dysfunction and PD pathogenesis. Moreover, we did not find changes in mitochondrial transcription factor A (TFAM) or peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1alpha) between +Dox and –Dox cells. In addition, we observed a reduction in SOD1 activity in mitochondrial fractions of –Dox cells, which was exacerbated by iron. In contrast, mitochondrial SOD2 activity was not affected under basal conditions in –Dox cells, but was highly increased upon iron treatment. GSH and GSSG remained decreased in mitochondrial fractions from –Dox cells in untreated conditions, while iron did not affect the levels of this endogenous antioxidant. These data show that enhanced alpha-syn in mitochondria may decrease the activity of mitochondrial complex I and promote mitochondrial ROS production, which may be boosted due to low antioxidant defenses in the organelle.

Overall, these studies add relevant information on a close relationship between overexpression and post-translational modifications (e.g. phosphorylation) of WT and mutant alpha-syn and oxidative stress-related mechanisms, including cellular and mitochondrial antioxidant defense pathways. Thus, the data reveal the importance of developing neuroprotective strategies targeted at the transcription level, in order to prevent the imbalance in the redox homeostasis that trigger subsequent neurodegenerative events in PD.

Resumo

A doença de Parkinson (PD, do inglês 'Parkinson's disease') é a doença neurodegenerativa mais comum associada ao movimento e à progressão da idade, que afeta atualmente mais de 1% da população acima dos 65 anos de idade. Em termos neurológicos, os doentes de Parkinson apresentam sintomas de tremor incontrolado, rigidez de movimentos, bradicinésia e instabilidade postural. As principais características neuropatológicas desta doença incluem uma progressiva degenerescência dos neurónios dopaminérgicos da *substantia nigra pars compacta* (SN) e a formação de inclusões intracitoplasmáticas denominadas corpos de Lewy, maioritariamente compostas por alfa-sinucleína (alfa-sin). Apesar dos esforços ao longo dos anos, a etiopatogénese da PD não está ainda completamente esclarecida. 95% dos casos são esporádicos e resultam de uma combinação entre fatores ambientais e suscetibilidades genéticas, enquanto os restantes 5% se devem a mutações genéticas, incluindo no gene da alfa-sin, que está envolvido numa das formas hereditárias autossómicas dominantes da PD. Não obstante, as formas esporádica e genética da PD partilham processos comuns, nomeadamente os relacionados com o stresse oxidativo e a disfunção mitocondrial, que têm constituído elevado interesse na compreensão dos mecanismos envolvidos na neurodegenerescência dopaminérgica na doença.

Neste trabalho foram modeladas situações de disfunção mitocondrial e de stresse oxidativo na PD, respetivamente, usando rotenona, um inibidor do complexo I da cadeia respiratória mitocondrial e ferro ($FeSO_4$), um indutor clássico de stresse oxidativo, numa linha celular humana de neuroblastoma (SH-SY5Y), em que se induziu a expressão de alfa-sin na sua forma *wild-type* (WT) ou mutante (A53T), utilizando dois sistemas diferentes de sobre-expressão de proteínas.

Na primeira parte do estudo, focámo-nos no efeito das modificações da alfa-sin mutante A53T *versus* WT em células SH-SY5Y transfetadas de forma transitória e analisámos a sua suscetibilidade a uma exposição prolongada (4 dias) a ferro ($FeSO_4$) e a rotenona na

produção de espécies reativas de oxigénio (ROS, do inglês 'reactive oxygen species') e a sua correlação com a morte celular, formação de inclusões e fosforilação do resíduo de serina (Ser) 129 da alfa-sin. Os resultados obtidos revelaram que a expressão de alfa-sin A53T mutante induziu um aumento dos níveis de ROS, de agregados compostos por alfa-sin e ubiquitina, da fosforilação da Ser129 e uma diminuição da atividade da proteína fosfatase 2A (PP2A, do inglês 'protein phosphatase 2A'), quando comparada com células que expressavam alfa-sin WT. Além disso, verificou-se um aumento exacerbado da formação de ROS em células que expressavam alfa-sin WT ou A53T mutante após a exposição prolongada a ferro ou rotenona, que se correlacionou com a formação de inclusões de alfa-sin, fosforilação da Ser129 e despolarização mitocondrial. Estes dados sugerem que a elevada produção de ROS está associada à agregação e fosforilação da alfa-sin no resíduo de Ser129, em particular no caso da alfa-sin A53T mutante, que pode eventualmente preceder a degeneração das células afetadas na PD.

Na abordagem seguinte do trabalho experimental utilizámos a linha celular humana de neuroblastoma (SH-SY5Y) que sobre-expressava de forma condicionada a alfa-sin, i.e., de forma regulada por doxiciclina (Dox) através de um sistema Tet Off. Neste caso, correlacionámos a ocorrência de stresse oxidativo em células que sobre-expressam alfa-sin WT (-Dox) antes e após uma exposição curta a ferro. Os resultados evidenciaram a produção elevada de ROS em condições basais em células que sobre-expressavam alfa-sin WT, comparativamente às células que expressavam a proteína endógena (+Dox), sem no entanto se verificarem alterações na capacidade redutora das células e nos níveis de proteínas apoptóticas. Além disso, o aumento dos níveis de ROS foi estreitamente relacionado com uma diminuição das atividades e dos níveis de proteínas envolvidas nas vias de defesa antioxidante, nomeadamente da superóxido dismutase de tipo 1 (SOD1), da SOD2, do glutatião reduzido (GSH) e oxidado (GSSG), enquanto as enzimas do ciclo redox do glutatião permaneceram inalteradas. De acordo com estes dados, os níveis da subunidade catalítica da glutamato cisteína ligase (GCLc) revelaram-se diminuídos. A análise de frações nucleares demonstrou que o fator de transcrição Nrf2 (do inglês 'nuclear factor

erythroid 2-related factor 2'), que ativa a transcrição dos genes destas enzimas antioxidantes após a formação de ROS intracelular, apresentava níveis mais baixos nas células –Dox. Por outro lado, o tratamento com ferro, embora tenha aumentado em grande medida a produção de ROS em ambas as células (+Dox e –Dox), induziu um aumento significativo dos níveis e atividades destas proteínas antioxidantes, aparentemente de uma forma independente da sobre-expressão da alfa-sin, sugerindo um mecanismo de defesa intrínseco e compensatório contra o estímulo oxidante.

Na parte final do trabalho investigámos o papel da expressão de alfa-sin WT na disfunção mitocondrial, fortemente associado à patologia da PD. Utilizando o mesmo modelo celular em que a expressão da alfa-sin foi regulada por Dox, mostrámos que as células que sobre-expressam alfa-sin, continham grandes quantidades de alfa-sin na mitocôndria, revelaram níveis mais elevados de anião superóxido mitocondrial, que, no entanto, não foram exacerbados após o tratamento com ferro. De acordo com o aumento dos níveis de ROS na mitocôndria, a análise da medição da atividade enzimática do complexo I da cadeia respiratória mitocondrial mostrou uma diminuição da atividade do mesmo, em conformidade com estudos anteriores que evidenciaram o papel da disfunção mitocondrial mediada por alfa-sin e a patogénese da PD. Além disso, não verificámos alterações dos níveis do fator de transcrição mitocondrial A (TFAM, do inglês 'mitochondrial transcription factor A'), nem do PGC-1alfa (do inglês 'peroxisome proliferator-activated receptor gamma coactivator 1-alpha') entre células +Dox e –Dox. Por outro lado, observámos uma redução da atividade da SOD1 em frações mitocondriais das células –Dox, que decresceu ainda mais após a exposição a ferro. Ao contrário da SOD1, a atividade da enzima SOD2 mitocondrial não se revelou alterada em condições basais nas células –Dox; contudo, observou-se um claro aumento da atividade da SOD2 após tratamento com ferro. As proteínas GSH e GSSG do ciclo do glutatião permaneceram diminuídas em frações mitocondriais das células –Dox em condições não tratadas, enquanto a exposição a ferro não afetou os níveis destes antioxidantes endógenos. Estes resultados mostram que a presença de altos níveis de alfa-sin na mitocôndria pode influenciar negativamente a atividade do complexo I mitocondrial

e promover a produção de ROS neste organelo, cujos níveis podem ser exacerbados devido a baixas defesas antioxidantes.

Em conjunto, este estudo vem adicionar informação relevante sobre a existência de uma estreita relação entre a sobre-expressão da alfa-sin WT e mutante e modificações pós-traducionais (e.g. fosforilação) destas proteínas e os mecanismos associados ao stresse oxidativo, incluindo as vias celulares e mitocondriais de defesas antioxidantes. Estes resultados revelam ainda a importância do desenvolvimento de estratégias neuroprotetoras direcionadas para a regulação do processo transcricional, com o objetivo de prevenir o desequilíbrio da homeostasia redox, que pode levar, subsequentemente, ao processo neurodegenerativo na PD.

CHAPTER 1

Introduction

Part of this chapter was based on: Rita Perfeito, Teresa Cunha-Oliveira, A. Cristina Rego (2012) Revisiting oxidative stress and mitochondrial dysfunction in the pathogenesis of Parkinson's disease – resemblance to the effect of amphetamine drugs of abuse. *Free Radical Biology and Medicine*; 53: 1791-1806.

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1.1. Overview of Parkinson's disease

Parkinson's disease (PD) is a chronic and progressive neurological disease associated to a loss of dopaminergic neurons in the *substantia nigra pars compacta* (SN), as well as with more-widespread neuronal changes that cause complex and variable motor and nonmotor symptoms. PD was first medically described as a neurological syndrome by James Parkinson in 1817 (in "An Essay on the Shaking Palsy"). At present, PD is the second most prevalent neurodegenerative brain disorder, affecting 1 to 2% of the population above 65 years of age and its prevalence increases to approximately 4% in individuals above 85 years of age (de Rijk et al., 2000; Bekris et al., 2010). The etiopathogenesis of PD is still not fully understood. In most cases the disease is sporadic: a multifactorial, idiopathic disorder that seems to arise from a combination of environmental exposures and genetic susceptibility. The remaining cases are the result of genetic inheritance. Moreover, 15 to 20% of the patients with PD report a family history of the disease, although monogenic forms of PD are relatively rare (Sellbach et al., 2006; Farrer, 2006; Bekris et al., 2010). Nevertheless, old age continues to be the main risk factor for the development of the disease (de Lau and Breteler, 2006; Mounsey and Teismann, 2010), making clear that during aging, our cells display a greater degree of dysfunction, leading to cell stress (including decreased capacity to cope with oxidative stress) and greater energy demand.

1.1.1. Clinical and pathological aspects

Clinically, PD is characterized by the presence of cardinal motor signs, namely resting tremor, rigidity, bradykinesia and postural instability. One of the main features affecting these patients includes a slowness of initiation of voluntary movement with a progressive reduction in speed and amplitude of sequential motor tasks (Dickson et al., 2009; Halliday et al., 2011). For a long time, PD was thought to involve a relatively simple neuropathological process primarily centered on the loss of dopaminergic neurons in the

SN. This results in the loss of dopaminergic transmission in the striatum, leading to the classic PD motor symptoms. The disease becomes evident when approximately 80% of striatal dopamine (DA) and 50% of nigral neurons are lost (Fearnley and Lees, 1991; Bekris et al., 2010; Dunning et al., 2011) (Figure 1.1A, B). However, recent evidence indicates that non-motor characteristics, such as autonomic insufficiency, cognitive impairment, olfactory deficits, sleep disturbance, depression and psychosis are very common during the course of the disease. The clinical diagnosis of PD is typically based on the presence of cardinal motor features, absence of atypical findings suggestive of an alternate diagnosis and response to Levo-3,4-dihydroxyphenylalanine (L-DOPA) (Samii et al., 2004; Bekris et al., 2010).

In addition to loss of dopaminergic neurons in the SN, PD is neuropathologically characterized by the presence of Lewy bodies (LBs) and Lewy neurites (LNs) in vulnerable populations of neurons. These are intracytoplasmic insoluble protein inclusions located in either the neuronal cell body or neuronal processes, respectively. The principal component of LBs and LNs is alpha-synuclein (alpha-syn), a small protein of 140 amino acids that is predominantly expressed in the neocortex, hippocampus, SN, thalamus and cerebellum (George, 2002). Effectively, the diagnosis of PD depends upon the presence of two neuropathological signs: 1) loss of neuromelanin-containing dopaminergic neurons of the SN and 2) LBs in the SN neurons, although it is clear that pathology is also present outside this area (Probst et al., 2008; Schapira and Jenner, 2011).

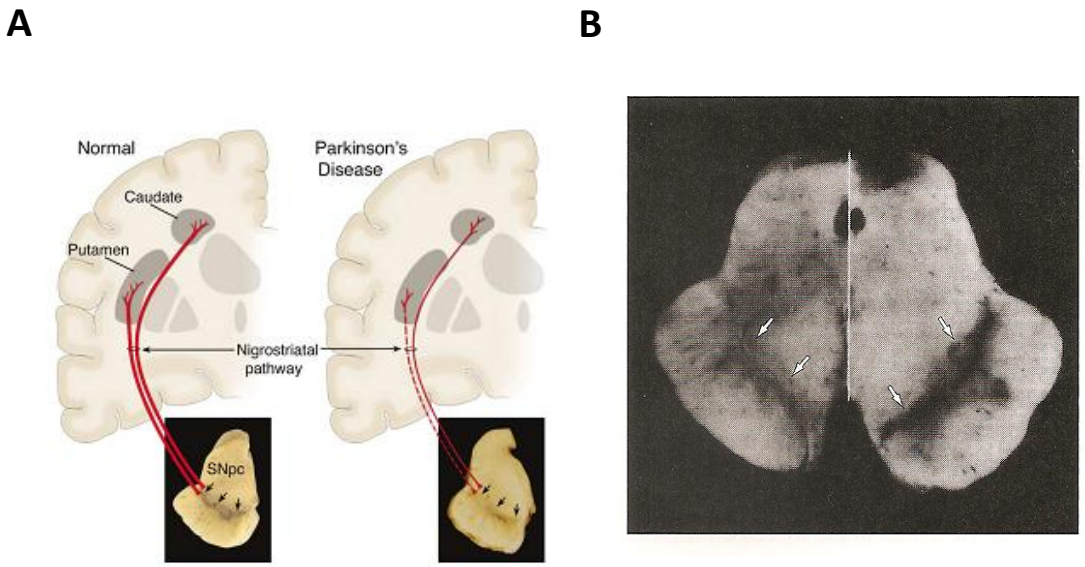

Figure 1.1. – Pathological changes in Parkinson's disease brain. (A) Schematic representation of the nigrostriatal pathway (in red) in normal (A left) and in PD (A right). Nigrostriatal pathway involves dopaminergic neurons whose cell bodies are located in the SN (arrows). These neurons project to the basal ganglia and synapse in the striatum (putamen and caudate nucleus). This pathway is disrupted in PD patients due to dopaminergic neuron loss. Photograph in **A left** represents the normal pigmentation of the SN produced by neuromelanin in dopaminergic neurons. Photograph in **A right** shows depigmentation (i.e., loss of dark-brown pigment neuromelanin) in the SN of a PD patient due to the marked loss of dopaminergic neurons. **(B)** *Left:* midbrain from a patient with PD. The SN (pigmented area) is largely absent in the region above cerebral peduncles (arrows). *Right*: midbrain from a normal subject, showing intact SN (arrows).
A – adapted from Dauer and Przedborski, 2003; **B** – from Bradley et al., 1991.

1.1.2. Parkinson´s disease – sporadic and genetic forms

Sporadic and inherited forms of PD share pathological, biochemical and clinical features, with dysfunction of mitochondria, increased oxidative stress levels and associated molecular pathways representing a link between the two forms of PD, as well as the natural

ageing process (Mounsey and Teismann, 2010). Environmental factors were long thought to be the principal cause of PD, particularly after the influenza pandemic of 1918, when a significant number of individuals developed postencephalitic parkinsonism. Infectious agents in the environment were then suspected to be the causal factors (Poskanzer and Schwab, 1963; Corti et al., 2011). This environmental theory was subsequently supported by the identification of *N*-methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) in the early 1980's, whose metabolite 1-methyl-4-phenylpyridinium (MPP^+), produced in glial cells, is responsible for the selective degeneration of the nigrostriatal pathways due to its neuronal uptake through the dopamine transporter (DAT). In dopaminergic neurons MPP^+ inhibits mitochondrial complex I of the respiratory chain and subsequently promotes oxidative stress. Moreover, MPP^+ was also shown to bind vesicular monoamine transporters (VMAT) and redistribute vesicular DA to the cytosol (Lotharius and O'Malley, 2001). Within the cytosol, MPP^+ may also interact with different enzymes, namely xanthine oxidase, aldehyde dehydrogenase and lipoamide dehydrogenase (Klaidman et al., 1993). MPTP leads to a parkinsonian syndrome in rodents (namely mice, since rats are generally resistant to systemic MPTP), primates and humans (Langston et al., 1983; Ross et al., 2008). A similar pathogenic mechanism was suggested for rotenone, a common naturally occurring botanical pesticide, which acts as an inhibitor of the mitochondrial respiratory chain complex I and leads to a failure of the mitochondrial energy supply of the cell. Betarbet and co-workers (2000) showed that chronic treatment with rotenone was sufficient to cause typical features of PD, including loss of dopaminergic neurons and appearance of alpha-syn positive inclusions in rodents (Betarbet et al., 2000; Sherer et al., 2003b). The involvement of oxidative damage caused by mitochondrial dysfunction after rotenone treatment was also demonstrated in *in vitro* models, suggesting specific neuronal death by rotenone (Sherer et al., 2003a). In this regard, a large number of environmental situations and agents have been identified, including exposure to farming and rural life, and/or industrial chemicals, metals and pesticides; however, no conclusive relations between individual studies have been found (Di Monte, 2003; Elbaz and Moisan, 2008; Corti et al., 2011).

Factors decreasing the risk of developing PD can also provide valuable clues to the understanding disease etiology. Evidence that cigarette smoking, caffeine and tea intake may reduce the risk for PD development appears clear, but there is still uncertainty over their exact role in the disease (Ascherio et al., 2001; Warner and Schapira, 2003; Costa et al., 2010).

Over the last two decades, the scientific view of PD etiology has dramatically changed. Although most patients with idiopathic or late-onset PD (90% of all cases) do not seem to have inherited the disease, a positive family history is frequently associated with a high risk of developing PD. Studies in families with rare Mendelian inheritance (5-10%), where several causative genes have been identified, show protein aggregation, abnormal handling of misfolded proteins by the ubiquitin (Ub) proteasome system (UPS), together with oxidative stress, mitochondrial dysfunction, alterations in autophagy-lysosomal pathways and kinase signaling pathways, suggesting that all these factors may play a major role in PD pathogenesis (Corti et al., 2011).

Molecular genetic analysis of PD families in the last years, in particular the mapping and the subsequent cloning of genes that cause inheritable forms of this disorder, provided important insights in the mechanisms underlying PD pathology. Particularly, it has been shown that PD is not a single clinical entity, but rather a heterogeneous group of diseases with different associated pathologies and a variable spectrum of clinical symptoms and signs. Furthermore, some familial forms include atypical clinical features, such as young-onset, onset with dystonia or the early occurrence of dementia and dysautonomia. Alternatively, PD may result from a combination between genetic predisposition and environmental factors, or, moreover, from common genetic variants in genes identified in monogenic forms that have been found to confer a risk of developing the sporadic disease.

Understanding the mechanisms underlying the initiation and progression of PD, came with the identification of mutations in the gene encoding alpha-syn (SNCA, PARK 1 locus) in an Italian family (the Contursi kindred) and the evidence that alpha-syn is the major component of LBs (Polymeropoulos et al., 1996; Polymeropoulos et al., 1997; Spillantini et

al., 1997; Corti et al., 2011). Since then, a total of 18 loci have been indicated through linkage analysis (PARK1-15) or genomewide association studies (GWASs) (PARK16-18) (Belin and Westerlund, 2008; Satake et al., 2009; Simon-Sanchez et al., 2009; Pankratz et al., 2009; Hamza et al., 2010; Paisan-Ruiz et al., 2009; Di et al., 2009; Hicks et al., 2002; Lautier et al., 2008; Leroy et al., 1998; Gasser et al., 1998; Strauss et al., 2005; Pankratz et al., 2003). Mutations within 11 genes of these loci have conclusively been demonstrated to cause familial parkinsonism in either autosomal dominant or recessive forms (Lesage and Brice, 2009; Bekris et al., 2010), including PARK1 and PARK4 [SNCA (alpha-syn)], PARK2 (parkin), PARK5 [ubiquitin C-terminal hydrolase L1 (UCHL1)], PARK6 [PTEN-induced putative kinase 1 (PINK1)], PARK7 (DJ-1), PARK8 [Leucine-rich repeat kinase 2 (LRRK2)], PARK9 [ATPase type 13A2 (ATP13A2]), PARK11 [Grb10-interacting GYF protein 2 (GIGYF2)], PARK13 [Omi/Htra2 (HTRA2)], PARK14 [phospholipase A2 group VI (PLA2G6)], and PARK15 [F-box protein 7 (FBXO7)] (Corti et al., 2011). Furthermore, common polymorphisms within two of these genes (SNCA and LRRK2) are now well-associated to risk factors for common sporadic PD (Paisan-Ruiz, 2009; Bekris et al., 2010), which suggests that the contribution of genetics to PD may be more relevant than previously thought.

1.1.3. Pathology spreading and neuronal circuits affected

Research in the last 25 years has confirmed that although the lesion in the SN is a key hallmark to the pathological confirmation of PD, the pathological lesions are much more extensive and involve a number of pathways in the brainstem and areas of the neocortex (Kosaka et al., 1988; Braak et al., 1995; Halliday et al., 2011). It is presently recognized that early neuronal loss occurs in other regions involved in motor control (Macdonald and Halliday, 2002; Henderson et al., 2000) and in neurons of the mesocortical system (McRitchie et al., 1997). The involvement of other neuronal populations takes place later in PD or only in certain clinical phenotypes and includes neuronal loss in the cholinergic basal forebrain (Bohnen and Albin, 2011), in the hypothalamic hypocretin system (Fronczek et al.,

2007; Thannickal et al., 2007) and in the upper brainstem serotonin system (Halliday et al., 1990). Neuronal loss is very restricted to areas containing LBs, such as the amygdala, the dorsal motor nucleus of the vagus nerve, locus coeruleus and the neocortex (Harding et al., 2002; Kingsbury et al., 2010; Hoogendijk et al., 1995; Pedersen et al., 2005). Although the pathology of PD affects a sort of neuronal systems, it does not cause substantial brain tissue loss (Double et al., 1996; Dalaker et al., 2010), since cell loss is limited to certain neuronal populations, including the SN (Fearnley and Lees, 1991).

Various types of studies suggest that PD is slow in progressing through the nervous system, with restricted pathology occurring before the characteristic symptoms. In fact, the onset of PD is so gradual, that it is frequently difficult to check out a patient when the disease first emerges, and by the time the diagnosis is made, pathology is likely to be widespread (Halliday et al., 2011). Many of the earliest symptoms are commonly misinterpreted, since some are linked to the normal aging process; these include depression, rheumatism, loss of elasticity and other secondary symptoms, namely fatigue, autonomic dysfunction, constipation or sleep disturbances, leading to insufficient information to make a definitive diagnosis. Indeed, the major symptoms associated to PD seem to appear after a significant neuronal cell loss.

The neuropathological distribution of the insoluble inclusions (LBs) constituted by misfolded alpha-syn throughout the brain, has gained importance over time. This is due to the work developed by Heiko Braak and colleagues, who proposed a theory for the progression of PD (Braak et al., 2002; Braak et al., 2003), based on LB pathology progression through cell-to-cell contact. They examined brains from patients with confirmed diagnoses of neurodegenerative disorder and made several observations, paying special attention to the development of PD neuropathology. In brain sections derived from autopsied PD patients, they detected an apparent correlation between the amount of alpha-syn deposits and the stage of the disease (Braak et al., 2002). Moreover, based on these initial observations, Braak's team performed anatomical neuropathological studies in order to characterize the cerebral progression of alpha-syn-positive LBs and LNs, as the disease

process continued. This led to the establishment of an association between LB pathology and clinical symptoms (Braak et al., 2002; Braak et al., 2003; Braak et al., 2004) and to the development of the Braak´s staging concept for PD, which includes six stages that represent pre-symptomatic (prior to the motor symptoms) and symptomatic phases (Braak et al., 2002).

In stage 1, Braak et al. (2004) suggested that LB pathology appears outside the SN. These initial alpha-syn-immunoreactive inclusions are found within the olfactory system, causing autonomic and olfactory deficits. In stage 2, the LBs and LNs are more widespread within the medulla oblongata, including monoaminergic areas. During these first stages, the individuals do not present any perceptible motor symptoms (Braak et al., 2004). Stage 3 is characterized by the progression of LB pathology into the brainstem. The alpha-syn-positive deposits are typically found in the midbrain and basal forebrain and later spread into the SN. In stage 4, cell loss into the SN is evident, as well as alpha-syn immunoreactivity in the cerebral cortex. At this time, patients may display the first symptoms consistent with parkinsonism. Finally, in end stages 5 and 6, few cells remain positive for neuromelanin in the SN, while alpha-syn-positive pathology begins to invade the neocortex. At these phases, the motor symptoms are clearly severe and cognitive dysfunction becomes evident (Braak et al., 2003; Duda et al., 2000b; Halliday and McCann, 2010; Hawkes et al., 2010; Dunning et al., 2011). In addition, Braak and colleagues (2003) proposed that projection neurons with no or limited myelination were particularly susceptible to the deposition of alpha-syn, suggesting that PD progression described above might evolve via these neuronal pathways, affecting more sensitive neurons before reaching the less vulnerable cells.

Recent studies demonstrating that misfolded aggregated alpha-syn can be transferred across synapses and spread within postsynaptic cells, gives strength to the concept of the cell-to-cell transfer (Brundin et al., 2008; Halliday et al., 2011). However, the most controversial aspect of the Braak PD's staging is identifying where LB pathology begins in the brain, since other studies on similar populations suggest that 7-8% of patients did not present medullary LBs (Kalaitzakis et al., 2008; Attems and Jellinger, 2008). Furthermore,

the lack of correlation between Braak staging and clinical severity has also been seen as problematic as the clinical deficits associated to PD appear to be more related to the degree of dopaminergic cell loss than to the severity of Lewy pathology.

1.2. Alpha-synuclein (SNCA) – the common link

Alpha-syn is a protein that is highly abundant in the brain (Iwai et al., 1995). Its dysfunction has been the central focus of several neurodegenerative disorders known as alpha-synucleinopathies, which include PD, dementia with LBs (DLB) and multiple system atrophy (MSA). These disorders are characterized by abnormal aggregation of alpha-syn in pathological hallmark inclusions in neurons (LBs and LNs) in PD and DLB and glial cytoplasmic inclusions in oligodendroglia in MSA (Dickson et al., 1999).

SNCA (also known as PARK1) was the first gene identified in a large Italian family (Contursi kindred) segregating a pathogenic missense mutation – A53T – causing dominantly inherited PD and LB pathology (Polymeropoulos et al., 1996; Polymeropoulos et al., 1997). Since then, two groups of disease-causing mutations have been described in the alpha-syn gene: 1) three missense point mutations (A53T, A30P and E46K) (Polymeropoulos et al., 1997; Kruger et al., 1998; Zarranz et al., 2004; Lesage and Brice, 2009) and 2) whole-locus multiplications, including duplications and triplications, leading to a pathogenic overexpression of the wild-type (WT) protein.

Human alpha-syn was first identified as the precursor protein for the non-amyloid beta-component (NAC) of Alzheimer's disease (AD) amyloid plaques (Ueda et al., 1993). Structurally, this small protein of 140 amino acids is characterized by: 1) a highly amyloidogenic domain, the NAC, which presents a high propensity for aggregation and to form oligomeric structures and insoluble fibers (Giasson et al., 2001); 2) seven imperfect repeats (KTKEGV) at the N-terminal, which form an amphipathic α-helical domain when the

protein interacts with lipid-containing membrane microdomains (Fortin et al., 2004) and 3) an acidic C-terminal region (Eliezer et al., 2001) (Figure 1.2).

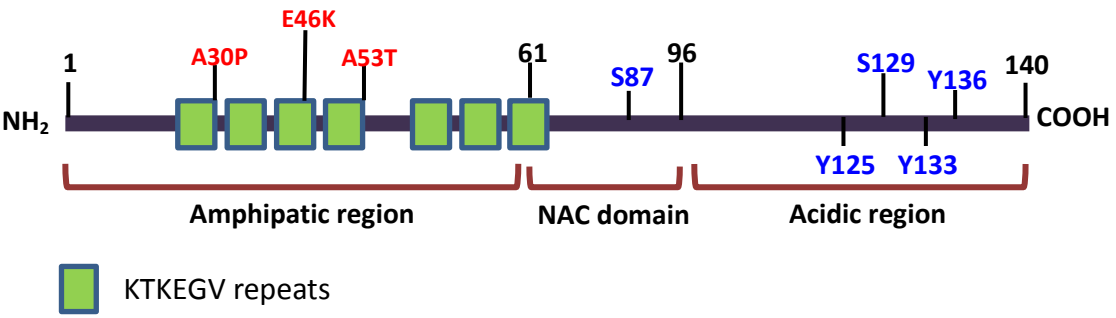

Figure 1.2. – Schematic representation of the alpha-syn protein. Alpha-syn is a 140 amino acid protein with seven imperfect repeats (KTKEGV) localized in the amino terminal. It has three distinct regions: the amphipatic region in the NH_2 terminus, a central hydrophobic NAC (non amyloid component) domain and an acidic COOH-terminal region. The three pathogenic missense mutations are represented in red. Missense mutations at residues 30 (A30P), 46 (E46K) and 53 (A53T) are shown in red. Phosphorylation sites at Tyr (125, 133 and 136) and Ser (87 and 129) are shown in blue. (Adapted from Perfeito et al., 2012.)

Alpha-syn is predominantly localized to presynaptic nerve terminals, close to synaptic vesicles, but can also be found in other neuronal compartments like the cytosol, mitochondria and nucleus. Although its physiological function is still unknown, it has been proposed to have a role in the integration of presynaptic signaling and neuronal plasticity (George et al., 1995). Prior findings are consistent with these roles, with alpha-syn appearing to control synaptic vesicle fusion and recycling. Most data indicate that alpha-syn inhibits synaptic transmission in an activity-dependent manner, as observed in alpha-syn deficient mice, exhibiting accelerated recovery of DA release when presented to multiple stimuli (Abeliovich et al., 2000). In addition, alpha-syn is also predicted to have specific effects in dopaminergic neurons, where it may negatively regulate the activity of TH, as well as the activity of DAT (Sidhu et al., 2004). Products resulting from the interaction between

DA and alpha-syn have been widely explored to address whether this interaction may produce toxic forms of alpha-syn (Sulzer, 2010). DA is prone to oxidation and its quinone derivatives react with proteins, in general via a covalent modification of a cysteine. Alpha-syn, however, does not possess a cysteine residue in its structure. Data from Norris et al. (2005) suggest an ionic interaction between DA-quinone and residues 125-129 of alpha-syn. DA modification of alpha-syn seems to maintain small alpha-syn oligomers in a reactive protofibril conformation by inhibiting progression to form less reactive aggregates and this effect was more evident for alpha-syn mutant forms (Volles et al., 2001; Sulzer, 2007a).

Fibrillization and aggregation of mutant alpha-syn play a central role in neurodegenerative mechanisms, either due to primary alterations in the peptide sequence or due to increased levels of physiological protein, possibly exceeding existing pathways of degradation. An increase in proteins that are determined to be degraded by the UPS or an impairment of the UPS function are contributive factors to proteolytic stress due to accumulation and aggregation of proteins in cytosol. How alpha-syn is degraded is still a matter of debate, but it is likely that both UPS and the lysosomal degradation pathway intervene in regulating the steady-state levels of the protein under normal physiological conditions. Degradation of alpha-syn by the proteasome (Bennett et al., 1999; Tofaris et al., 2001) and the formation of inclusions in neuronal cells treated with proteasome inhibitors have been described (Rideout et al., 2001; Rideout and Stefanis, 2002). On the other hand, the involvement of autophagy and lysosomes has also been demonstrated (Paxinou et al., 2001; Webb et al., 2003; Lee et al., 2004; Cheung and Ip, 2009). Evidence has been shown for mutant, and possibly excessive normal alpha-syn facilitating its pathological accumulation by impairing the function of protein degradation processes (Cuervo et al., 2004; Petrucelli et al., 2002; Snyder et al., 2003; Tanaka et al., 2001). Cuervo et al. (2004) identified in the alpha-syn sequence, the pentapeptide motif required to target cytosolic proteins to lysosomal degradation via chaperone-mediated autophagy (CMA) (Cuervo et al., 2004). In cellular models and rodents, alpha-syn was shown to interact with lysosome-associated membrane protein 2A (LAMP-2A), a transmembrane receptor of substrates for lysosomal degradation,

giving evidence to the theory that CMA is involved in its clearance (Cuervo et al., 2004; Vogiatzi et al., 2008). Accordingly, LAMP-2A gene silencing slowed the degradation of monomeric and oligomeric species of alpha-syn in primary neurons of the ventral midbrain (Vogiatzi et al., 2008). CMA, however, was not the only pathway for alpha-syn degradation. Vogiatzi et al. (2008) also reported that clearance of WT alpha-syn was in part mediated by macroautophagy. The study involved the selective macroautophagy inhibitor 3-methyladenine (3-MA), which led to a considerable increase of the steady state levels of alpha-syn in PC12 cells and in primary cortical and ventral midbrain neurons, suggesting that dysfunction of this degradation process could also contribute to the gradual accumulation of endogenous WT alpha-syn in sporadic PD. Furthermore, oxidative stress linked to mitochondrial dysfunction may also increase misfolded proteins and therefore lead to aggregation of alpha-syn and subsequent death of dopaminergic neurons (Zhang et al., 2000). Thus, alpha-syn aggregation itself may also contribute to increased oxidative stress levels, leading to a vicious cycle in the cell (Hashimoto et al., 1999). Pesticides like paraquat and maneb were found to lead to proteasomal dysfunction and nitrative/oxidative damage causing an upregulation and subsequent fibrillization of recombinant alpha-syn (Norris et al., 2007; Manning-Bog et al., 2002). MPTP (previously described in section 1.1.2) creates an oxidative stress environment that enhances alpha-syn aggregation and consequent death of dopaminergic neurons (Hashimoto et al., 1999). Moreover, Dauer and colleagues (2002) investigated the toxic effect of MPTP in mice lacking SNCA gene and concluded that these animals were resistant to MPTP, giving support to alpha-syn critical role in the pathogenesis of toxin-induced dopaminergic neuron death. A recent study performed by Kalivendi et al. (2010) demonstrated that oxidant-induced alternative splicing of alpha-syn plays a crucial role in the mechanism of DA neuron cell death, contributing to PD. The authors provided evidence that the parkinsonian mimetic MPP^+ is able to induce alternative splicing of alpha-syn, leading to the deletion of exon-5, which resulted in the generation of a 112 amino acids protein, instead of the 140 amino acids WT alpha-syn. This process was deleterious to DA cells, since the expression of

the 112 amino acids form of alpha-syn exhibited a more pronounced effect on proteasomal dysfunction and enhanced cell death, when compared to WT alpha-syn.

Although alpha-syn function is not yet defined, there seems to be a reciprocal relationship between the activity of this protein and mitochondrial function. Alpha-syn contains an N-terminal mitochondrial-targeting sequence (Devi et al., 2008), and cytosol acidification or alpha-syn overexpression can lead the protein to localize to mitochondria (Cole et al., 2008; Shavali et al., 2008), resulting in complex I impairment and increased ROS production (Devi et al., 2008), increased protein tyrosine nitration and decreased mitochondrial transmembrane potential (Parihar et al., 2009). In addition, human embryonic kidney cells overexpressing alpha-syn showed enhanced susceptibility to cell death and lower ATP levels compared to control cells (Shavali et al., 2008). In brainstem neurons of mice that overexpress human A53T mutant alpha-syn, there is evidence of degenerating and dysmorphic mitochondria with DNA damage (Martin et al., 2006). Furthermore, in both SH-SY5Y neuroblastoma cells and isolated rat mitochondria, alpha-syn seems to induce mitochondrial release of cytochrome c (cyt c), increased mitochondrial calcium and NO, and oxidative modification of mitochondrial components. These findings suggest a pivotal role for mitochondria in oxidative stress and apoptosis induced by alpha-syn (Parihar et al., 2008). In a model of aging yeast, functional mitochondria are required for alpha-syn toxicity (Buttner et al., 2008).

Besides all these facts, it has been recognized that alpha-syn is one of the most abundant components of LBs; alpha-syn can aggregate under a variety of post translational modifications, namely phosphorylation, ubiquitination, C-terminal truncation and nitration (Anderson et al., 2006; Baba et al., 1998; Giasson et al., 2000; Hasegawa et al., 2002; Spillantini et al., 1997; Tofaris et al., 2003; Fujiwara et al., 2002). Indeed, alpha-syn primary structure includes several sites susceptible to undergo post-translational modifications (reviewed in Perfeito and Rego, 2008). Nevertheless, it is still controversial whether these modifications contribute to progressively conduct the protein into insoluble fibrils and modulate toxicity.

1.2.1. Alpha-synuclein phosphorylation

Protein phosphorylation is the most studied and probably the most important post translational modification, although other modifications, including ubiquitination and truncation, may also have functional consequences. Alpha-syn can be phosphorylated *in vitro* at several residues, including Ser 87 and 129 (Chen and Feany, 2005; Okochi et al., 2000; Pronin et al., 2000) and three C-terminal tyrosine (Tyr) residues (125, 133 and 136) (Ellis et al., 2001) (Figure 1.2). *In vivo* phosphorylation at Ser87, Ser129 and Tyr125 have been identified, thus making these phosphorylation sites the major focus of study. *Post-mortem* human brain studies have shown that alpha-syn is hyperphosphorylated at Ser129 in LBs (Fujiwara et al., 2002). In addition, evidence for alpha-syn phosphorylation at Ser129 has been observed in transgenic mice (Wakamatsu et al., 2007; Schell et al., 2009; Lee et al., 2011) and in primates overexpressing alpha-syn through a viral vector in the ventral midbrain (Eslamboli et al., 2007). Together, data from *post-mortem* human studies of alpha-synucleinopathies and animal models suggest that phosphorylation of alpha-syn at Ser129 is strongly linked to disease progression.

The role of phosphorylation at Ser129, presumably the dominant pathological modification occurring in human synucleinopathies (Anderson et al., 2006; Fujiwara et al., 2002), has been studied in a variety of models, but with contradictory results (Azeredo da et al., 2009; Chen and Feany, 2005; Gorbatyuk et al., 2008; McFarland et al., 2009; Sugeno et al., 2008). A mass spectrometry analysis revealed that alpha-syn is selectively and extensively phosphorylated at Ser129 in synucleinopathy lesions and that this protein modification promoted fibril formation *in vitro* (Fujiwara et al., 2002). Chen and Feany (2005) demonstrated that alpha-syn carrying a negatively charged residue aspartate at position 129 (S129D) to mimic constitutive phosphorylation enhanced toxicity in dopaminergic neurons of a transgenic *Drosophila* model, whereas a nonphosphorylated alanine residue at the same position (S129A) suppressed toxicity. In this study, the authors could not find a correlation between alpha-syn propensity to aggregate and its toxicity. Similar results were observed in SH-SY5Y cells (Sugeno et al., 2008), however in two independent viral vector-

based rat models, S129A alpha-syn variant dramatically increased alpha-syn toxicity, whereas S129D alpha-syn was less toxic than the normal protein (Gorbatyuk et al., 2008; Azeredo da et al., 2009). Another study with viral vector-mediated delivery of WT, S129A and S129D alpha-syn to the rat SN induced similar pathological changes (McFarland et al., 2009). In cell models, phosphorylation of Ser129 seems to increase the tendency of alpha-syn to form inclusions (Smith et al., 2005; Sugeno et al., 2008). However, *in vitro* analysis by biophysical methods of the structural properties of aggregating alpha-syn demonstrated that phosphorylation at Ser129 inhibits alpha-syn fibrillization, suggesting that P-Ser129 alpha-syn in LBs may not correspond to the fibrillar species that accumulate in these models or that phosphorylated alpha-syn fibrillization *in vivo* may be modulated by other factors (Paleologou et al., 2008). This study was of great importance in providing evidence that the phosphorylation mimics S129D/E do not accurately reproduce the effects of phosphorylation, so the use of these models should be carefully considered.

Alpha-syn phosphorylation is modulated by enzymes that have been of major interest, as they may constitute useful therapeutic targets. *In vitro* and *in vivo* systems have convincingly demonstrated that polo-like kinases (PLKs), particularly PLK2 and PLK3, phosphorylate alpha-syn at Ser129 (Inglis et al., 2009; Mbefo et al., 2010), although *in vitro* and cellular studies have also evidenced casein kinases (CKs) 1 and 2 (Okochi et al., 2000; Braithwaite et al., 2012) and G-protein receptor coupled kinases (GRKs) 1, 2, 5 and 6 (Pronin et al., 2000; Braithwaite et al., 2012). Phosphorylation of Tyr residues can be achieved by the Src family of protein tyrosine kinases, including c-Src and Fyn (Nakamura et al., 2001), as well as Syk (Negro et al., 2002). This redundancy in the large number of different kinases that can phosphorylate alpha-syn may be considered as a cellular mechanism to ensure that phosphorylation can be regulated at multiple steps. However, this may bring difficulties to find a therapeutic strategy as inhibition of multiple kinases may be required to reduce phosphorylation. Nevertheless, each of these kinases can provide important information on the role of alpha-syn phosphorylation in disease pathogenesis and may be manipulated for disease modification.

Protein phosphorylation is reversed by phosphatases. Despite the interest in the phosphorylation state of alpha-syn, relatively few studies have been performed to determine the specific phosphatases that dephosphorylate alpha-syn. So far, protein phosphatase 2A (PP2A) has been demonstrated to dephosphorylate alpha-syn at Ser129 (Lee et al., 2011), although other reports have suggested that it is not active on the protein (Waxman and Giasson, 2008; Lou et al., 2010). One study reported PP2C to be active at dephosphorylating alpha-syn at this site, but only of soluble, not fibrillar forms (Waxman and Giasson, 2008).

Hyperphosphorylation of key proteins appears to be a common event in multiple neurodegenerative disorders. Therefore, understanding the consequences of alpha-syn phosphorylation may help clarifying particular aspects of PD pathology and a range of related conditions. Unlike tau, for example, which phosphorylation at certain residues is critical for its normal function in binding to microtubules and regulating axonal transport (Avila et al., 2004), it is unclear whether alpha-syn phosphorylation plays a physiological role. Nevertheless, several studies have demonstrated how phosphorylation can affect alpha-syn aggregation and toxic properties, both of which seem to be determinant in PD.

1.3. Genetics in familial PD – other proteins involved

As described before, heritable cases of PD constitute early onset forms of the disorder (Lucking et al., 2000; Bonifati et al., 2003b; Valente et al., 2004a). Previous studies have shown evidence that the products of PD-associated genes have crucial effects on mitochondrial morphology, function and oxidative stress (Henchcliffe and Beal, 2008), which are further described in section 1.4.

1.3.1. Autosomal dominant forms of PD

Dominant mutations often act through a gain-of-function mechanism. Besides SNCA (described above, and which is also involved in sporadic PD, as the main component of LBs), to date, at least one more gene, LRRK2, has been shown to cause autosomal dominant PD, with a mutation frequency of 5% (reviewed in Corti et al., 2011).

1.3.1.1. Leucine-rich repeat kinase 2 (LRRK2)

Mutations in the LRRK2 represent the most common known cause of familial PD and are inherited in autosomal dominant form, but with a significant variation in penetrance. The resulting disease phenotype is identical to sporadic PD, except for a slightly lower age at diagnosis (Healy et al., 2008), which has increased the interest in this gene. The LRRK2 gene encodes a large multidomain protein with 2527 amino acids, the physiological function of which is currently unknown. However, there are numerous functional domains associated with this protein, including a leucine-rich repeat (LRR) domain, a Ras of complex protein (Roc) GTPase domain followed by its associated C-terminal of Roc (COR) domain, a kinase domain of the tyrosine kinase-like (TKL) subfamily, homologous to other mitogen-activated protein kinase kinase kinase (MAPKKK), and a C-terminal WD40 domain (Mata et al., 2006). This combination of motifs is highly conserved in vertebrates (Bosgraaf and Van Haastert, 2003), however, LRRK2 is unusual, since it encodes two distinct enzymes, kinase and GTPase in the same molecule. Initial biochemical studies on LRRK2 suggested that this protein is able to undergo phosphorylation and to phosphorylate generic substrates such as moesin and myelin basic protein (MBP) (Greggio et al., 2008; Imai et al., 2008; Jaleel et al., 2007; West et al., 2005) and that the kinase activity is mildly regulated by the GTPase domain (Guo et al., 2007; Ito et al., 2007; Burbulla and Kruger, 2011; Smith et al., 2006). The presence of multiple protein interaction domains, namely armadillo (ARM), ankyrin (ANK), LRR and WD40 suggests that in addition to its predicted protein kinase and GTPase activities, LRRK2 might work as a multiprotein signaling complex.

The single most common mutation occurring in LRRK2, G2019S, is present in the kinase domain of the protein and augments the kinase activity (West et al., 2005). This mutation is responsible for both familial (4% worldwide) and apparently sporadic (1% worldwide) PD cases, and has a very low frequency in healthy populations (<0.1% worldwide) (Di et al., 2005; Gilks et al., 2005; Healy et al., 2008; Nichols et al., 2005). In particular, most patients with the G2019S mutation exhibited alpha-syn-positive LBs as in sporadic PD (Giasson et al., 2006; Gilks et al., 2005; Ross et al., 2006). Tong and collaborators (2010), showed that accumulation and aggregation of alpha-syn and ubiquitinated proteins occurred in aged LRRK2 germ-line deletion mice with great loss of LRRK2. The autophagy-lysosomal pathway was impaired together with an increase in apoptotic cell death, inflammatory response and oxidative stress.

Although LRRK2 is mainly localized to the cytosol, approximately 10% of the protein is associated with the outer mitochondrial membrane (West et al., 2005), raising an important question of whether mutant LRRK2 kinase increased activity might directly affect mitochondrial function. Furthermore, impaired mitochondrial function and an increased susceptibility to apoptosis were observed in *ex vivo* models of fibroblasts from PD patients carrying the G2019S mutation in the LRRK2 gene or based on induced pluripotent stem (iPS) cells that carry the G2019S mutation (Mortiboys et al., 2010; Nguyen et al., 2011). In addition, dopaminergic neurons derived from G2019S-iPS cells showed an increase of key oxidative stress response genes and pathogenomic accumulation of alpha-syn (Nguyen et al., 2011).

These studies support an involvement of LRRK2 in mitochondrial dysfunction and impaired protein degradation pathways with alpha-syn accumulation and speculate on the kinase activity of the protein, which seems to be required for cellular toxicity and neuronal death.

1.3.2. Autosomal Recessive Forms of PD

Most recessive alleles result in the absence of the encoded protein or inactive protein, and consequently to a loss of function. Homozygous or heterozygous mutations in the recessive genes parkin (PARK2), PTEN-induced putative kinase 1 (PINK1) (PARK6) and DJ-1 (PARK7) are unequivocally associated with heritable, early age at onset, in most cases before age 40 and no atypical signs (Criscuolo et al., 2006; Lucking et al., 2001; Maruyama et al., 2000). Furthermore, autosomal recessive PD is characterized by: 1) slowly progressive disease course; 2) excellent response to L-DOPA, although early L-DOPA induced dyskinesias; and 3) minimal cognitive decline and dysautonomia.

1.3.2.1. Parkin

Mutations in the parkin gene at PARK2 are the most frequent known cause of early-onset (<40-50 years) PD (10-20% worldwide; 50% of recessive familial forms) (Lucking et al., 2003; Periquet et al., 2003). Pathological changes include significant loss of dopaminergic neurons in the SN and milder changes in the locus coeruleus. Initially LBs were thought to be absent in the brain (Takahashi et al., 1994), but LB-positive cases have been reported in a reduced number of patients with parkin mutations (Farrer et al., 2001).

The parkin gene encodes a protein of 465 amino acids that contains an N-terminal ubiquitin-like (UBL) domain followed by three RING finger domains (RING 0-2) and separated by an in-between-ring (IBR) domain localized at the C-terminal. Like other RING finger proteins, parkin acts as an Ub E3 ligase. It has an important role in the function of Ub, which is primarily involved in the targeting of aggregation-prone substrates for degradation by the UPS, by conferring substrate specificity (Moore, 2006). Many ubiquitination substrates have been proposed for parkin, including the aminoacyl-tRNA synthetase cofactor, p38, and a rare 22 kDa glycosylated form of alpha-syn (Shimura et al., 2001; Corti et al., 2003; von et al., 2004). Parkin is predominantly a cytosolic protein, but also co-localizes to synaptic vesicles, the Golgi complex, endoplasmic reticulum and the

mitochondrial outer membrane (Shimura et al., 2000; von et al., 2004; Kubo et al., 2001; Mouatt-Prigent et al., 2004; Darios et al., 2003). Many PD-linked point mutations alter WT parkin cellular localization, solubility or propensity to aggregate (Cookson et al., 2003; Gu et al., 2003; Wang et al., 2005). Other mutations, including insertions or deletions, lead to parkin loss-of-function, specifically loss of its ligase activity, resulting in accumulation of toxic substrates and degeneration (Moore, 2006). However, the most possibly relevant activity of parkin is related to its neuroprotective function against a variety of pathogenic factors. Particularly, this neuroprotection is given by delaying mitochondrial swelling and rupture and the subsequent cyt c release and caspase 3 activation, as shown in cells overexpressing parkin (Darios et al., 2003). As mentioned above, parkin can associate to mitochondrial outer membrane, suggesting a direct and local protective effect (Kuroda et al., 2006). The protein is involved in the regulation of transcription and replication of mtDNA in proliferating cells, stimulating this organelle's biogenesis – an effect blocked by parkin short-interfering RNA (siRNA) knockdown (Kuroda et al., 2006). Furthermore, in a knockout mouse line for parkin, reduced number of mitochondrial oxidative phosphorylation proteins, decreased mitochondrial respiratory capacity, age-dependent increased oxidative damage and increased protein and lipid peroxidation were reported (Palacino et al., 2004), leading to nigrostriatal damage. Changes in mitochondrial morphology have been also observed, but this led only to disruption of complex I function in nigral mitochondria and did not result in cell death (Stichel et al., 2007). Thus, mitochondrial defects and an increased susceptibility to oxygen radical damage were also reported in a parkin knockout model of *Drosophila*, suggesting that abnormalities in parkin ubiquitination function might be secondary in the course of pathogenic events (Greene et al., 2003; Pesah et al., 2004). *In vitro* studies of PARK2-knockdown SH-SY5Y neuroblastoma cell line showed apoptotic cell death and high levels of autoxidized forms of L-DOPA and DA, suggesting that parkin might have important antioxidant properties (Machida et al., 2005). Rothfuss and co-workers (2009) confirmed that the association between parkin and mtDNA led to protection from oxidative stress and stimulation of mitochondrial genome

repair. These functions were damaged in parkin-deleted human fibroblasts (Rothfuss et al., 2009).

Overall, functional parkin seems to be crucial in the maintenance of mitochondrial antioxidant defenses and protection of mtDNA.

1.3.2.2. PTEN-induced putative kinase 1 (PINK1)

PINK1 gene (PARK6) mutations are the second most common cause of autosomal recessive, early-onset parkinsonism (Valente et al., 2004a). Although age at onset for PINK1-related PD is usually in the fourth to fifth decade, clinical features are similar to late onset PD, with slow progression, excellent response to L-DOPA, and in some cases, dementia (Valente et al., 2001; Bonifati et al., 2005; Tan et al., 2006; Hatano et al., 2004). Nigrostriatal neuronal loss has been observed in *post-mortem* brains of PD patients with PINK1 mutations (Gandhi et al., 2006).

PINK1 is a putative serine/threonine kinase, constituted by 581 amino acids, which is expressed ubiquitously. Until present, homozygous missense and nonsense mutations affecting the kinase domain, as well as insertions, deletions and truncation of the protein, were observed, all of them supposedly impairing the kinase activity (Exner et al., 2007; Atsumi et al., 2006).

Due to its N-terminal mitochondrial targeting sequence, PINK1 is localized to the mitochondrial intermembrane space and bound to mitochondrial membranes. However, as PINK1 was also identified as a component of LBs in patients with sporadic PD (Gandhi et al., 2006), its final destination within mitochondria is not entirely defined.

Physiological role of PINK1 in mitochondria is thought to include the phosphorylation of mitochondrial proteins in response to cellular stress and the protection against mitochondrial dysfunction (Burbulla and Kruger, 2011). There is evidence that WT PINK1 in contrast to mutant PINK1 may protect neurons from stress-induced mitochondrial dysfunction and apoptosis (Valente et al., 2004b). Furthermore, loss of PINK1 function in

human cell lines originated morphological changes of mitochondria and impaired energy metabolism, showed by a decreased mitochondrial membrane potential (Exner et al., 2007). Cells isolated from individuals with a PINK1 mutation that causes a G309D substitution demonstrate reduced complex I activity and evidence of increased oxidative damage compared with cells from age matched controls (Hoepken et al., 2007). Furthermore, PINK1 deficiency in a *Drosophila* model results in the loss of dopaminergic cells, as well as increased susceptibility to oxidative stress and reduced ATP levels (Clark et al., 2006). These mutants also showed reduced mitochondrial mass with disorganized morphology. Moreover, PINK1 seems to exert neuroprotective properties, as shown by Petit et al. (2005). These authors showed that WT, but not mutant, PINK1 attenuated staurosporine-induced apoptosis and reduced mitochondrial cyt c release when overexpressed in SH-SY5Y cells. Also, silencing of PINK1 increased susceptibility to MPP^+ or rotenone (Petit et al., 2005). In this context, decreased expression of PINK1 in human dopaminergic neurons led to reduced long-term survival, along with morphological/structural mitochondrial abnormalities and higher levels of oxidative stress (Wood-Kaczmar et al., 2008).

1.3.2.3. DJ-1

Mutations in DJ-1 gene, also known as PARK7, are the least common cause of autosomal recessive PD (approximately 1% of early-onset PD). The DJ-1-related phenotype, with early-onset and slow disease progression, closely matches that of patients with parkin or PINK1 mutations, but genotype-phenotype correlations could not be precisely performed, due to the small number of DJ-1 patients (Bonifati et al., 2003b). DJ-1 gene encodes a protein of 189 amino acids, belonging to the peptidase C56 family of proteins (Moore et al., 2003). It is a cytosolic protein, but under stress conditions can also translocate to the mitochondria and nucleus (Zhang et al., 2005; Bonifati et al., 2003b). WT DJ-1 appears to exert an antioxidant function (Nagakubo et al., 1997; Abou-Sleiman et al., 2003; Canet-Aviles et al.,

2004; Moore et al., 2005), while deletions and point mutations in the DJ-1 gene cause a loss of its physiological function and therefore, lead to neurodegeneration in rare families (Bonifati et al., 2003b; Bonifati et al., 2003a; Taira et al., 2004; Hering et al., 2004). Knockdown of DJ-1 by siRNA in SH-SY5Y human neuroblastoma cell line, enhanced susceptibility to several oxidative insults, including H_2O_2, MPP^+ and 6-OHDA (Taira et al., 2004). Reciprocally, DJ-1 overexpression in these cells resulted in increased resistance to these insults and reduced intracellular ROS formation (Lev et al., 2008). This protection seems to be selective against environmental oxidative stress *in vivo*, as shown in DJ-1 null *Drosophila* treated with paraquat and rotenone (Meulener et al., 2005). Thus, DJ-1β-deficient flies displayed a locomotor deficit that was exacerbated by oxidative stress (Park et al., 2005). Furthermore, the levels of DJ-1 modification increase with age, also leading to significant increments in oxidative stress and inactivation of its own function (Meulener et al., 2006). DJ-1-deficient mice demonstrated hypersensitivity to MPTP and this was observed by increased dopaminergic neuronal loss and striatal denervation (Kim et al., 2005). In embryonic cortical neurons, an increased sensitivity to oxidative stress and proteasomal inhibition has been also demonstrated, ending in apoptotic cell death (Kim et al., 2005; Martinat et al., 2004). All impairments were reversed by restitution of DJ-1 expression. DJ-1 is normally activated by an oxidative cytoplasmic environment (Shendelman et al., 2004). These authors described a role for DJ-1 as a redox-sensitive molecular chaperone, which was able to inhibit alpha-syn aggregate formation. Another study in DJ-1 deficient mice showed that the complex I inhibitor paraquat decreased proteasome activity concomitantly with decreased ATP and regulatory subunit levels (Yang et al., 2007). In addition to these effects, the levels of Nrf2, which activates cytoprotective genes, were reduced. This provided evidence for a role of DJ-1 as a regulator of transcription. The mechanism by which DJ-1 protects against oxidative stress was described by Im et al. (2010). In this study, the authors demonstrated that DJ-1 regulates the mitogen-activated protein 3 (MAP3) kinase apoptosis signaling-regulating kinase 1 (ASK1)/ thioredoxin (Trx) 1 complex. ASK1 is a major effector of cell death induced by oxidative

stress and is physiologically inhibited by Trx1. Oxidative insults disrupt this complex, and DJ-1 null cells are more susceptible to this dissociation, leading to increased activation of downstream cell death mediators.

A mitochondria-DJ-1 association was also established. Lev et al. (2008) described a cellular redistribution of DJ-1 in cells exposed to neurotoxins. This work was extended by Hayashi et al. (2009), who showed DJ-1 binding to NADH dehydrogenase (ubiquinone) 1 α-subcomplex 4 (NDUFA4) and to mitochondrial encoded NADH dehydrogenase 1 (ND1), nuclear and mitochondrial DNA-encoding subunits of complex I, respectively, validating the importance of DJ-1 in mitochondrial function. Recently, a direct link between loss of DJ-1, impaired mitochondrial stress response and reduced clearance of mitochondria by lysosomal degradation was described (Krebiehl et al., 2010). An accumulation of fragmented and dysfunctional mitochondria upon reduced basal autophagy contributed to the loss of function phenotype in cells from DJ-1 knockout mouse and human carriers of the E64D mutation in DJ-1 gene (Krebiehl et al., 2010).

In summary, PD individuals with autosomal recessive inheritance differ generally from those with idiopathic PD, although cases with clinical evolution similar to that of the typical disease have been reported. A raising body of recent information provides strong support to the idea that mitochondrial dysfunction may be central to the pathophysiology of familial PD, particularly to that of parkinsonian syndromes with autosomal recessive inheritance that directly associate with mitochondria, such as parkin, PINK1 and DJ-1.

Table 1.1 briefly summarizes the genes described in sections 1.2 and 1.3, which are predominantly involved in sporadic and inherited forms of PD.

Table 1.1. – Summary of the major genes involved in sporadic and inherited forms of PD.

PARK loci	Gene	Map position	Forms of PD	Mutations
PARK1/PARK4	SNCA	4q21	Early-onset PD Autosomal dominant Sporadic	A30P, E46K, A53T Genomic duplications/triplications
PARK8	LRRK2	12q12	Late-onset PD Autosomal dominant Sporadic	7 pathogenic mutations, including the common G2019S
PARK2	Parkin	6q25-q27	Juvenile and early-onset PD Autosomal recessive Sporadic	100 mutations (point mutations, exonic rearrangements)
PARK6	PINK1	1p35-36	Autosomal recessive PD	40 point mutations, rare large deletions
PARK7	DJ-1	1p36	Early-onset PD Autosomal recessive	10 mutations and large deletions

1.4. Mechanisms of PD pathology

Despite the efforts and advances made over the years, the pathogenesis of idiopathic PD still remains unclear. Nevertheless, it is safe to assign a major role for oxidative stress and mitochondrial dysfunction, at least in the neurodegeneration of dopaminergic neurons.

Investigations into *post-mortem* PD brains, particularly, in the SN, have consistently demonstrated abnormalities in mitochondrial function and increased levels of oxidative stress (Schapira, 2009; Schapira et al., 1990; Dexter et al., 1992; Owen et al., 1996; Mann et al., 1992b; Schapira, 1995; Gu et al., 1998; Schapira et al., 1989). Furthermore, there is

evidence of inflammation through microglia activation in the SN. The finding that alpha-syn is the major component of LBs directed studies on protein metabolism and defects in protein degradation through the UPS and autophagy pathways as contributory factors to PD pathogenesis. These cellular pathways are interconnected, since mitochondrial dysfunction, namely complex I inhibition, leads to increased free radical generation, which further evokes deficits of the respiratory chain. Importantly, the UPS is dependent on oxidative phosphorylation for energy production and oxidatively damaged proteins increase the bulk of substrates to be degraded by the UPS. Moreover, this leads to increased cell dysfunction and a lowered threshold to apoptosis (Schapira, 2011), a type of programmed cell death characterized by membrane blebbing, shrinking of organelles, and chromatin condensation and fragmentation (e.g. Rego and Oliveira, 2003).

1.4.1. Mitochondrial dysfunction

Mitochondria are the main producers of reactive oxygen species (ROS) and therefore may play a central role in increased oxidative stress in neurodegenerative diseases. It has been shown that during aging, deletions in mitochondrial DNA (mtDNA) accumulate and lead to a decline in mitochondrial function and a subsequent increase in ROS production (Lin and Beal, 2006). Free radicals may thus be generated by affected neurons as a result of impaired electron transport chain during mitochondrial respiration (Andersen, 2004).

The direct relationship between mitochondrial dysfunction and PD came with the description of complex I deficiency in the SN of patients who had died with PD (37, 38 de Schapira 2011), and was followed by subsequent reports of mitochondrial defects in skeletal muscle and platelets of PD patients (Mann et al., 1992a). In the brain, mitochondrial deficiency appeared to be confined to the SN (Schapira et al., 1990). These mitochondrial abnormalities, identified in pathologically confirmed, apparently sporadic PD, were seen under a background of increased oxidative stress and elevated iron levels,

emphasizing the importance of interconnecting pathways (Dexter et al., 1992; Mann et al., 1994; Gu et al., 1998; Schapira, 2007).

Additional support for the involvement of mitochondria in PD pathogenesis came with the identification of genetic causes responsible for inherited forms of PD, since multiple genes in which mutations or polymorphisms increase the risk of PD, are linked to mitochondrial function (Navarro and Boveris, 2009).

Moreover, mitochondrial toxins were described to induce a phenotype similar to PD (Betarbet et al., 2000). Complex I impairment seems to be important for the pathogenesis of PD since exposure to inhibitors of this complex, such as MPP^+ or rotenone, as described before in this chapter, reproduces the clinical symptoms of PD observed in human subjects (Bougria et al., 1995; Gomez et al., 2007). Indeed, MPP^+ generates free radicals, including nitric oxide (Hantraye et al., 1996) that can in turn inhibit mitochondrial function (Cleeter et al., 1994). As stated above, rotenone is commonly used in the United States as a pesticide. Treatment of rodents with low doses of rotenone over 1 month induced nigrostriatal cell death and Lewy-like inclusions (Betarbet et al., 2000). Both rotenone and MPP^+ lead to dopaminergic cell death through induction of apoptosis, acting on the mitochondrial transition pore (Seaton et al., 1998).

Complex I is the largest of the electron transport chain (ETC) complexes, consisting of 46 subunits, 7 of which are encoded by mtDNA (Henchcliffe and Beal, 2008) and the major site of superoxide production in the ETC (Kudin et al., 2004). Complex I activity and expression are decreased in the SN (Janetzky et al., 1994; Mann et al., 1992a; Schapira et al., 1990; Schapira et al., 1989) and cortex (Navarro et al., 2009) of PD patients to a greater extent than would be expected from normal aging (Henchcliffe and Beal, 2008). Oxidized, functionally impaired and misassembled complex I subunits, have also been reported in PD (Keeney et al., 2006).

1.4.2. Oxidative stress

Oxidative stress and the production of ROS are involved in the pathogenesis of several chronic neurodegenerative diseases, including PD. Taking into account that age is a great risk factor for these neurodegenerative disorders and the fact that ROS increase with age in the brain, there is a strong support for the involvement of ROS in neurodegeneration (Mariani et al., 2005). Indeed, the free radical theory of aging hypothesizes that the aging process is associated with an increase in the generation of ROS together with a decrease in the defense capacities against oxidative stress, thus leading to the accumulation of ROS-modified macromolecules (Perry et al., 1998; de Vries et al., 2008).

Analysis of biochemical markers of oxidative damage in tissue samples from patients or in *post-mortem* brains provided direct evidence for oxidative stress in PD. Increased levels of lipid peroxidation were found in the SN, as suggested by decreased levels of polyunsaturated fatty acids (substrates for lipid peroxidation) and increased levels of malondialdehyde (MDA) (Dexter et al., 1989a) and 4-hydroxynonenal (HNE)(Yoritaka et al., 1996). Nucleic acid oxidation product 8-hydroxyguanosine (8OHG) is also elevated in the neurons affected in the disease, compared to surrounding brain regions in the PD brain, as well as in comparison to the SN of age-matched controls (Alam et al., 1997b; Zhang et al., 1999). Lipid and DNA oxidation were also found to be systemically elevated in PD (Seet et al., 2010). Oxidative and nitrative post translational modifications have been identified on proteins that may affect the disease progression (Danielson and Andersen, 2008). This includes increased levels of oxidized protein carbonyls in the SN of PD patients (Alam et al., 1997a; Floor and Wetzel, 1998) and nitration of tyrosine residues within LBs in the PD brain (Good et al., 1998; Duda et al., 2000a). Increased 3-nitrotyrosine (3-NT) levels have been also detected following systemic administration of the PD-inducing toxin MPTP in baboons (Ferrante et al., 1999) and mice (Pennathur et al., 1999). Along with the increase in oxidative damage, decreased levels of the antioxidant glutathione (GSH) (described below) were found in the SN of PD patients (Perry et al., 1982; Perry and Yong, 1986; Pearce et al., 1997).

SN is also particularly vulnerable to toxic effects caused by nitric oxide (NO), leading to neurodegeneration of dopaminergic and catecholaminergic cells, probably due to the presence of high concentrations of 6-hydroxydopamine (6-OHDA) in this structure, which readily reacts with NO generating DA semiquinones and increasing intramitochondrial and cytosolic peroxynitrite ($ONOO^-$) formation (Navarro and Boveris, 2009). Indeed, NO is known to readily react with superoxide anions ($O_2^{\bullet -}$) to form the highly toxic $ONOO^-$, which induces cellular damage through protein nitration, lipid peroxidation and DNA fragmentation (Szabo et al., 2007). Apart from the recognized $^{\cdot}NO$ control of complex IV activity and cell respiration, both $^{\cdot}NO$ and $ONOO^-$ have been reported as direct inhibitors of complex I, probably due to S-nitrosylation and Fe-nitrosation of complex I subunits (Brown and Borutaite, 2004). Tyrosine nitration, protein oxidation and damage to iron sulfur centers with sustained complex I inhibition are associated with increased generation of $O_2^{\cdot -}$ by complex I (Navarro and Boveris, 2009).

1.4.2.1. Dopamine

Even though neuronal alterations observed in PD are not restricted to dopaminergic neurons, these neurons of the nigrostriatal pathway are the major site of neuronal degeneration in PD (Sulzer, 2007b). Selective degeneration of the dopaminergic neurons of the SN suggests the possibility that DA itself may be contributing to the neurodegenerative process in PD (Hastings, 2009). Moreover, the loss of dopaminergic neurons in the SN, leading to the degeneration of the nigrostriatal pathway and resulting in the marked reduction in striatal DA, appears to be crucial for the development of the clinical features of the disease, as demonstrated by the remarkable response of most of the motor symptoms to the DA precursor L-DOPA (DA replacement therapy). Thus, DA may play a role in the selective degeneration of this brain area in PD. Under normal conditions, DA is synthesized from tyrosine by tyrosine hydroxylase (TH), the rate-limiting enzyme in DA biosynthesis, and aromatic amino acid decarboxylase. Once formed, DA is safely stored in high millimolar

concentrations in synaptic vesicles following uptake by VMAT2 (Staal et al., 2004). Impairment of vesicular storage of DA, which may be due to the presence of alpha-syn protofibrils, oxidative stress or weak base compounds such as methamphetamine (Caudle et al., 2008), leads to increased DA levels in the cytoplasm. Concordantly, alpha-syn overexpression was shown to increase cytosolic catecholamine concentration (Mosharov et al., 2006). MPP^+ interaction with VMAT and redistribution of vesicular DA to the cytosol also causes intracellular DA oxidation (Lotharius and O'Malley, 2001). Under conditions of oxidative stress, DA has the potential to form reactive metabolites by enzymatic and non-enzymatic mechanisms that may further contribute to mitochondrial dysfunction and oxidative damage, accelerating dopaminergic cell death in PD. DA may be metabolized intracellularly by monoamine oxidase (MAO), a mitochondrial enzyme present in the cytoplasmic side of the outer mitochondrial membrane and aldehyde dehydrogenase, producing 3,4-dihydroxyphenylacetic acid (DOPAC) and hydrogen peroxide (H_2O_2). MAO exists in two isoforms, MAO-A and MAO-B, which have different substrate specificity. Both isoforms oxidize DA, but in humans DA is preferentially oxidized by MAO-B, whereas in rodents it is predominantly oxidized by MAO-A (Shih et al., 1999).

In addition, autoxidation of DA originates the electron-deficient DA quinones or semiquinones, which is facilitated by the presence of transition metal ions (Hastings, 2009). DA quinones readily react with cellular nucleophiles, such as the reduced sulfhydryl group on protein cysteinyl residues, and covalently modify protein structure (Hastings, 2009). These cysteinyl residues are often at the active site of proteins and, thus, covalent modification by DA quinones often results in inactivation of protein function, which may result in compromised cell survival. This mechanism may play a role in the degenerative process in PD (Stokes et al., 1999). DA was found to modify proteins associated with the genetic forms of PD, such as alpha-syn (Mazzulli et al., 2006), parkin (LaVoie et al., 2005), LRKK2 or UCH-L1 (Van Laar et al., 2009). Other proteins that are modified by DA oxidation include TH (Kuhn et al., 1999), superoxide dismutase 2 (SOD2), mitochondrial creatine kinase, mitofilin and mitochondrial heat-shock protein 70 (Van Laar et al., 2009). Alpha-syn

was shown to interact with DA-quinones stabilizing alpha-syn protofibrils (Conway et al., 2001) and promoting alpha-syn aggregation; interestingly, this process may also prevent DA-quinones from producing greater oxidative damage to dopaminergic neurons (Mauceli et al., 2006). Excess alpha-syn also seems to potentiate the production of ROS by endogenous DA and cell death, involving the formation of soluble alpha-syn-14-3-3 protein complexes (Xu et al., 2002). Moreover, direct binding and functional coupling of alpha-syn to DAT (responsible for DA re-uptake from the synaptic cleft) was reported to accelerate DA uptake, increasing DA levels in the cytosol and DA-induced apoptosis (Lee et al., 2001).

1.4.2.2. Iron deregulation

An additional pathological feature of PD is the abnormal accumulation of iron in the affected neurons. Iron plays a vital role in several physiological functions including DNA synthesis, mitochondrial respiration and oxygen transport. In the central nervous system (CNS), iron is involved in myelination and neurotransmission and is the most abundant metal in the brain. Iron acts as a cofactor for TH, the rate-limiting enzyme in DA synthesis, as demonstrated by *in vitro* studies showing that TH activity is stimulated by iron in a dose dependent manner (Rausch et al., 1988). While iron is physiologically important in these processes, in excess, this metal can be toxic through oxidative stress. Increased levels of iron have been found in the SN of PD patients (Dexter et al., 1987; Sofic et al., 1988; Sian-Hulsmann et al., 2011) and are implicated in numerous neurological disorders with parkinsonian symptoms (Gerlach et al., 2006). It is not clear whether it is a cause or develops secondarily to neuronal degeneration (Kaur and Andersen, 2004). However, infusion of ferric iron into the SN can be used to create models of dose-related, progressive parkinsonism, including a reduction in dopaminergic activity (Sengstock et al., 1993). This can be attenuated by treatment with lazaroid U-74389G, which is an inhibitor of lipid membrane peroxidation (Wesemann et al., 1995), suggesting that iron may play a causative

role in neuronal death through oxidative stress and lipid peroxidation. Furthermore, chronic exposure (more than 20 years) to iron and other metals leads to an increase in the risk of developing PD (Gorell et al., 1997), corroborating the hypothesis that excess iron may, at least in part, contribute to PD pathogenesis.

Increased iron content is caused by several factors, including a disturbed blood-brain barrier (BBB), occupational exposure (Gorell et al., 1997) and disruption of the body's iron storage and transport mechanisms. Iron distribution and storage is tightly regulated in the body since iron homeostasis deregulation (deficiency or overload) can have deleterious effects to the organism. This complex equilibrium is maintained by differential expression of proteins that regulate its cellular uptake, utilization and storage.

The access of iron to cells is controlled primarily by transferrin receptors; it is further stored by the protein ferritin and neuromelanin, a by-product of DA oxidation and an excellent binder of metal ions, especially iron (reviewed in Berg et al., 2001) in a process that generates ROS (Zecca et al., 2001). Iron binds to transferrin after carefully controlled absorption from the duodenum and circulates in the blood. It is taken into cells via transferrin receptors and stored in metalloproteins. Excess iron is stored as ferritin, which levels are crucial, since iron is relatively nontoxic when bound to ferritin. The second important storage protein, neuromelanin, is closely related to nigral neurons, which are the most affected neurons in PD (Hirsch, 1992; Sulzer, 2007b) and produce this dark pigment. The presence of high amounts of iron in PD SN (Dexter et al., 1989b), which contribute to the production of free radicals via the Fenton-Haber Weiss reaction, has also been pointed out as a cause for selective degeneration of these neurons. In this reaction, H_2O_2 generated during normal metabolism, through electron transport in mitochondria, or by pathogenic mechanisms, can be subsequently converted to the extremely toxic $^{\bullet}OH$, which may elicit cellular damage, lipid peroxidation and eventually apoptosis (Gutteridge, 1994). Thus, elevated levels of iron observed in the SN of PD patients may reflect a dysfunction of brain iron homeostasis. Alternatively, the excess in iron content may also be due to brain iron deposition associated with aging (Sian-Hulsmann et al., 2011).

As a sum up, the combination of mitochondrial dysfunction, oxidative stress and DA oxidation likely increases the vulnerability of dopaminergic neurons to degeneration in PD (reviewed in Greenamyre and Hastings, 2004) (Figure 1.3). DA oxidation contributes to additional factors implicated in PD, such as protein aggregation, endoplasmic reticulum stress and lysosomal dysfunction (Hastings, 2009). Moreover, oxidative damage, for which iron is also implicated, is known to impair ubiquitination and degradation of proteins by the proteasome (Jenner, 2003), thus boosting the accumulation of protein aggregates.

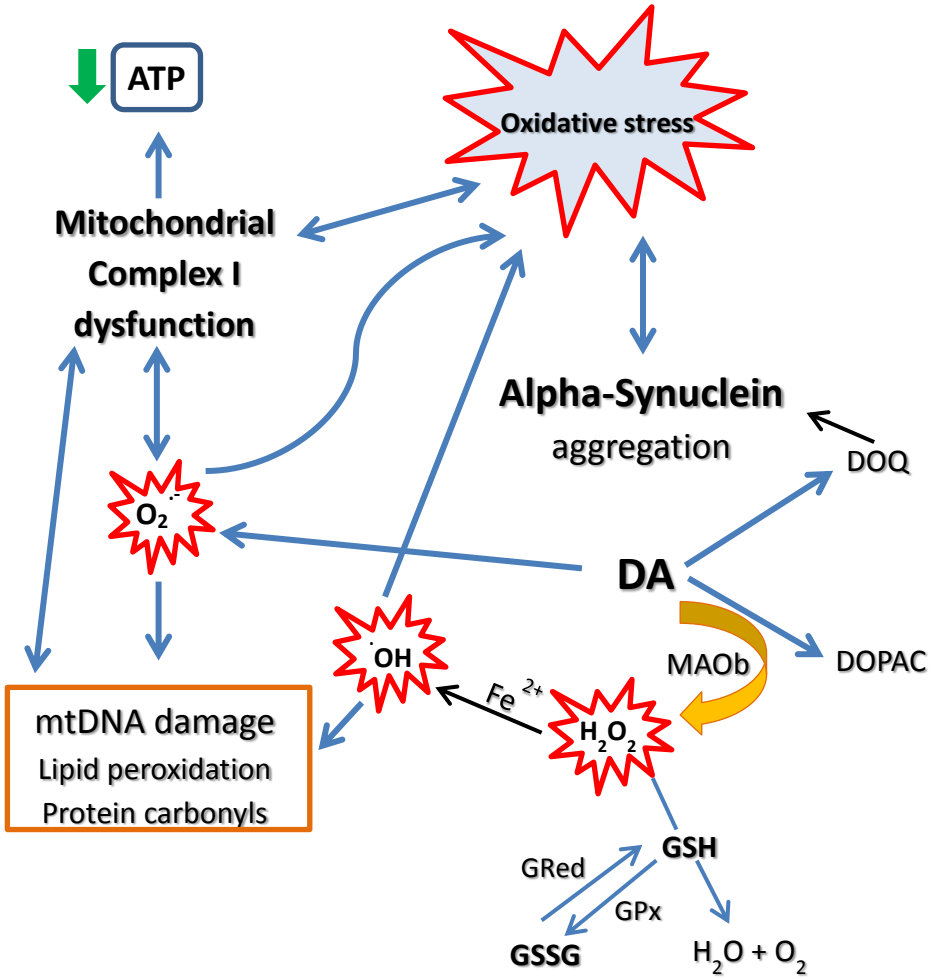

Figure 1.3. – Major sources of oxidative stress within dopaminergic neurons. Abbreviations: ATP – adenosine triphosphate; DA – dopamine; DOPAC – dihydroxyphenylacetic acid; DOQ – dopamine quinone; Fe^{2+} - ferrous iron; GRed – glutathione reductase; GPx – glutathione peroxidase;

GSH – reduced glutathione; GSSG – oxidized glutathione; H_2O_2 – hydrogen peroxide; MAOb – monoamine oxygenase B; $O_2^{\cdot-}$ - superoxide anion; $^{\cdot}OH$ – hydroxyl radical; mtDNA – mitochondrial DNA. (Adapted from Clark and Simon, 2009.)

1.5. Superoxide dismutases and glutathione-related antioxidants – two main cellular antioxidants

To ameliorate and deal with injury caused by oxidative damage and maintain redox homeostasis, aerobic organisms developed efficient defense systems, including enzymatic and nonenzymatic antioxidants. Here, we describe two main cellular antioxidants, where we focused part of the work presented in this thesis.

1.5.1. Superoxide dismutases

The superoxide dismutase (SOD) family is specialized in eliminating $O_2^{\cdot-}$ radicals derived from extracellular stimuli, including ionizing radiation and oxidative insults, together with those primarily produced within the mitochondrial matrix as by-products of oxygen metabolism through the electron transport chain (McCord and Fridovich, 1969; Miao and St Clair, 2009). SODs are very efficient in catalyzing the dismutation of $O_2^{\cdot-}$ radical through a redox reaction into molecular oxygen (O_2) and H_2O_2. Three distinct isoforms of SOD have been identified and characterized in mammals: copper-zinc superoxide dismutase (Cu/ZnSOD, encoded by the sod1 gene), manganese superoxide dismutase (MnSOD, encoded by the sod2 gene) and extracellular superoxide dismutase (ECSOD, encoded by the sod3 gene). Although with similar functions, the three SODs hold different characteristics on their protein structure, chromosome localization, gene distribution, metal cofactor requirements and cellular compartmentalization that distinguish them from one another (reviewed in Parge et al., 1992).

Cu/ZnSOD or SOD1 was the first SOD characterized in eukaryotes and it has been found in cytoplasm, nucleus, lysosomes, peroxisomes and also in the mitochondrial intermembrane space (Okado-Matsumoto and Fridovich, 2001). Mammalian SOD1 is highly expressed in the liver and in the kidney (Asayama and Burr, 1985; Culotta et al., 2006) and is also abundant in motor neurons (Pardo et al., 1995). Knockout studies indicate that elimination of SOD1 gene in rodents is associated with vulnerability to motor neuron loss after axonal injury (Reaume et al., 1996) and decrease in life span (Elchuri et al., 2005), among many other consequences. However, the most widely studied connection between SOD1 and neurological diseases has been established in amyotrophic lateral sclerosis (ALS). Indeed, SOD1 mutations have been associated to inherited ALS, a fatal, adult-onset, neurodegenerative disease primarily affecting motor neurons in the brain, brain stem and spinal cord (Orrell et al., 1995).

Among the three SOD isoforms, MnSOD or SOD2 is the only one that has proven to be essential for the survival of aerobic organisms (Carlioz and Touati, 1986; Miao and St Clair, 2009). SOD2 is found in the matrix of mitochondria only (Weisiger and Fridovich, 1973; Holley et al., 2010). The physiological role of MnSOD as a cytoprotective enzyme has been clearly confirmed by the extremely short life span of MnSOD-knockout mice, which die shortly after birth with dilated cardiomyopathy, neurodegeneration, metabolic acidosis and lipid accumulation in both skeletal muscle and liver (Li et al., 1995). Decreased expression of MnSOD in *Drosophila* causes an increase in oxidative DNA damage in the brain, as well as increased neuronal death and decreased olfactory capacity (Paul et al., 2007). MnSOD knockout mice also presented death of basal ganglia and brain stem neurons, as well as severe anemia (Lebovitz et al., 1996). Overexpression of Cu/ZnSOD does not rescue MnSOD knockout mice from early death caused by a lack of this enzyme, supporting the importance of mitochondrial ROS scavenging in aerobic organisms (Copin et al., 2000). Heterozygous MnSOD knock-out mice have an approximately 50% reduction in this enzyme activity in all tissues tested and presented a substantial decrease in mitochondrial membrane potential in isolated mitochondria from liver, compared to the control groups at all ages tested

(young, middle-aged and old mice). Furthermore, these animals showed an age-dependent increase in oxidative DNA damage in both nuclear and mitochondrial DNA (Van et al., 2003).

A distinct SOD activity is observed in the circulatory system of many mammals. This activity arises from a secreted copper and zinc containing enzyme encoded by the human sod3 gene that is related to the dimeric Cu/Zn family. ECSOD or SOD3 is typically produced in cells of the vascular smooth muscle and secreted into the extracellular environment, where it binds to extracellular matrix and endothelial surface components (Stralin et al., 1995). SOD3 functions include prevention of superoxide-dependent inactivation of endothelial cell enzymes and products, including NO (Culotta et al., 2006).

Very few studies directed their investigation on SOD enzymes and PD. Navarro et al. (2009) analyzed several mitochondrial parameters in frontal cortex samples from frozen human brains of patients with PD and dementia with LBs (DLB). The authors verified that mitochondrial O_2 uptake and complex I activity were significantly lower in PD and DLB, whereas cytochrome c content, oxidative damage and expression of MnSOD were significantly higher in the frontal cortex of these patients. Baillet and colleagues (2010) examined oxidative stress markers in patients with PD and concluded that there was an increase in Cu/ZnSOD activity compared to the healthy controls.

1.5.2. Glutathione redox cycle and synthesis

Cells have many antioxidant mechanisms to counteract the deleterious effects of ROS. L-γ-glutamyl-L-cysteinylglycine (GSH, glutathione) is a major component of cellular antioxidant defenses against exogenous and endogenous toxins, including ROS and reactive nitrogen species (RNS) (Garrido et al., 2011).

Radical species can be removed via nonenzymatic reduction with GSH, whereas the removal of hydroperoxides requires enzymatic catalysis by glutathione peroxidase (GPx) (Dickinson and Forman, 2002; Zeevalk et al., 2008; Dringen, 2000; Griffith, 1999). Both reactions lead to the production of glutathione disulfide (GSSG, or oxidized glutathione), which is reduced back to GSH by glutathione reductase (GRed) that uses NADPH from the pentose phosphate shunt (Dickinson and Forman, 2002). Conjugation of GSH with electrophilic compounds mediated by glutathione-S-transferases (GSTs) and subsequent excretion of these conjugates from the cell also constitute a protective mechanism against toxins (Dickinson and Forman, 2002; Dringen, 2000; Griffith, 1999).

When the redox state of a cell is modified, increased GSH utilization occurs and while the generated GSSG can be reduced back to GSH, the formation and export of GSH conjugates leads to GSH depletion. This depletion may then be attenuated by *de novo* synthesis of GSH (Dickinson and Forman, 2002; Griffith, 1999). GSH synthesis in neurons and in the supporting glial cells is a 2-step ATP-dependent process (Meister and Anderson, 1983; Lu, 2009). The first of these steps forming L-γ-glutamyl-L-cysteine from glutamate and cysteine, is rate limiting, being the supply of cysteine the critical factor. This step is also influenced by GSH levels, as GCL, the enzyme catalyzing this reaction, is inhibited by GSH in a negative feedback manner. The second step, the addition of glycine to generate GSH is catalyzed by glutathione synthase (GS) (reviewed in Lu, 2009). Astrocytes synthesize the vast majority of brain GSH and secrete the peptide, but neurons can take up only the precursors derived from extracellular catabolism and must synthesize their own GSH (reviewed in Martin and Teismann, 2009).

Glutathione functions to maintain the cellular redox equilibrium, which can be expressed as GSSG:2GSH (Schafer and Buettner, 2001). Oxidative stress results when this redox balance is destabilized in favor of GSSG, which can be due to a decrease in the reducing capacity of the cell or an increase in the reduction potential (Genestra, 2007).

GSH deficiency is a very early finding in the SN of PD patients (Dexter et al., 1994). This depletion in GSH observed in PD may result from a decrease in synthesis and/or recycling

under normal redox conditions. To determine why this occurs, it is necessary to evaluate the enzymes and substrates involved in GSH synthesis and metabolism. If changes in GSH synthesis are the main reason for GSH depletion observed in PD, one would anticipate that the activity of the rate-limiting enzyme in GSH synthesis, GCL, would be decreased. GCL is a dimeric protein composed of a catalytic (GCLc) and a modulatory (GCLm) subunit. The modulatory subunit increases the affinity of the catalytic subunit for its substrate glutamate, and renders the holoenzyme less sensitive to feedback inhibition by GSH (Garrido et al., 2011). It has been reported that GCL levels are reduced throughout the brain as a consequence of the aging process and this reduction was attributed to a decrease in the levels of the modulatory subunit of GCL (Sian et al., 1994b). Diminishment of GCL activity was observed in immortalized N27 mesencephalic dopaminergic cells after administration of the parkinsonian toxin MPP^+ (Drechsel et al., 2007). However, activity of GCL was unaffected in *post-mortem* brain samples from PD patients (Sian et al., 1994b). In addition, circulating levels of cysteine, which is the critical amino acid for GSH synthesis, have been described to be decreased with age (Droge and Schipper, 2007), which can also contribute to GSH depletion observed in PD. Excitatory amino acid carrier 1 (EAAC1), which is a glutamate transporter expressed on mature neurons in the CNS, constitutes the primary route for uptake of the neuronal cysteine needed to produce GSH (reviewed in Maragakis and Rothstein, 2004). Aoyama et al. (2008) reported that MPTP-treated mice showed decreased GSH levels, EAAC1 translocation to the membrane and increased levels of nitrated EAAC1, which leads to carrier dysfunction. The findings suggested that oxidative stress induced by MPTP may reduce dopaminergic neuronal cysteine supply, via EAAC1 dysfunction, leading to impaired GSH synthesis and consequent GSH depleted content. Another possible explanation to GSH depletion may be caused by oxidized DA. Nigrostriatal region is rich in DA and, indeed, intrastriatal injection of DA in rats induces a significant decrease in GSH levels (Rabinovic and Hastings, 1998), probable through the interactions of DA quinone with GSH and/or cysteine (Spencer et al., 1998; Hirrlinger et al., 2002).

Other theories for GSH depletion observed in PD involve mitochondrial dysfunction and complex I impairment. It has been suggested that a prolonged mild inhibition of complex I leads to increased ROS production and a subsequent decrease in GSH levels (Greenamyre et al., 2001), implying that GSH depletion would be secondary to complex I inhibition. However, decreased levels of GSH are also observed in incidental LB disease, a presymptomatic precursor of PD, in the absence of complex I inhibition (Sian et al., 1994a). Therefore, it remains unclear whether GSH depletion or complex I inhibition comes first. Several studies have shown that decreased GSH can impair complex I function and may even precede complex I inhibition. Hsu and collaborators (2005) demonstrated that reducing GSH by ~50% in dopaminergic PC12 cells resulted in 50% decreased complex I activity. This cellular model, genetically engineered to permit inducible inhibition of GCL, therefore reducing GSH, led to changes in GSH and GSSG similar to those observed in PD patients (Sofic et al., 1992; Jha et al., 2000). These effects of GSH depletion in complex I activity were then replicated in N27 cells using a pharmacological inhibitor of GCL, L-buthionine-S-sulfoximine (BSO) for 7 days, to mimic a chronic GSH depletion as in the case of PD (Chinta and Andersen, 2006). Clementi et al. (1998) previously demonstrated that persistent inhibition of complex I occurred with prolonged exposure to NO and that this condition was accelerated by GSH depletion.

Interestingly, Seaton et al. (1996) did not find changes in complex I activity in the cerebral cortices of rats treated with BSO, suggesting that these GSH depletion-induced reduction in complex I activity could primarily be confined to dopaminergic cells. Also corroborating these results, Vasquez and colleagues (2001) showed that GSH depletion in glial cultures caused complex I activity up-regulation and enhanced levels of mRNA of the NADH-ubiquinone oxidoreductase chain 6 (ND6) subunit of this mitochondria respiratory complex. This suggests that the capacity of GSH depletion to inhibit complex I could be specific to dopaminergic cells and may, at least in part, contribute for their vulnerability in PD progression.

Nevertheless, GSH depletion and oxidative stress are undoubtedly linked and one may conclude that the decreased levels in GSH that affect complex I activity may result from multiple processes. Inhibition of complex I activity has several downstream effects, including ROS production that may also lead to GSH depletion and a reduction in ATP synthesis by the electron transport chain. These effects would then influence the other pathogenic processes that are involved in PD pathogenesis.

1.6. Transcription factors relevant for antioxidant defense and mitochondrial biogenesis

1.6.1. Nuclear factor erythroid 2-related factor 2 (Nrf2)

Genes coding for proteins involved in ROS detoxification share a common promoter element, the antioxidant response element (ARE). ARE-mediated gene activation is coordinated by the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), which, upon exposure to electrophiles or ROS, translocates to the nucleus, where it binds ARE (Itoh et al., 2003; Kobayashi and Yamamoto, 2006; Motohashi and Yamamoto, 2004; Itoh et al., 2004). Under normal homeostatic conditions, Nrf2 is repressed by its negative regulator Kelch-like ECH-associated protein 1 (Keap1) in the cytosol (Itoh et al., 2003). Keap1 protein contains four particularly reactive cysteine residues, which are likely candidates for being the direct sensors of oxidative stress. Recent studies on Nrf2 activation suggest that the oxidant sensor function of Keap1 may be primarily to slow the ubiquitination and subsequent degradation of Nrf2 under higher levels of oxidative stress, leading to more Nrf2 accumulation in the cell under these conditions. Nrf2 itself may hold an oxidant sensor that facilitates nuclear translocation, but this function is still poorly understood (Hu et al., 2010). Thus, the phosphorylation of Nrf2 at serine (Ser) 40 by protein kinase C (PKC) appears to be an important event in the release of Nrf2 from Keap1 and its

translocation to the nucleus (Huang et al., 2002; Bloom and Jaiswal, 2003). Many early studies interpreted the role of Nrf2 activators to be mediated solely via adduct formation with, or by oxidation of, the reactive cysteine residues of Keap1; however, it seems more likely that kinase signaling pathways could also be involved, with phosphorylation of Nrf2 ultimately responsible for its migration to the nucleus (Hybertson et al., 2011).

Nrf2 belongs to a subset of basic leucine-zipper (bZip) genes that share a conserved structural domain, called the cap'n'collar (CNC) domain. This CNC family in mammals is composed of four closely related proteins: p45-NFE2, Nrf1, Nfr2 and Nrf3 that share the CNC-like bZip domain and require heterodimerization with small musculoaponeurotic fibrosarcoma oncogene (MAF) proteins to bind the MAF recognition element (MARE) and activate transcription of the target gene (Kensler et al., 2007; Itoh et al., 2004). The MARE is a long palindromic DNA sequence [TGCTGAC(G)TCAGCA] (Kataoka et al., 1993), where is included, among others, the ARE sequence (TGACNNNGC). Nrf2 binds to ARE to regulate the transcription of phase II antioxidant enzymes, including SOD1 or Cu/ZnSOD (McCord and Edeas, 2005), heme oxygenase-1 (HO-1) (Prestera et al., 1995), nicotinamide adenine dinucleotide phosphate (NADPH):quinone oxidoreductase-1 (NQO1) (Favreau and Pickett, 1995; Wang and Williamson, 1994), GSTs (Rushmore and Pickett, 1990), GCL, GPx, GRed (Galloway et al., 1999; Galloway et al., 1997; Mulcahy and Gipp, 1995) and Trx (Hintze et al., 2003) and peroxiredoxin (Prx) families (Ishii et al., 1999) (Figure 1.4).

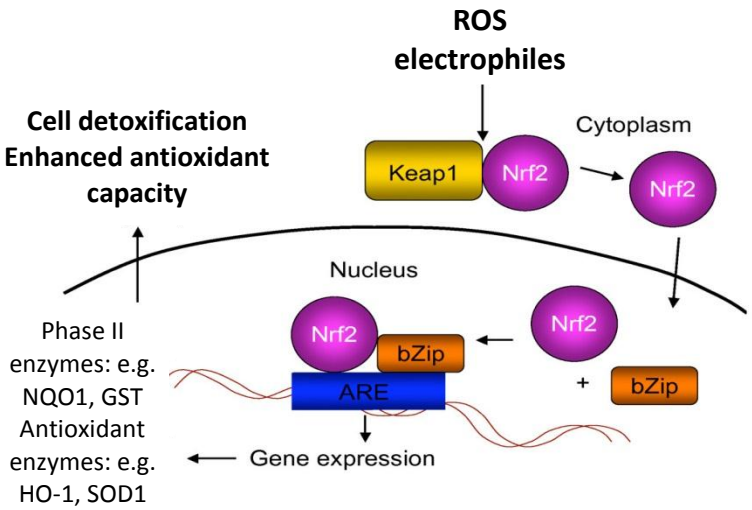

Figure 1.4. – Schematic representation of Nrf2-ARE pathway. In the cytoplasm, under normal conditions, newly synthesized Nrf2 is constitutively bound to Keap1 forming a dimer, Nrf2-Keap1. When oxidants such as ROS, RNS or electrophiles react with redox reactive cysteines in Keap1, Nrf2 will be released from Keap1, hence allowing the transcriptional factor Nrf2 to translocate to the nucleus. In the nucleus, Nrf2 dimerizes with basic leucine zipper partners (bZip) such as small MAF-family proteins and bind to ARE, which is located in the promoter of the phase II and antioxidative genes, triggering the transcription of ARE-regulated genes (adapted from Saw et al. 2010).

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Currently, information about the functional status of the Nrf2-ARE system in PD is scarce. Activation of the Nrf2-ARE system in PD was suggested by van Muiswinkel and colleagues (2004) who demonstrated a strong upregulation in the immnunoreactivity of ARE-regulated proteins, namely NQO1 in *post-mortem* brain sections. Other authors verified almost the same pattern of immunoreactivity for HO-1 (Schipper, 2004; Riedl et al., 1999). However, this increased expression of NQO1 and HO-1 seemed largely restricted to astrocytes and was almost absent in dopaminergic neurons. In contrast, investigation of the localization of Nrf2 in the SN of PD brains showed that besides cytoplasm, a strong nuclear immunoreactivity was observed in neurons (Ramsey et al., 2007). Data concerning a possible role for the Nrf2-ARE pathway in PD has been obtained from the use of several *in vitro* and *in vivo* models. Thus, the highly neurotoxic DA analogue 6-OHDA was found to

activate the Nrf2-ARE system as part of a cellular defense mechanism to protect against ROS-mediated damage (Jakel et al., 2005). Conversely, the loss of Nrf2 increased both *in vitro* and *in vivo* vulnerability to 6-OHDA, whereas upregulation of Nrf2 using tert-butylhydroquinone (tBHQ), a phenolic antioxidant, was protective against 6-OHDA-induced cell death *in vitro* (Jakel et al., 2005). Furthermore, transplantation into mouse brain of astroglial cells overexpressing Nrf2 resulted in a significant reduction in the susceptibility to 6-OHDA neurotoxicity (Jakel et al., 2007). In another study, deprenyl, a drug applied in the treatment of PD, was reported to stimulate Nrf2 activity as part of its cytoprotective mechanism of action (Nakaso et al., 2006). Moreover, the flavonoid luteolin protected against the mitochondrial toxin MPP+ by activating the Nrf2 pathway in cultured neural PC12 and glial C6 cells (Wruck et al., 2007). In this model, knockdown of Nrf2 annulled this effect, thereby demonstrating that luteolin-induced protection was mediated through Nrf2. Similar effects in PC12 cells were reported upon use of the Nrf2 transcriptional activator tBHQ to protect against the toxicity of another PD-associated neurotoxin, deltamethrin (Li et al., 2007).

1.6.2. Peroxisome proliferator-activated receptor (PPAR) γ co-activator 1 alpha (PGC-1alpha)

Nuclear receptors (NRs) are members of a large superfamily of ligand-regulated DNA-binding transcription factors that perform a broad range of physiological activities, namely by transducing steroid, retinoid, thyroid and lipophilic endocrine hormone signals into specific patterns of gene expression (reviewed in Lonard et al., 2007)). NRs regulate gene expression through their capacity to bind to specific sequences in the promoters of their target genes. Transcriptional co-regulators – co-activators and co-repressors – are protein factors directly recruited by NRs to modulate NR-regulated gene expression without binding to DNA (Tsunemi and La Spada, 2012). In humans there are nearly 300 transcriptional co-regulators that control transcriptional activity of NRs, suggesting that

carefully coordinated regulation of NR activity is of crucial importance to execute complex physiological functions. Thus, mutations occurring in more than 100 co-regulators are sufficient to lead to the development of specific human diseases (reviewed in Lonard et al., 2007).

Peroxisome proliferator activated receptor (PPAR) γ co-activator 1 alpha (PGC-1alpha) is a key transcriptional regulator of antioxidant defense systems that is mainly expressed in brown adipose tissue, heart, skeletal muscle, kidney and brain – all highly oxidative tissues (Esterbauer et al., 1999; Lin et al., 2002). PGC-1alpha is a multifunctional protein that activates most NRs and also functions as a co-activator to many transcription factors (Puigserver and Spiegelman, 2003). It is a large ~100 kDa protein with a number of functional domains that bind several protein complexes. The N-terminal of PGC-1alpha contains an activation domain, which interacts with c-AMP response element-binding (CREB) binding protein (CBP)/p300 (Puigserver et al., 1999). In the C-terminal of PGC-1alpha there is a serine/arginine-rich domain and a putative RNA recognition motif that are both involved in RNA binding and splicing (Graveley, 2000; Monsalve et al., 2000). PGC-1alpha associates with the elongating form of RNA polymerase II and binds proteins that regulate RNA splicing (Knutti and Kralli, 2001), contributing to PGC-1alpha activity as a highly potent co-activator of transcription. Furthermore, PGC-1alpha is a critical regulator of metabolism, linking metabolic activity to relevant environmental stimuli in multiple pathways, including those responsible for adipogenesis, gluconeogenesis, myogenesis and mitogenesis (reviewed in Handschin and Spiegelman, 2006). In addition, PGC-1alpha can coordinate the expression of many antioxidant programs in response to oxidative stress (Anderson et al., 2008; Pessayre, 2007; St-Pierre et al., 2006; Valle et al., 2005). As a co-activator, PGC-1alpha also interacts with a variety of other transcription factors, including nuclear respiratory factors 1 and 2 (NRF1 and NRF2) and nuclear hormone receptors, namely estrogen-related receptor α (ERRα) and thyroid receptor, leading to the initiation of a diverse set of metabolic programs in different tissues (Lin et al., 2005). NRF1, NRF2 and ERRα are primarily responsible for regulation of the expression of many nuclear-encoded

mitochondrial genes, including cyt c, the components of complexes I-V and mitochondrial transcriptional factor A (TFAM) (Kelly and Scarpulla, 2004). PGC-1alpha increases upon a number of external stimuli, including cold in brown adipose tissue, exercise and decreased ATP levels in skeletal muscle and fasting in the liver. Furthermore, PGC-1alpha has an important role in the regulation of the metabolism and survival of GABAergic neurons in the developing brain (Cowell et al., 2007). Indeed, PGC-1alpha remains highly expressed in the adult brain; however, its activities and functions remain yet to be fully clarified. Nevertheless, it is believed that PGC-1alpha in neurons may be involved in regulating the cellular response to oxidative stress (St-Pierre et al., 2006). ROS induce the expression of several genes coding for antioxidant enzymes in 10T1/2 cells, including SOD1, SOD2 and GPx1. RNA interference (RNAi) knock-down of PGC-1alpha prevented the induction of these genes by ROS, suggesting that PGC-1alpha mediates these protective responses (St-Pierre et al., 2006).

To better understand the role of PGC-1alpha in metabolism, the Spiegelman laboratory generated PGC-1alpha knockout mice (Lin et al., 2004). Studies in these transgenic animals revealed neurological abnormalities, including myoclonus, dystonia, exaggerated startle responses and clasping. Furthermore, neuropathological examination evidenced the degeneration in cortex, thalamus, basal ganglia and hippocampus, with the most pronounced degeneration observed in the striatum. In addition, this lesion was accompanied by massive gliosis and significant neuronal loss. Also in the PGC-1alpha knockout mice, baseline levels of SOD1 and SOD2 (but not GPx) appeared decreased (Lin et al., 2004).

Recent investigations have suggested the involvement of impaired PGC-1alpha in PD pathogenesis (Shin et al., 2011; Zheng et al., 2010). A combination of several microarray studies performed on PD, including *post-mortem* PD SN, *post-mortem* PD with LBs and SN dopaminergic neurons isolated by laser microdissection, demonstrated a strong evidence for PGC-1alpha transcription interference, as gene set enrichment analysis revealed a down-regulation of 425 PGC-1alpha regulated genes in PD samples (Zheng et al., 2010).

These findings indicated that defects in mitochondrial electron transport, glucose usage and glucose sensing may occur early in PD pathogenesis. Furthermore, activation of PGC-1alpha could rescue dopaminergic cell loss induced by mutant alpha-syn or rotenone in primary neuronal cultures (Zheng et al., 2010).

1.6.3. Mitochondrial transcription factor A (TFAM)

As described above in section 1.5.1, evidences from different research fields point to the involvement of mitochondrial dysfunction in PD (reviewed in Schapira, 2008). TFAM is a highly conserved 25 kDa protein (Reyes et al., 2002) that interacts with light and heavy strand promoters in mtDNA. It is a nuclear-encoded transcription factor and is imported to mitochondria, where it is necessary for mitochondrial transcription. TFAM is also required for mtDNA maintenance in mammals; it stabilizes mtDNA, regulates mtDNA copy number *in vivo* and is essential for mitochondrial biogenesis (Larsson et al., 1998; Galter et al., 2010). There is growing evidence for the possible involvement of TFAM in PD pathogenesis. Apart from controlling mtDNA transcription and replication, TFAM was also implicated in the recognition of mtDNA damage and repair (Yoshida et al., 2002). *In vivo* and *in vitro* models demonstrated both direct and indirect (through protection from oxidative stress and enhancement of mitochondrial respiratory functions) involvement of TFAM in PD pathogenesis. The conditional selective inactivation of both TFAM alleles in the SN of transgenic mice (the MitoPark mice) led to Parkinson-like phenotype characterized by progressive motor dysfunction with adult onset accompanied by intraneuronal inclusions and respiratory chain deficiency (Ekstrand et al., 2007). TFAM overexpression in rat myoblasts accelerated mtDNA recovery after peroxide injury (Noack et al., 2006), whereas in HeLa cells it was able to inhibit ROS generation upon rotenone treatment. Transgenic mice overexpressing TFAM presented lower oxidative stress and mtDNA damage levels, while higher activities of complex I and IV were observed in the brain compared to control

animals (Hayashi et al., 2008). Furthermore, TFAM complexed with parkin, a protein implicated in familial PD, bound to mtDNA control region and enhanced mitochondrial biogenesis (Kuroda et al., 2006). In addition, gene therapy based on transfection of either recombinant TFAM or mtDNA-complexed TFAM to PD cybrid cells resulted in marked improvement of several mitochondrial functions (Keeney et al., 2009).

1.7. Therapeutic strategies in PD

Before the introduction of levodopa or L-DOPA as a therapy, PD was essentially a motor disorder. After the arrival of levodopa, the development of motor complications and psychiatric manifestations – such as hallucinations and delirium – became an important focus of attention and also turned into dominant clinical problems in PD (Obeso et al., 2010). A more prudent use of levodopa, the introduction of DA agonists and atypical neuroleptics, and the possibility of treating severely affected patients with surgery (e.g. deep brain stimulation of the subthalamic nucleus) have reduced the urgency of these problems. Indeed, there is general consensus that new PD treatments should undertake two unresolved problems: moving from symptom-alleviating to disease-modifying therapies, and reducing the growing prevalence of nonmotor disease symptoms such as loss of equilibrium, autonomic dysfunction and cognitive impairment, which are the main causes of disability in long-term PD. Progress will certainly rely on understanding genetic mutations and/or susceptibility factors that lead to PD and better translation between preclinical animal models and clinical research.

Based on the main focus of this thesis, in this section we describe possible new therapeutic approaches that may be developed, taking into account the molecular mechanisms associated to PD pathology and disease progression.

1.7.1. Modulation of antioxidant levels – role of Nrf2 and PGC-1alpha

Dysfunction of mitochondria and increased oxidative stress are dominant topics associated to PD pathogenesis, as described previously in this thesis. PGC-1alpha and Nrf2 are complementary and overlapping regulators of the antioxidant defense system. The majority of antioxidant enzymes found to be regulated by PGC-1alpha function within mitochondria. Similarly, Nrf2 is a regulator of both cytosolic and mitochondrial antioxidant mechanisms, including the antioxidant enzyme NQO1 which may be important in detoxifying potentially electrophilic DA quinones, as NQO1 is a two-electron reductase, thus preventing the one electron reduction of quinones that results in the production of radical species. Therefore, in dopaminergic neurons, Nrf2 function seems to be quite necessary for the clearance of ROS produced through the autoxidation of DA and also for the detoxification of quinone-end products (Jia et al., 2008) to maintain the redox balance of these vulnerable cells.

A molecular interaction between PGC-1alpha and Nrf2 is not well established. The PGC-1alpha promoter contains an ARE (St-Pierre et al., 2006), although it is not known whether this is functional. Even without a direct interaction between Nrf2 and PGC-1alpha (whereby PGC-1alpha forms part of a co-activator complex to upregulate Nrf2 transcription or Nrf2 binds to the PGC-1alpha promoter), it seems possible that the expression of one gene may regulate the expression of the other via redox signaling. Both genes are induced by oxidative stress and upregulate downstream antioxidant enzymes, which in turn reduce the levels of free radicals and ROS. Therefore, any PD therapeutic strategy that combines Nrf2 and PGC-1alpha activation or expression must be able to reduce oxidative stress and restore intracellular redox homeostasis.

A probable increased risk of cancer associated with the constitutive systemic overexpression of antioxidant defense master regulators, such as Nrf2, has been described (Padmanabhan et al., 2006; Singh et al., 2006; Wang et al., 2008; Clark and Simon, 2009). However, Nrf2 knockout mice proved to be more susceptible to carcinogens than WT animals (Ramos-Gomez et al., 2001), indicating that low Nrf2 levels may also promote tumor formation. Hence, these observations suggest that is crucial to maintain a correct

balance of Nrf2 activity in the organism. One way to face this issue could be achieved by expressing Nrf2 or PGC-1alpha using a cell-type specific promoter. In the particular case of PD, expression of Nrf2 and PGC-1alpha using a TH promoter would ensure that the gene was expressed in dopaminergic neurons. An additional strategy could be based on the use of an inducible promoter that had the possibility to be "switched on" under conditions of oxidative stress. Hurttila and co-workers (2008) have already tested this methodology by using a double ARE to overexpress HO-1 in human endothelial cells positively transduced by a lentiviral vector. Applying similar strategies to Nrf2 or PGC-1alpha expression could provide the upregulation of multiple antioxidant activities in specific tissues or cells that are subjected to oxidative stress (Figure 1.5).

Further studies on the mechanisms of transcriptional control of antioxidant defenses need to be performed, as they may give clues to understand mechanisms associated to the pathogenesis of PD. Thus, the ability to manipulate the activity of transcriptional regulators of the antioxidant defense pathways, such as Nrf2 and PGC-1alpha, may provide promising strategies for achieving neuroprotective therapies.

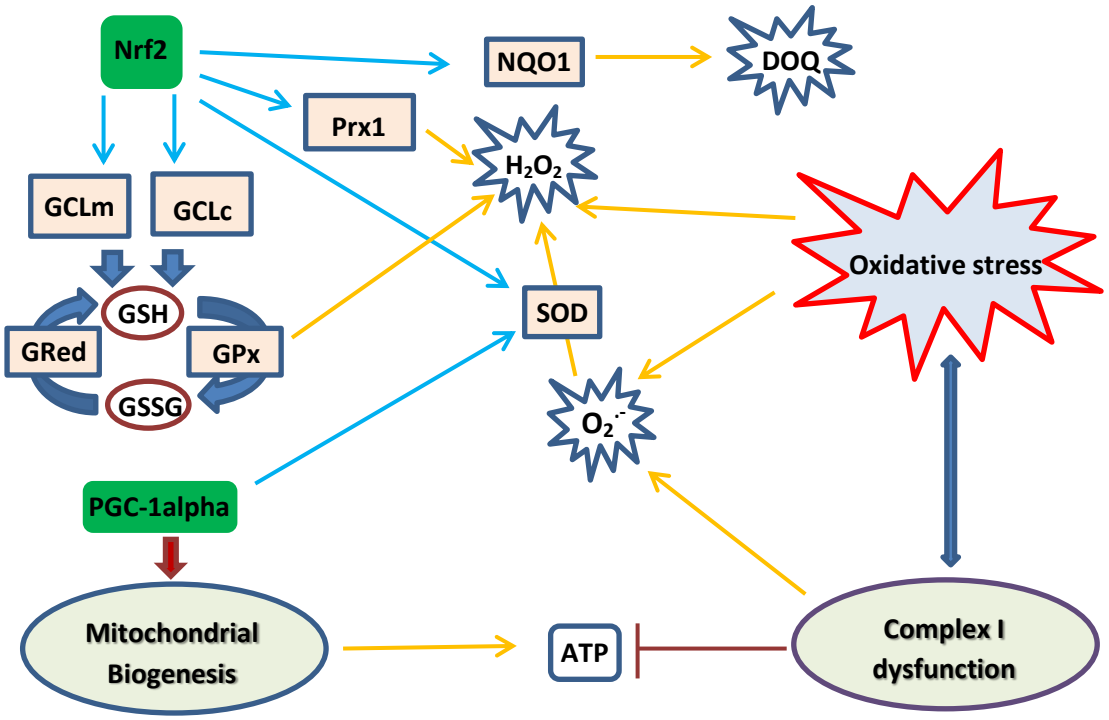


Figure 1.5. – Schematic representation of potentially decreased oxidative stress within dopaminergic neurons as a result of PGC-1alpha and Nrf2 upregulation. Abbreviations: ATP – adenosine triphosphate; DOQ – dopamine quinone; GCLc – glutamate cysteine ligase catalytic subunit; GCLm – glutamate cysteine ligase modulatory subunit; GPx – glutathione peroxidase; GRed – glutathione reductase; GSH – reduced glutathione; GSSG – oxidized glutathione; H_2O_2 – hydrogen peroxide; $O_2^{\cdot-}$ - superoxide anion; NQO1 – NADPH dehydrogenase quinone-1; Prx1 – peroxiredoxin 1; SOD – superoxide dismutase

1.7.2. Sequestering excess iron

The correlation between iron accumulation in the brain and PD has led to the theory that iron chelators could help slowing the development of the disease by clearing the unbound, free radical-enhancing iron in the brain. This mode of action has already a precedent, since effective removal of neuronal copper in Wilson's disease was obtained with copper chelation using D-penicillamine (Leggio et al., 2005). Potential chelators must selectively scavenge excess intracellular iron and turn it into a nontoxic product, which can be safely excreted. Importantly, chelators should only access the intracellular excess free iron pool, i.e., iron that is not bound by ferritin, which is essential for normal physiological functions. Another important factor is the need for chelators to readily cross the BBB, which means that the size of the potential chelator should not exceed 300 Da. There are several substances that were proven to have neuroprotective effects in conditions of iron accumulation; however, this is mainly due to their antioxidant properties and/or increased mitochondrial activity (reviewed in Mounsey and Teismann, 2012).

Iron chelators can be classified in two classes: 1) chemical chelators (e.g. desferal, deferiprone, apomorphine, among others), which are already available clinically for various conditions (Figure 1.6). Their use in neurodegenerative disorders has been restricted primarily by their capacity to cross the BBB barrier in therapeutically efficacious concentrations; 2) natural chelators, which include components of plant polyphenols, such

as epigallocatechin gallate (EGCG), a green tea extract, and phytic acid (Figure 1.6). These compounds have been used over many years due to their antioxidant properties and have been investigated for their potential use in diverse areas, including oncology, cardiology and neurology.

A great advantage of these new pharmacotherapies is the variety of neuroprotective actions they can exert. In addition to iron chelation, these therapies may also inhibit MAO-B, as well as having antioxidant properties. Antioxidant actions of natural substances, such as the green tea extract EGCG, have been coupled with iron chelation activities to provide natural alternatives.

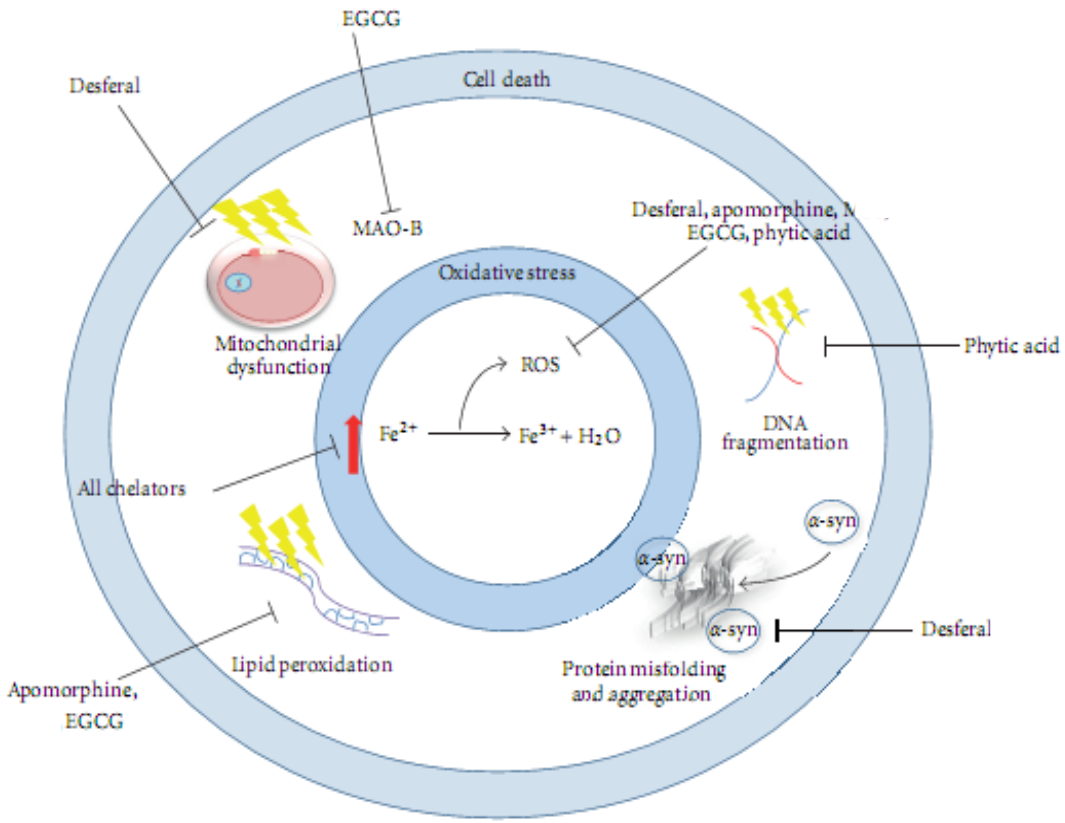


Figure 1.6 – Summary of iron chelators action targeting PD. Iron chelators remove excess free, reactive iron that produces ROS, such as ·OH, through the Fenton reaction. ROS induce several

deleterious intracellular insults, which can lead to cell death. Chelators with antioxidant properties can also inhibit ROS production in an environment with lower antioxidant activity. MAO-B, the DA-oxidizing enzyme, localized in the outer mitochondrial membrane can also be inhibited by natural chelators. (Adapted from Mounsey and Teismann 2012).

Despite the advantages one can take from these promising therapies, it is important to consider whether iron chelators will provide the cellular specificity required to remove excess iron from the affected tissue, without impacting on systemic iron homeostasis. The dose of chelator is a crucial factor to be considered in treatment, so that the correct tissues are penetrated and systemic iron levels are not altered.

In conclusion, reversing the redox imbalance caused by increased levels of iron observed in the affected areas of PD patients is an important aim for developing neuroprotective therapies. Iron chelators, initially used for other medical applications, have been improved. With some treatments now reaching clinical trials, it is expected that an efficacious and tolerable iron chelation therapy will be effective at attenuating neurodegeneration in PD.

1.7.3. Targeting alpha-syn phosphorylation

Alpha-syn is closely linked to PD pathogenesis, thus it becomes an attractive target for therapeutic intervention. As stated before in this thesis, alpha-syn phosphorylation has complex consequences that remain to be fully understood. However, it is also clear that hyperphosphorylation, particularly at Ser129, has a correlation with disease progression, is present in the main pathological hallmark lesions of alpha-synucleinopathies and can have undesirable functional consequences. Therefore, reducing this Ser129 phosphorylation by inhibiting the relevant kinases involved in alpha-syn phosphorylation pathways has been a primary focus. As an example, treatment with the specific PLKs inhibitor BI2356 in mice inhibited alpha-syn phosphorylation at Ser129 (Inglis et al., 2009). However, there have

been no further reports on the functional effects of this inhibition or even long-term safety of this approach. Kinases that phosphorylate alpha-syn have ubiquitous distribution and several molecular targets, and there is clear redundancy with multiple kinases phosphorylating the same residue. Consequently, it may be difficult to generate a safe and efficacious agent that has activity in the brain. Kinases are a critical class of targets for CNS disorders; however, problems need to be faced regarding pharmacological selectivity and BBB permeation, which are probably the major challenges in the future. Another viable approach to modulate alpha-syn phosphorylation could be the use of allosteric activators to increase Ser129 phosphatase activity. This has already been achieved *in vivo* by treatment with eicosanoyl-5-hydroxytryptamide (EHT) which inhibits PP2A demethylation, thereby enhancing its phosphatase activity towards alpha-syn (Lee et al., 2011). In alpha-syn transgenic mice, which diet contained EHT, Ser129 phosphorylation was reduced and alpha-syn aggregation was inhibited with concomitant improved neuronal integrity, reduction of inflammation and recovery of behavioral deficits (Lee et al., 2011). In this particular case, this mechanism appears to be more safe and selective, since it only modulates a pool of PP2A, regulating a specific set of substrates. Finally and quite important, PP2A is highly methylated in healthy cells and methylation seems to be only deficient in states of cellular stress associated to disease or injury.

Thus, efforts must converge to understand the functional consequences of alpha-syn phosphorylation, the interplay between different phosphorylation sites and the pathways that modulate this mechanism, so it can be possible to develop crucial, novel and efficacious pharmacological therapies for these disabling diseases, such as PD.

1.8. Objectives

During the past years, several mechanisms have been proposed to underlie PD pathogenesis. However, it remains unclear how neurodegeneration occurs in the disease, leading to the disabling typical symptoms of PD. The main aim of this thesis was to study the impact of alpha-syn on cellular susceptibility to oxidative stress, its modulation by transcription factors and the role of mitochondria in cellular models of PD.

To achieve this objective we used: i) a human neuroblastoma cell line (SH-SY5Y) expressing alpha-syn, namely human WT or A53T mutant alpha-syn (Chapter 3) and ii) the same human SH-SY5Y cell line, genetically modified to conditionally express human WT alpha-syn in a doxycycline (Dox) regulated manner under a Tet Off system (Chapters 4 and 5).

Specific objectives, delineated in Chapters 3-5, were depicted as follows:

AIM 1 - To examine the interplay between formation of ROS and alpha-syn aggregation and phosphorylation following expression of mutant alpha-syn in transiently transfected SH-SY5Y cells (Chapter 3).

AIM 2 - To define cellular and mitochondrial oxidative stress, the modulation of antioxidant activity and the role of related transcription factors in SH-SY5Y cells conditionally overexpressing wild-type alpha-syn (Chapters 4-5).

In Chapter 3 we aimed to assess ROS formation and its correlation with cell death, alpha-syn aggregation and phosphorylation at Ser129 of A53T mutant *versus* wild-type (WT) alpha-syn in transiently transfected SH-SY5Y cells, further subjected to prolonged (4 days) exposure to ferrous iron ($FeSO_4$, a potent oxidative stress inducer) and rotenone (complex I inhibitor), respectively, to mimic oxidative stress and mitochondrial dysfunction. We characterized this PD cell model by analyzing markers of necrotic cell death, generation of endogenous ROS and changes in mitochondrial morphology and mitochondrial membrane potential. We also correlated WT and A53T mutant alpha-syn modifications, namely phosphorylation at Ser129 and inclusion formation, with oxidative stress-induced

conditions in situations mimicking sporadic and familial forms of PD caused by alpha-syn (Chapter 3).

Because it is still unknown whether oxidative stress is a cause or a consequence of the mechanisms underlying PD progression, in Chapter 4 we defined the influence of WT alpha-syn overexpression (mimicking the multiplication of WT alpha-syn gene associated to familial forms of PD) on oxidative stress, antioxidant regulation and the role of the nuclear transcription factor Nrf2. Using this paradigm, we also analysed the effects of short $FeSO_4$ exposure. In this study, we used SH-SY5Y cells maintained in a doxycycline (Dox) regulated manner in a Tet Off system, taking advantage of a model where human WT alpha-syn expression was regulated and the whole cell population expressed the protein. Furthermore, we correlated the high levels of alpha-syn with susceptibility to oxidative stress, apoptotic cell death and cellular reducing capacity. Under these conditions, the role of proteins involved in the intracellular antioxidant defense system and nuclear transcriptional factors were also examined (Chapter 4).

In the last part of the thesis (Chapter 5), we aimed to establish a relationship between overexpression of WT alpha-syn and mitochondrial redox status and activity to clarify a possible correlation between high levels of WT alpha-syn in mitochondria and PD-associated mitochondrial dysfunction linked to ROS generation. Thus, we investigated the influence of WT alpha-syn overexpression on mitochondrial activity by assessing the activity of mitochondrial complex I, mitochondrial biogenesis-related transcription factors and further analysed mitochondrial ROS generation and antioxidant-associated detoxification pathways localized in mitochondria (Chapter 5).

CHAPTER 2

Materials & Methods

2.1. Cell culture, transient transfection and incubation of SH-SY5Y cells

SH-SY5Y neuroblastoma cell line was cultured in DMEM-F12 medium supplemented with 10% fetal bovine serum (FBS) and kept at 37°C in an atmosphere of 95% air and 5% CO_2. Cells were plated at $1.4x10^5$ cells/cm^2 on 16 mm coverslips, 96, 48, 12 or 6-well plates. 24 hours after plating, SH-SY5Y cells were transfected with Fugene 6 transfection reagent at a 6:1 ratio (according to the manufacturer's instructions) with wild type (WT) or A53T alpha-synGFP plasmids, which encoded a fluorescent fusion protein composed of full length human alpha-syn, with green fluorescent protein (GFP) attached to its C-terminal (McLean et al., 2001). SH-SY5Y cells expressed neuronal markers as assessed by immunofluorescence using anti-MAP2 (microtubule-associated protein) and anti-TH (tyrosine hydroxylase) (Figure 2.1). During the four days after transfection, cells were incubated daily with $FeSO_4$ (1 mM and 2.5 mM) or rotenone (10 nM and 100 nM). Except for some experiments related with reactive oxygen species (ROS) generation, transfection with WT or A53T alpha-synGFP was performed for 24 h and cells were incubated the next day for 15 min – 2 h with the toxic stimuli. $FeSO_4$ stock solution was prepared in sterile water, while rotenone stock solution was prepared in dimethyl sulfoxide (DMSO, final percentage in the culture medium at 0.001%).

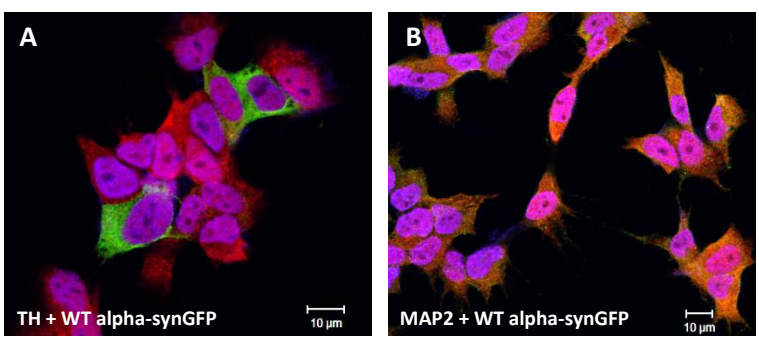


Figure 2.1 – Confocal images of SH-SY5Y cells transfected with WT alpha-synGFP (green). Cells were treated as described in section 2.11. Levels of expression of WT alpha-synGFP are defined in Figure 3.1 (Chapter 3). Images show TH (red, **A**) and MAP2 (red, **B**) immunofluorescence.

2.2. Cell culture and incubation of stable SH-SY5Y cell lines

The generation of a stable cell line inducibly expressing WT alpha-syn was previously described by Vekrellis et al. 2009. Briefly, naïve SH-SY5Y cells were transfected with the Tet-Off vector (Clontech) and selected with 500 μg/ml geneticin (G418). Induction of G418 resistant clones was determined by transient transfection of a pTRE-LUC vector, in the presence or absence of Dox (2 μg/mL). One clone (2-22) was further used for generation of stable pTRE-alpha-syn expression. WT alpha-syn was subcloned into the *HindIII* and *XbaI* sites of the pTRE-2 vector (Clontech) and co-transfected with the pTK-Hygromycin vector (Clontech). Selection was performed with 250 μg/mL G418 and 50 μg/mL Hygromycin B (Vekrellis et al., 2009).

Stable SH-SY5Y cell lines conditionally expressing WT alpha-syn (SH-SY5Y WT alpha-syn) were kindly donated by Dr. Leonidas Stefanis (Division of Basic Neurosciences, Biomedical Research Foundation of the Academy of Athens, Soranou Efesiou, Athens, Greece), and maintained in culture as follows: cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 250 μg/mL G418 and 50 μg/mL hygromycin B and kept at 37ºC in an atmosphere of 95% air and 5% CO_2. Alpha-syn overexpression was switched off with 2 μg/mL doxycycline (Dox). Thus, cell cultures were kept in the presence of Dox and further plated at $1.4x10^5$ cells/cm^2 on 48-well plates, 16 mm coverslips or 100 mm petri dishes. For induction of WT alpha-syn overexpression, in all the experiments cells were kept during 5 days in the absence of Dox. The cells showed little basal expression in the presence of Dox (2 μg/ml) and robust expression of the transgene in the absence of dox at day five, as shown in Figure 2.2A. Cells overexpressing WT alpha-syn after 5 days without dox (-Dox) and immunostained for alpha-syn revealed a greater red fluorescence when compared to cells expressing the endogenous protein (+Dox) (Figure. 2.2B). Incubation with rotenone (10 μM) or $FeSO_4$ (500 μM) was performed during 2 hours at day 5 in the absence of Dox. Apparently, no significant differences in alpha-syn protein levels were observed after iron exposure (data not shown). For some experiments, SH-SY5Y WT alpha-syn cells were

differentiated using retinoic acid (RA) 20 µg/ml (in DMSO, final percentage in the culture medium at 0.1%) for 10 days, the last 5 days coinciding with the absence of Dox.

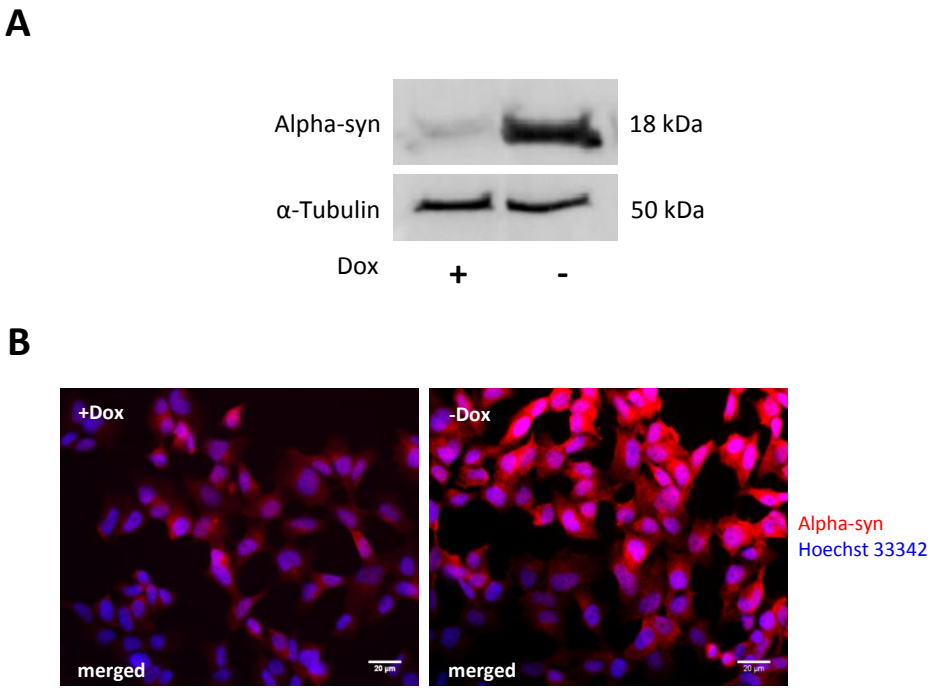


Figure 2.2 – Characterization of Tet-inducible SH-SY5Y cells expressing WT alpha-syn. SH-SY5Y WT alpha-syn cells were cultured for 5 days in the presence or in the absence of dox. Levels of alpha-syn were confirmed by western blotting in total lysates (**A**) and fluorescence microscopy (**B**), using an antibody against alpha-syn. Nuclei were stained with Hoechst 33342.

2.3. Reactive oxygen species measurements

2.3.1. Analysis of intracellular hydroperoxides

At the day of the experiments, the culture medium was removed and cells were washed with phosphate buffered solution (PBS, in mM: 137 NaCl, 2.7 KCl, 1.4 K_2HPO_4, 4.3 Na_2HPO_4, at pH 7.4) twice and then incubated for 30 min with 20 µM 2',7'-dichlorodihydrofluorescein

diacetate (H_2DCFDA), a stable non-fluorescent cell permeable compound, at 37ºC in Krebs medium (in mM: 135 NaCl, 5 KCl, 0.4 KH_2PO_4, 1.8 $CaCl_2$, 1 $MgSO_4$, 20 HEPES and 5.5 glucose, pH 7.4). When internalized by the cell, H_2DCFDA is hydrolysed by intracellular esterases to form H_2DCF and rapidly converted by endogenous hydroperoxides to DCF - a highly green fluorescent compound. Intracellular levels of peroxides were measured by following DCF fluorescence (488 nm excitation, 530 nm emission) at 37ºC, continuously for 1 h, using a Microplate Spectrofluorometer Gemini EM (Molecular Devices, USA). In order to correct the DCF fluorescence values for variations in total protein content in the wells, cell protein in each well was quantified by the BioRad protein assay. The values obtained were expressed as RFU (Relative Fluorescence Units) per minute and per mg protein for each condition.

2.3.2. Analysis of intracellular superoxide ions

Superoxide formation was determined using the fluorescent probe dihydroethidium (DHE). DHE is a permeable-cell membrane fluorescent dye that, once in the cytoplasm is oxidized to fluorescent ethidium bromide by superoxide, which intercalates with DNA. DHE itself shows a blue fluorescence (355 nm excitation, 420 nm emission) in cell cytoplasm until oxidation to form ethidium, which becomes red fluorescent (518 nm excitation, 605 nm emission), upon DNA intercalation. Briefly, 5 µM of DHE dissolved in Krebs medium was incubated in cells during 1 h at 37ºC. Ethidium bromide fluorescence was measured continuously for 1 h at 37ºC and the relative levels of superoxide production quantified, using a Microplate Spectrofluorometer Gemini EM (Molecular Devices, USA). At the end of the experiment, the cells were lysed to quantify cell protein in each well, using the BioRad protein assay. Ethidium fluorescence was corrected for variations in total protein between wells.

2.3.3. Analysis of mitochondrial superoxide ions

Mitochondrial superoxide formation was determined using the fluorescent probe MitoSox™ Red, a live-cell permeant that is rapidly and selectively targeted to the mitochondria, where it is oxidized by superoxide [but not by other ROS and reactive nitrogen species (RNS)], exhibiting red fluorescence (510 nm excitation, 580 nm emission). The chemical reactivity of mitochondria-targeted hydroethidine, MitoSox Red, with superoxide is similar to the reactivity of DHE with superoxide (e.g. Zielonka and Kalyanaraman, 2010). The oxidation product becomes highly fluorescent upon binding to nucleic acids. Briefly, 5 µM of Mitosox Red dissolved in Krebs medium was incubated in cells during 10 min at 37ºC. Fluorescence was measured continuously for 1 h at 37ºC and the relative levels of mitochondrial superoxide production quantified, using a Microplate Spectrofluorometer Gemini EM (Molecular Devices, USA). At the end of the experiment, the cells were lysed to quantify cell protein in each well, using the BioRad protein assay. The slope obtained for MitoSox spectra was corrected for variations in total protein in each well.

2.3.4. Electron Paramagnetic Resonance (EPR) experiments

SH-SY5Y cells were treated with iron and further incubated with 5,5-dimethyl pyrroline *N*-oxide (DMPO) (100 mM), a commonly-used spin trap that reacts with O-, N-, S-, and C-centered radicals (Chamulitrat et al., 1992). Cells were scraped in the culture medium and frozen/unfrozen three times in liquid nitrogen. The same treatment was performed in cells transfected with WT or A53T alpha-synGFP and further incubated with iron or rotenone plus DMPO. EPR spectra were recorded with a Bruker EMX spectrometer at room temperature in a continuous flow system using open Pasteur pipette inserted in the EPR cavity and directly connected to a 1 ml mixing cell. Appropriate controls without iron or rotenone were also run.

The instrument settings for iron and rotenone were: microwave frequency, 9.7 GHz; microwave power, 20 mW; modulation amplitude, 2 G; time constant, 0.16 s.

2.4. Measurement of glutathione levels

SH-SY5Y_WT alpha-syn cells were washed twice in PBS and lysed in 15 mM Tris, pH 7.4, and the intracellular levels of reduced and oxidized glutathione (GSH and GSSG, respectively) were determined using a fluorimetric assay, according to a previously described method (Hissin and Hilf, 1976). GSH levels were measured in samples after the addition of *ortho*-phethaldialdehyde (1 mg OPA/ml methanol) and 100 mM NaH_2PO_4. After 15 min incubation, the fluorescence was measured using an excitation wavelength of 350 nm and an emission wavelength of 420 nm. The experimental procedure for GSSG was similar, although the samples were mixed during 30 min with *N*-ethylmaleimide (5 mg NEM/ml methanol) which forms adducts with GSH. Then, the mixture was incubated for 15 min in 100 mM NaOH plus OPA (1 mg OPA/ml methanol), and the fluorescence was measured with excitation at 350 nm and emission at 420 nm in a Microplate Spectrofluorometer Gemini EM (Molecular Devices, USA). The results were calculated as RFU per mg protein.

2.5. Measurement of Glutathione Peroxidase (GPx) and Glutathione Reductase (GRed) activities

The cells were washed twice in PBS and lysed in 25 mM Tris-HCl (pH 7.4) and then centrifuged at 20,800×*g* for 10 min, at 4ºC (Eppendorf Centrifuge 5417R). The supernatant was used for protein quantification using the BioRad protein assay (Bradford method) and for measuring GRed and GPx activities, spectrophotometrically, at 340 nm, through the analysis of NADPH oxidation, as described previously (Paglia and Valentine, 1967) with some modifications. Briefly, the activity of GPx in samples was measured upon a 5 min incubation, in the dark, with phosphate buffer containing 0.25 M KH_2PO_4, 0.25 M K_2HPO_4 and 0.5 mM EDTA, pH 7.0, 10 mM GSH and GRed (1 unit). The reaction occurred after the addition of 2.5 mM NADPH and 12 mM *tert*-Butyl hydroperoxide. For the activity of GRed, each sample was incubated with a phosphate buffer containing 0.2 M K_2HPO_4 and 2 mM

EDTA, pH 7.0, plus 2 mM NADPH. The measurements were initiated with the addition of 20 mM GSSG. GRed and GPx activities were determined using a Microplate Spectrophotometer SpectraMax Plus^{384} (Molecular Devices, USA). Results were calculated as milliunits (mU) per mg protein.

2.6. Determination of Glutamate Cysteine Ligase (GCL) activity

Glutamate-cysteine ligase (GCL) activity was measured according to White and collaborators (White et al., 2003), with some minor modifications (Zhu et al., 2007). Cells were washed twice with PBS and lysed with 25 mM Tris-HCl (pH 7.4) plus 0.1 mM EDTA. Cellular extracts were centrifuged at 20,800×*g* for 10 min at 4ºC, and protein content analysed by the BioRad protein assay. 50 µL of sample was added to 50 µL of GCL reaction buffer containing (in mM): 100 Tris, 10 ATP, 20 L-glutamic acid, 2 EDTA, 20 sodium borate, 2 serine, and 40 $MgCl_2$, and incubated at 37ºC during 5 min. The GCL reaction was initiated by adding 50 µL of 2 mM L-cysteine. After 20 min incubation, the GCL reaction was stopped by adding 50 µL of 200 mM 5-sulfosalicylic acid. Samples were vortexed, incubated on ice for 20 min, and centrifuged at 660×*g* at 4°C, for 5 min. Then, 20 µL of supernatant were transferred into a 96-well plate and 180 µL of 2,3-naphthalenedicarboxaldehyde (NDA) derivatization solution (50 mM Tris, pH 10, 0.5 M NaOH and 10 mM NDA in DMSO, v/v/v 1.4/0.2/0.2, respectively) was then added to each well to form NDA-γ-GC fluorescent complexes. The fluorescence was detected at 472 nm excitation and 528 nm emission, for 30 min, using a Microplate Spectrofluorometer Gemini EM (Molecular Devices, USA). The results were calculated as RFU per min per mg protein.

2.7. Measurement of Superoxide Dismutase (SOD) activity

Determination of SOD activity was performed according to the manual provided by the SOD Assay Kit (Sigma). This method allows SOD assaying by utilizing Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with O_2 is linearly related to the xanthine oxidase (XO) activity and is inhibited by SOD. Therefore, the IC_{50} (50% inhibition activity of SOD) can be determined by this colorimetric method. In order to measure only SOD2 activity, 2 mM potassium cyanide (KCN) was incubated in the cell samples.

2.8. Caspase-3 activity assay

Caspase-3 activity was determined using the fluorogenic substrate Ac (N-acetyl)-Asp-Glu-Val-Asp-AFC (7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC). After washing two times with PBS, cells were lysed in a buffer containing (in mM): 25 HEPES, 2 $MgCl_2$, 1 EDTA and 1 EGTA and 0.04% Triton X-100, supplemented with 2 mM DTT and 1 µg/ml protease inhibitor cocktail (chymostatin, pepstatin A, leupeptin and antipain). Then, a reaction buffer composed by 25 mM HEPES, 10% sucrose and 0.1% CHAPS, supplemented with 2 mM DTT and 15 µM Ac-DEVD-AFC was added to cell samples. The fluorescence was taken during 1 h at 37ºC, with 400 nm excitation and 505 nm emission, using a Microplate Spectrofluorometer Gemini EM (Molecular Devices, USA). After the readings, cell samples were used to determine protein content. The values were obtained as RFU per minute per mg protein for each condition.

2.9. Assessment of necrotic cell death

Necrotic cells were characterized using the fluorescent probe Hoechst 33342 and propidium iodide (PI)-double staining, with some modifications. Briefly, cells were loaded with 7.5 µg/ml Hoechst 33342 and 4 µg/ml PI in PBS for 5 min in the dark. The cells (viable or necrotic) were observed with a fluorescence microscope (Axioskop 2 Plus, Carl Zeiss, Jena, Germany), using a 40x objective and the images were acquired with the Axiovision software 4.2. The cells showing PI labelling due to the rupture of the plasma membrane were defined as necrotic. Viable cells displayed no signs of red PI staining and showed intact nuclei with diffuse Hoechst labelling. The results were expressed as the percentage of total cells. For each coverslip, 100-500 cells were counted in five different fields.

2.10. Alamar Blue assay

Alamar Blue is a cell viability indicator that uses the natural reducing power of living cells to convert resazurin to the fluorescent molecule, resorufin. The active component of Alamar Blue (resazurin) is a nontoxic, cell permeable compound that is blue in color and virtually nonfluorescent. Upon entering cells, resazurin is reduced to resorufin, which produces very bright red fluorescence. Viable cells continuously convert resazurin to resorufin, thereby generating a quantitative measure of viability and thus also cytotoxicity.

Resazurin (1 mg/ml) diluted in RPMI 1640 medium containing the selection antibiotics G418 (250 µg/ml) and hygromycin B (50 µg/ml), was added to SH-SY5Y WT alpha-syn cells in the presence or in the absence of Dox during 2 h at 37ºC. Fluorescence was detected at 530 nm excitation and 590 nm emission, using a Microplate Spectrofluorometer Gemini EM (Molecular Devices, USA). The values obtained were expressed as RFU (Relative Fluorescence Units) per mg protein for each condition.

2.11. Immunocytofluorescence

The culture medium was removed and the cells were washed twice with PBS. Cells were incubated with MitoTracker Red (0.5 μM), a red fluorescent dye that stains mitochondria in live cells and whose accumulation is dependent upon the mitochondrial membrane potential, in sodium saline solution (in mM: 132 NaCl, 4 KCl, 1.2 NaH_2PO_4, 1.4 $MgCl_2$, 10 Hepes, 6 glucose and 1 $CaCl_2$, pH 7.4) during 45 min at 37ºC, in the dark. Cells were washed three times and then fixed with 4% paraformaldehyde in PBS, for 10 min at 4ºC. Three more washes were performed with PBS and then the cell membrane was permeabilized with 0.2% (vol/vol) Triton X-100 (in PBS) during 2 min and blocked with a 3% BSA solution in PBS, for 30 min. The primary antibodies rabbit anti-ubiquitin (Ub) (Dako Cytomation, 1:500), rabbit anti-alpha-syn (Cell Signaling, 1:200), rabbit MAP2 (Chemicon, 1:200) and rabbit TH (Cell Signaling, 1:200) were prepared in PBS 3% BSA and incubated for 1 h at room temperature. Cells were then washed three more times with PBS and the secondary antibody anti-rabbit IgG Alexa-fluor 594 (1:200) was diluted in PBS 3% BSA and incubated for 1 h, at room temperature in the dark. The cells were stained with Hoechst 33342 (4 μg/ml) in PBS for 8 min before the coverslips being mounted using DAKO solution. In experiments performed for analysis of mitochondrial depolarization, cells were visualized with a confocal microscope LSM 510 Meta (Carl Zeiss, Jena, Germany), using a 63x objective. Immunofluorescence for inclusion formation detection was performed by cell staining with primary antibody rabbit anti-Ub (method described above) and cells were visualized with a fluorescence microscope (Axioskop 2 Plus, Carl Zeiss, Jena, Germany), using a 63x objective and the images were acquired with the Axiovision software 4.2.

2.12. PP2A activity assay

Samples from SH-SY5Y cells were sonicated in 20 mM imidazole-HCl, 2 mM EDTA, 2 mM EGTA, pH 7.0, with 1 µg/ml each of pepstatin A, leupeptin, chymostatin, antipain and soybean trypsin inhibitor, 1 mM benzamidine and 1 mM PMSF and centrifuged at 2000 *xg* for 5 minutes, at 4ºC. The supernatant was used to assay phosphatase activity. Triplicate samples were assayed spectrophotometrically at 650 nm relative to known standards using the malachite green assay (Millipore/Upstate, Billerica, MA, USA). Protein concentrations were determined by the BioRad protein assay and data were calculated as pmol/min/µg of protein.

2.13. Subcellular fractionation

2.13.1. Total cell lysates

Cells were washed twice in ice-cold PBS and lysed with a buffer containing (in mM): 100 NaCl; 20 Tris (pH 7.0); 2 EDTA; 2 EGTA and supplemented with 1% Triton X-100, 1 mM PMSF, 1 mM DTT, 50 mM NaF, 1.5 mM sodium orthovanadate and 1 µg/ml protease inhibitor cocktail (chymostatin, leupeptin, antipain and pepstatin A). The lysates were frozen/unfrozen three times in liquid nitrogen and centrifuged (14,000 rpm for 10 min, Eppendorf Centrifuge 5417R) to remove insoluble material. The supernatants were collected, assayed for protein content using the BioRad reagent, according to the manufacturer's instructions and stored at -80ºC.

2.13.2. Nuclear extracts

Briefly, cells were washed twice in ice-cold PBS, resuspended in buffer A (in mM: 10 HEPES, 10 NaCl, 3 $MgCl_2$, 1 EGTA, 0.1% Triton X-100, pH 7.5) supplemented with 50 mM NaF, 1.5

mM sodium orthovanadate, 1 mM DTT, 1 mM PMSF and 1 µg/ml protease inhibitor cocktail (chymostatin, leupeptin, antipain and pepstatin A) and incubated on ice for 40 min. The nuclei were pelleted by centrifugation at 4,700 rpm for 12 min at 4ºC and resuspended in buffer B (in mM: 25 HEPES, 300 NaCl, 5 $MgCl_2$, 1 EGTA, 20% glycerol, pH 7.4) supplemented with 50 mM NaF, 1 mM DTT, 1 mM PMSF plus 1 µg/ml protease inhibitor cocktail. After incubation on ice for 60 min, the lysates were centrifuged at 10,600 rpm for 20 min, at 4ºC. The supernatants (nuclear extracts) were collected, assayed for protein content using the BioRad reagent and stored at -80ºC.

2.13.3. Mitochondrial and cytosolic fractions

Cells were washed twice in sucrose medium (in mM: 250 sucrose, 20 HEPES, 10 KCl, 1.5 $MgCl_2$, 1 EGTA and 1 EDTA, pH 7.4) and resuspended in ice-cold sucrose buffer supplemented with 50 mM NaF, 1.5 mM sodium orthovanadate, 1 mM DTT, 1 mM PMSF and 1 µg/ml protease inhibitor cocktail (chymostatin, leupeptin, antipain and pepstatin A) at 4ºC. Lysates were homogenized in a potter (120 strokes) and centrifuged at 2,300 rpm for 12 min at 4ºC to pellet the nuclei and cell debris. The supernatant was further centrifuged at 10,600 rpm for 20 min (4ºC). The resulting pellet (mitochondrial fraction) was resuspended in supplemented sucrose buffer containing protease inhibitors. Trichloroacetic acid (TCA) was added to the latter supernatant and further centrifuged at 12,400 rpm for 10 min at 4ºC. The resulting pellet (cytosolic fraction) was resuspended in supplemented sucrose buffer and the pH was adjusted to 7.0 with KOH. Protein concentration was determined using the BioRad reagent and samples were stored at -80ºC.

2.14. NADH-ubiquinone oxidoreductase and citrate synthase assays

Culture media was removed, cells were washed and resuspended in sucrose medium, manually homogenized in a potter (120 strokes) and further centrifuged at 2,300 rpm for 12 min at 4ºC to precipitate nuclei and cell debris. Complex I activity was determined at 340

nm by following the decrease in NADH absorbance due to ubiquinone reduction to ubiquinol. The reaction was started by adding the sample to the reaction mixture (in mM: K_2HPO_4, pH 7.2, 10 $MgCl_2$, 0.15 NADH, 2.5 mg/ml BSA fatty-acid free, 1 KCN) containing 50 µM decylubiquinone, at 30ºC. Complex I activity was expressed in nanomoles/minute/milligram protein and corresponds to the rotenone sensitive rate. The enzyme activity was corrected for citrate synthase (CS) activity.

CS activity was performed at 412 nm following the reduction of 0.2 mM 5,5'-dithio-bis(2-nitrobenzoic acid) in the presence of acetyl-CoA and 0.1 mM oxaloacetate in a medium with 100 mM Tris-HCl, pH 8.0 and 0.1% Triton X-100. CS activity was expressed in nanomoles/minute/milligram of protein.

2.15. Western blotting analysis

Equivalent amounts of protein were separated on SDS-PAGE gels and electroblotted onto polyvinylidene difluoride (PVDF) membrane in CAPS/methanol 10% at 0.75 A. The membranes were blocked with 5% skim milk or 5% BSA in TBS (in mM: 25 Tris-HCl, 150 NaCl, pH 7.6,)/0.1% Tween (vol/vol), during 60 min at room temperature and incubated overnight at 4ºC with agitation with antibodies directed against P-Ser129-alpha-syn (Wako Chemicals, 1:1000), alpha-syn (Cell Signaling, 1:1000), AIF (Cell Signaling, 1:1000), Bak (Abcam, 1:2500), Bax (Cell Signaling, 1:500), Bcl-2 (Santa Cruz Biotechnology Inc., 1:500), Cytochrome c (BD Pharmingen, 1:500), P-CREB (Abcam, 1:500), CREB (Cell Signaling, 1:1000), CBP (Cell Signaling, 1:1000), NF-kB p65 (Cell Signaling, 1:500), GCLc (Abcam, 1:1000), 60 kDa heat shock protein (HSP60; Millipore, 1:500), 30 kDa subunit of complex I (Molecular Probes, 1:250), Nrf2 (Abcam, 1:500), PGC-1 alpha (Santa Cruz Biotechnology Inc., 1:500), TBP (Abcam, 1:1000), TFAM (Santa Cruz Biotechnology Inc., 1:500), SOD1 (Abcam, 1:2000), SOD2 (Abcam, 1:5000) alpha-tubulin (Sigma-Aldrich, 1:20,000). The day

after, the membranes were washed three times with 1% TBS-T containing skim milk or BSA, and further incubated with alkaline phosphatase-conjugated secondary antibody (1:20000) during 1 h at room temperature. Proteins were visualized by using an enhanced chemifluorescence reagent (ECF) and the bands were detected with the BioRad VersaDoc 3000 Imaging System.

2.16. Statistical analysis

Data were expressed as the mean±SEM of the number of experiments indicated in the figure legends. Comparisons among multiple groups were analysed by two-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test. Comparisons between two groups/conditions were performed with two tailed Student´s t test. $P<0.05$ was considered significant. Data were analyzed using GraphPad Prism version 5.0 for Windows (Graph Pad Software, San Diego, CA).

2.17. Material

Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM F/12), Optimem, 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), penicillin/streptomycin (P/S), hygromycin B, doxycycline (Dox) and alamar blue (resazurin) were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Fugene 6 was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Secondary antibodies for western blotting and the chemifluorescence reagent (ECF) were acquired from GE Healthcare (Uppsala, Sweden). Fluorogenic caspase-3 substrate VII Ac-Asp-Glu-Val-Asp-AFC (Ac-DEVD-AFC) was from Calbiochem (Darmstadt,

Germany). The fluorescence probes dihydroethidium (DHE), 2',7'-dichlorodihydrofluorescein ($DCFH_2$-DA), MitoSox Red, Hoechst 33342 and MitoTracker Red and the secondary antibody for immnunocytochemistry anti-rabbit Alexa fluor 594 were obtained from Molecular Probes (Invitrogen, USA). All other reagents were of analytical grade.

CHAPTER 3

Linking reactive oxygen species formation to mutant alpha-synuclein aggregation and phosphorylation in SH-SY5Y cells

3.1. Summary

Alpha-synuclein (alpha-syn) is a soluble protein highly enriched in presynaptic terminals of neurons. Moreover, accumulation of alpha-syn as intracellular filamentous aggregates is a pathological feature of sporadic and familial forms of Parkinson's disease (PD). Changes in alpha-syn post-translational modifications, as well as mitochondrial dysfunction and oxidative stress constitute key pathogenic events of this disorder. Here we assessed reactive oxygen species (ROS) formation and its correlation with cell death, alpha-syn aggregation and phosphorylation at serine 129 (Ser129) of A53T mutant *versus* wild-type (WT) alpha-syn in transiently transfected SH-SY5Y neuroblastoma cell lines subjected to prolonged exposure to ferrous iron ($FeSO_4$) and rotenone (complex I inhibitor). Under basal conditions, prolonged expression of A53T mutant alpha-syn increased superoxide formation, ubiquitin-labelled alpha-syn aggregates and phosphorylation at Ser129, which was linked to decreased activity of protein phosphatase 2A (PP2A). Exposure to $FeSO_4$ or rotenone enhanced the production of hydroperoxides and superoxide anion in both types of cells, along with alpha-syn aggregate formation, Ser129 phosphorylation and mitochondrial depolarization. These changes were highly evident in A53T mutant alpha-syn expressing cells and were associated with slight increased cell death following incubation with $FeSO_4$. The data suggest that stimuli that promote ROS formation highly correlate with mutant alpha-syn aggregation and phosphorylation at Ser129, which may precede cell degeneration in sporadic and familial forms of PD.

3.2. Introduction

Parkinson's disease (PD) is the most common age-related neurodegenerative movement disorder, affecting 1-3% of the population over the age of 65 (de Rijk et al., 2000). PD is slowly progressive and is characterized by a loss of dopaminergic neurons in the *substantia nigra pars compacta* (SN) and depletion of dopamine (DA) in the striatum (Dawson and Dawson, 2003). The major pathological hallmark found in PD patients is the presence of cytosolic alpha-synuclein (alpha-syn)-positive inclusions, named Lewy Bodies (LBs) in surviving neurons, although the mechanism that underlies LB biogenesis is poorly understood (Olanow et al., 2004; Shults, 2006). Immunohistochemical and biochemical studies have shown that approximately 90% of alpha-syn deposited in LBs is phosphorylated at serine 129 (Ser129) (Fujiwara et al., 2002; Anderson et al., 2006). In contrast, the proportion of phosphorylated alpha-syn in normal brains is only 4% of total alpha-syn, suggesting that accumulation of phosphorylated Ser129 is involved in the formation of LBs and in the neurodegenerative process associated to PD (Fujiwara et al., 2002).

Alpha-syn is implicated in both sporadic and familial forms of PD, the latter representing a minor proportion of all PD cases. Mutations in the alpha-syn gene lead to autosomal dominant PD and are of crucial relevance to predict the susceptibility to develop the disease. A missense mutation in this protein, A53T, was the first defined genetic lesion in familial PD (Polymeropoulos et al., 1997). Two additional point mutations linked to autosomal dominant early-onset PD have subsequently been described, namely A30P (Kruger et al., 1998) and E46K (Zarranz et al., 2004). Therefore, it is important to understand the molecular mechanisms involved in wild-type (WT) and mutant alpha-syn toxicity and particularly the effect of prolonged exposure to neurotoxic stimuli that have been hypothesized to modify disease progression. Although the defined mechanisms by which neurons die in PD is still unknown, it has been suggested that mitochondrial dysfunction (Schapira, 2008; Schapira, 2004), oxidative stress (Jenner, 2003), inflammatory

response (McGeer and McGeer, 2004) and protein misfolding may play an important role in the pathogenesis of sporadic PD (Greenamyre et al., 2003).

Mitochondrial dysfunction has long been implicated in PD pathogenesis due to reduced activity in complex I (Olanow and Tatton, 1999; Lin and Beal, 2006). The administration of rotenone, an inhibitor of mitochondrial complex I, induced the major pathological and behavioral features of PD in animal and cellular models (Betarbet et al., 2000; Sherer et al., 2007; Sherer et al., 2003; Matsuzaki et al., 2004). In addition, multiple studies suggested an association between pesticides and metals and the incidence of PD (Gorell et al., 1998; Gorell et al., 1999). Several lines of evidence indicate that iron ions play an important role in PD pathogenesis, where the most severely affected neurons are located in the SN and *locus coeruleus*. Interestingly, these brain areas are enriched with neuromelanin that sequesters reactive metals, mainly iron. Indeed, a correlation between increased iron levels and the severity of neuropathological changes in PD patients was observed (Gotz et al., 2004). Additionally, high levels of ferric ions have been found in LBs (Gaeta and Hider, 2005). An increase in iron levels seems to be an early event in patients at risk for developing PD and precedes loss of dopaminergic neurons (Berg, 2007; Michaeli et al., 2007; Oakley et al., 2007; Tofaris et al., 2007). Recent studies demonstrated that high concentrations of iron induce alpha-syn aggregation in dopaminergic cells (Li et al., 2011) and that a specific interaction between iron toxicity and alpha-syn aggregation occurs in a neuroblastoma cell line overexpressing this protein (He et al., 2011). However, the mechanisms underlying iron-induced alpha-syn aggregation are still not clear.

Phosphorylation of alpha-syn at Ser129 was previously described to play a critical role in the pathological mechanisms of neuronal death (Sugeno et al., 2008). Furthermore, *in vitro* studies demonstrated that oxidative stress induces alpha-syn phosphorylation at Ser129, an effect blocked by treatment with the antioxidant ascorbic acid (Takahashi et al., 2007). However, there has been some controversy regarding the role of phosphorylated alpha-syn at Ser129 in fly and rat models. In a transgenic *Drosophila* model of PD, Chen and Feany showed that alpha-syn phosphorylation at Ser129 by G-protein-coupled receptor kinase 2

(GRK2) homolog enhanced alpha-syn neurotoxicity (Chen and Feany, 2005). On the other hand, in rat recombinant adeno-associated virus (rAAV)-based models of PD, the expression of phosphorylation mimic S129D mutant alpha-syn did not display toxicity in dopaminergic neurons (Gorbatyuk et al., 2008; Azeredo da et al., 2009; McFarland et al., 2009). Sugeno and colleagues (2008) further demonstrated that alpha-syn phosphorylation at Ser129 induced intracellular aggregate-formation and endoplasmic reticulum stress in alpha-syn overexpressing cells exposed to low doses of rotenone(Sugeno et al., 2008). More recently, Sato et al. showed that phosphorylated alpha-syn at Ser129 accelerated A53T mutant alpha-syn neurotoxicity in the rAAV-based rat model (Sato et al., 2011). The kinases and phosphatases that modulate alpha-syn phosphorylation have been of major interest, in part as they may provide therapeutic targets. Protein phosphatase 2A (PP2A) is a ubiquitous serine/threonine phosphatase with a wide range of substrates and functional roles. Importantly, the PP2A isoform that contains the Bα subunit appears to have a strong ability to dephosphorylate alpha-syn at Ser129 (Lee et al., 2011; Braithwaite et al., 2012).

In the present work, we analysed the effects of prolonged exposure to oxidative stress and mitochondrial dysfunction, induced by iron and rotenone, respectively, in a neuroblastoma SH-SY5Y cell model, expressing WT or mutant A53T alpha-syn. Since previous results demonstrated a correlation between the effects caused by ferrous iron and WT alpha-syn (Li et al., 2011), we compared the susceptibility caused by $FeSO_4$ in WT and mutant A53T alpha-syn expressing cells; moreover, the classical inhibitor of mitochondrial complex I, rotenone, is also known to induce oxidative stress (Greenamyre et al., 2003; Sugeno et al., 2008). We report effects of increased reactive oxygen species (ROS) production, which correlate with mitochondrial dysfunction, alpha-syn inclusion formation and phosphorylation at Ser129, along with a decrease in PP2A activity (particularly in the case of iron). Furthermore, we demonstrate a higher susceptibility of mutant A53T alpha-syn expressing cells for these stimuli.

3.3. Results

We initiated the work by evaluating the expression of alpha-syn constructs in SH-SY5Y cells. Both WT alpha-synGFP and A53T alpha-synGFP expression vectors were highly expressed for up to five days in culture, with similar transfection efficiencies after this period of time (Figure. 3.1A, B).

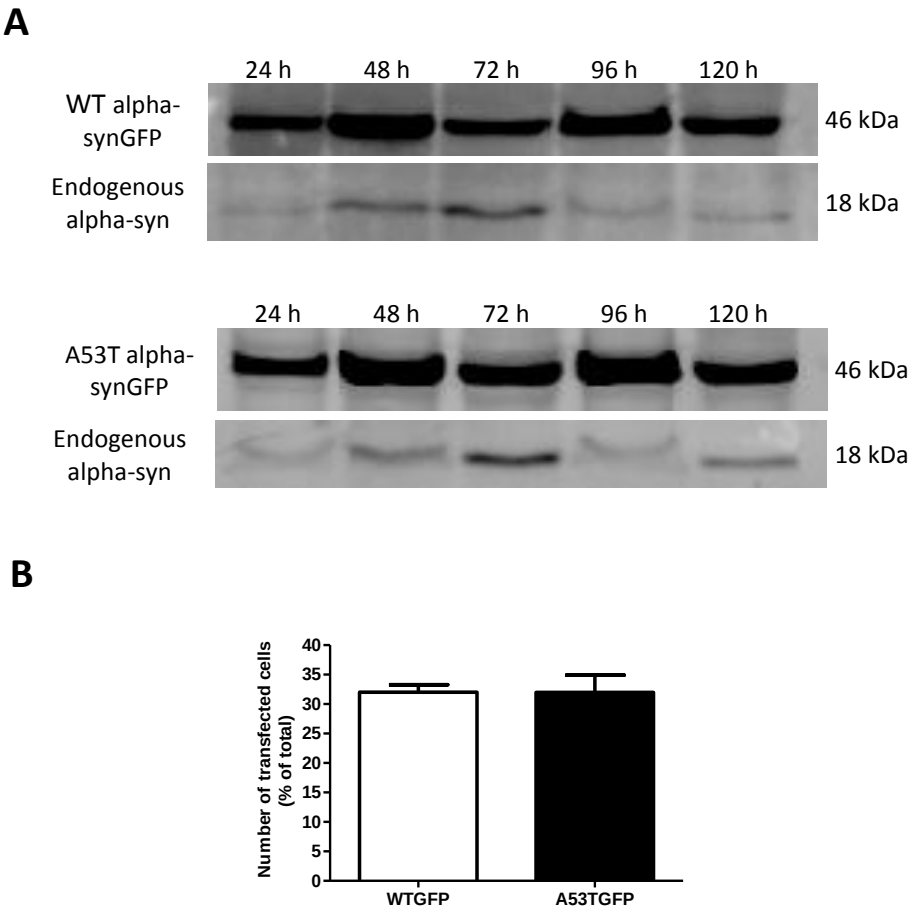


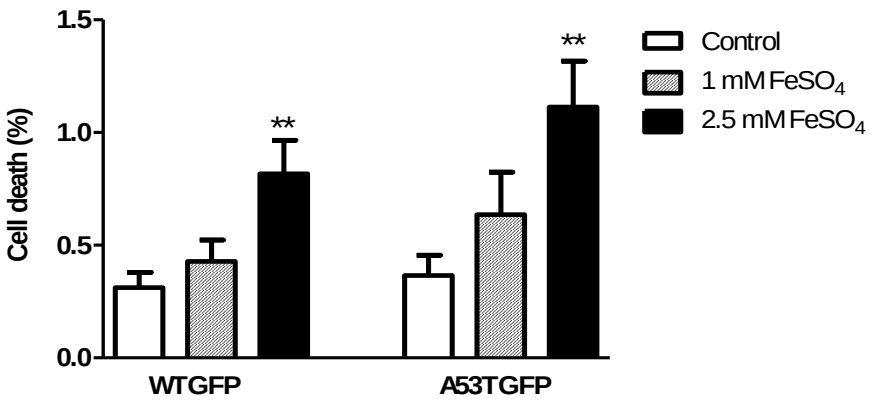
Figure 3.1. Levels of WT or A53T alpha-synGFP expression in a transiently transfected PD cellular model. SH-SY5Y neuroblastoma cells were transiently transfected with WT or A53T alpha-synGFP during 1 to 5 days and total cellular extracts were immunoblotted to confirm protein

overexpression over time (**A**). In **B**, SH-SY5Y cells expressing WT or A53T alpha-synGFP were fixed, nuclear stained with Hoechst 33342 and observed by fluorescent microscopy at day 4 after transfection. The graph plots the percentage of cells expressing WT or mutant (A53T) alpha-synGFP fluorescent fusion proteins in three independent transfections.

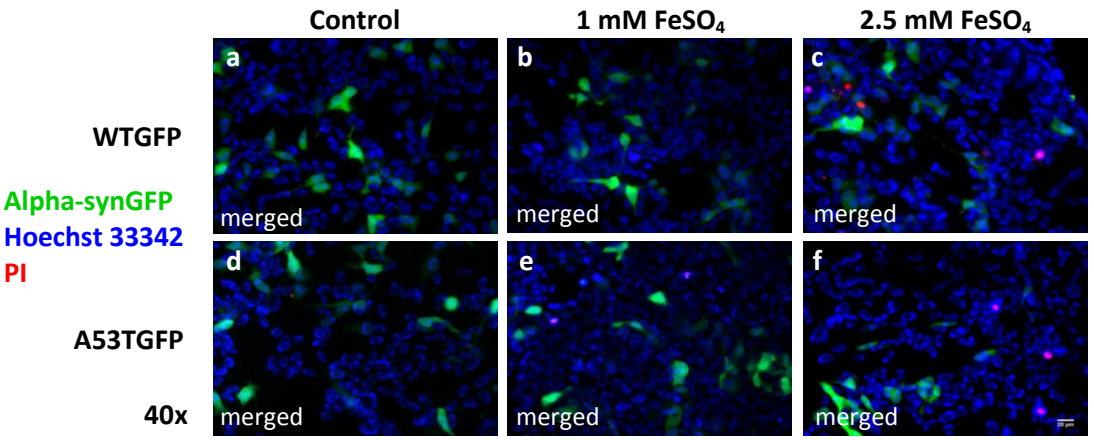
3.3.1. Effect of iron and rotenone on the viability of SH-SY5Y cells overexpressing alpha-syn

In order to investigate the susceptibility to prolonged (4 days) exposure to iron or rotenone, we evaluated cell death occurring by necrosis, by fluorescence microscopy of Hoechst and PI in SH-SY5Y cells transfected with WT or A53T alpha-synGFP (Figure. 3.2). Based on previous studies involving the effects of oxidative stress and PD, we selected a range of concentrations between 1 to 2.5 mM for iron and 10 to 100 nM for rotenone (Sherer et al., 2001; Takahashi et al., 2007). These concentrations of rotenone were previously described to inhibit complex I activity by approximately 50% (Sherer et al., 2001). In SH-SY5Y cells expressing WT alpha-synGFP, the percentage of total necrotic cells was significantly increased after exposure to 2.5 mM $FeSO_4$ (Figure 3.2A and 3.2Bc) when compared to control WT alpha-syn cells. Notably, the percentage of cell death was less than 1% (0.82%). In SH-SY5Y cells expressing A53T alpha-synGFP, the percentage of total necrotic cells significantly raised to 1.1% after incubation with 2.5 mM $FeSO_4$ (Figure 3.2A and 3.2Bf), when compared with untreated A53T alpha-syn cells. Exposure to rotenone (10 and 100 nM) did not cause substantial cell death in either WT or A53T alpha-syn transfected SH-SY5Y cells (Figure 3.2C and 3.2D).

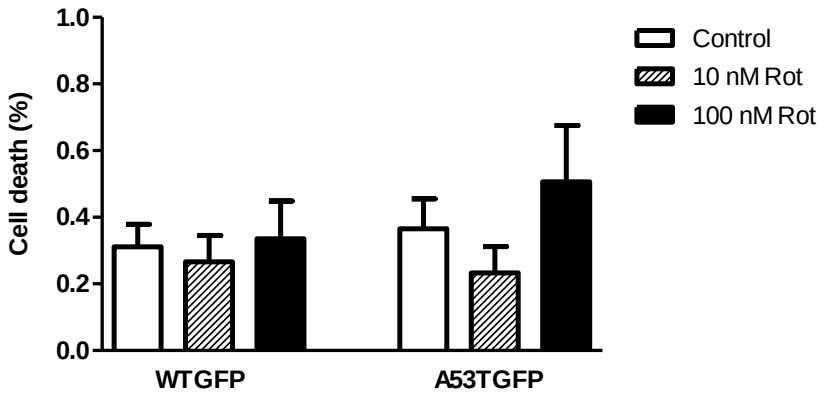
A


Cell death (%)
1.5
1.0
0.5
0.0
**
**
WTGFP
A53TGFP
Control
1 mM FeSO4
2.5 mM FeSO4

B


Control
1 mM FeSO4
2.5 mM FeSO4
WTGFP
Alpha-synGFP
Hoechst 33342
PI
A53TGFP
40x
a
b
c
d
e
f
merged

C


Cell death (%)
1.0
0.8
0.6
0.4
0.2
0.0
WTGFP
A53TGFP
Control
10 nM Rot
100 nM Rot

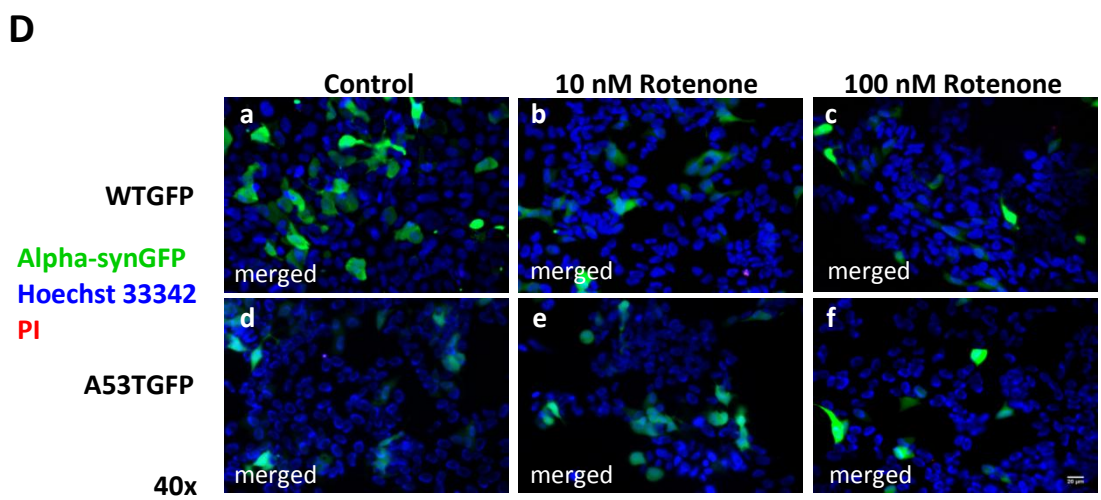


Figure 3.2. Increased necrotic cell death caused by exposure to high iron concentration – relevance for A53T mutation. Cells were transfected with WT or A53T alpha-synGFP and incubated with $FeSO_4$ (**A**, **B**) or rotenone (**C**, **D**) for four days. At the day of the experiment, cells plated in coverslips were incubated with Hoechst 33342 and propidium iodide (PI), visualized by fluorescence microscopy and counted in five different fields. Results from iron incubations are shown in **A**, while the results from rotenone are depicted in **C**. **B** and **D** show representative images of the cells. Data are the mean±SEM of three independent experiments performed in triplicates. Statistical analysis: ** $p<0.01$ compared to the respective control (transfected cells not exposed to iron or rotenone).

These results show that high $FeSO_4$ concentrations (2.5 mM) significantly affected SH-SY5Y cells viability following overexpression of WT or A53T alpha-synGFP, although significant necrotic cell death never raised above 1.5%, and PI labelling cells rarely co-localized with alpha-synGFP transfected cells, suggesting that these stimuli were not sufficient to cause massive necrosis. Features of apoptotic cell death, namely Hoechst-stained chromatin condensation/fragmentation or cell shrinkage were not observed throughout the experiments in WT or A53T alpha-syn expressing cells exposed to iron or rotenone either. Notably, no cell death could be attributed to transfected (WT or A53T alpha-synGFP) *versus* non-transfected cells in the absence of the toxic stimuli (not shown).

3.3.2. Reactive oxygen species production after exposure to $FeSO_4$ and rotenone

In order to evaluate features of oxidative stress induced by both iron and rotenone and to explain the slightly higher susceptibility of alpha-synGFP transfected SH-SY5Y cells exposed to iron, we examined the production of endogenous ROS. The levels of hydroperoxides and superoxide anion were analysed by measuring $DCFH_2$-DA and hydroethidium fluorescence, respectively.

We first analysed hydroperoxide generation by following DCF fluorescence in non-transfected SH-SY5Y cells, in comparison with cells transiently expressing WT or A53T alpha-synGFP. As shown in Figure 3.3A no significant changes in ROS were detected between the three conditions tested. Although we did not observe significant differences in ROS formation between untreated WT and A53T alpha-synGFP cells, the levels of hydroperoxides increased upon prolonged (4 days) exposure to 1 or 2.5 mM $FeSO_4$ in both WT and A53T alpha-synGFP cells, when compared to the respective controls (Figure 3.3B). Endogenous ROS production was largely increased in SH-SY5Y cells transfected with A53T alpha-synGFP, when compared to the untreated condition (control), after prolonged exposure to the complex I inhibitor rotenone at 10 and 100 nM (Figure 3.3C). Taking into account the effects generated by the highest concentrations of iron and rotenone, we further analysed ROS formation after short exposure (up to 2h) to these stimuli in cells expressing WT and A53T alpha-synGFP for 24 h. Interestingly, we detected a significant increase in DCF fluorescence after 2 h of incubation with 2.5 mM $FeSO_4$ or 100 nM rotenone in A53T alpha-synGFP cells only (Figure 3.3D and 3.3E).

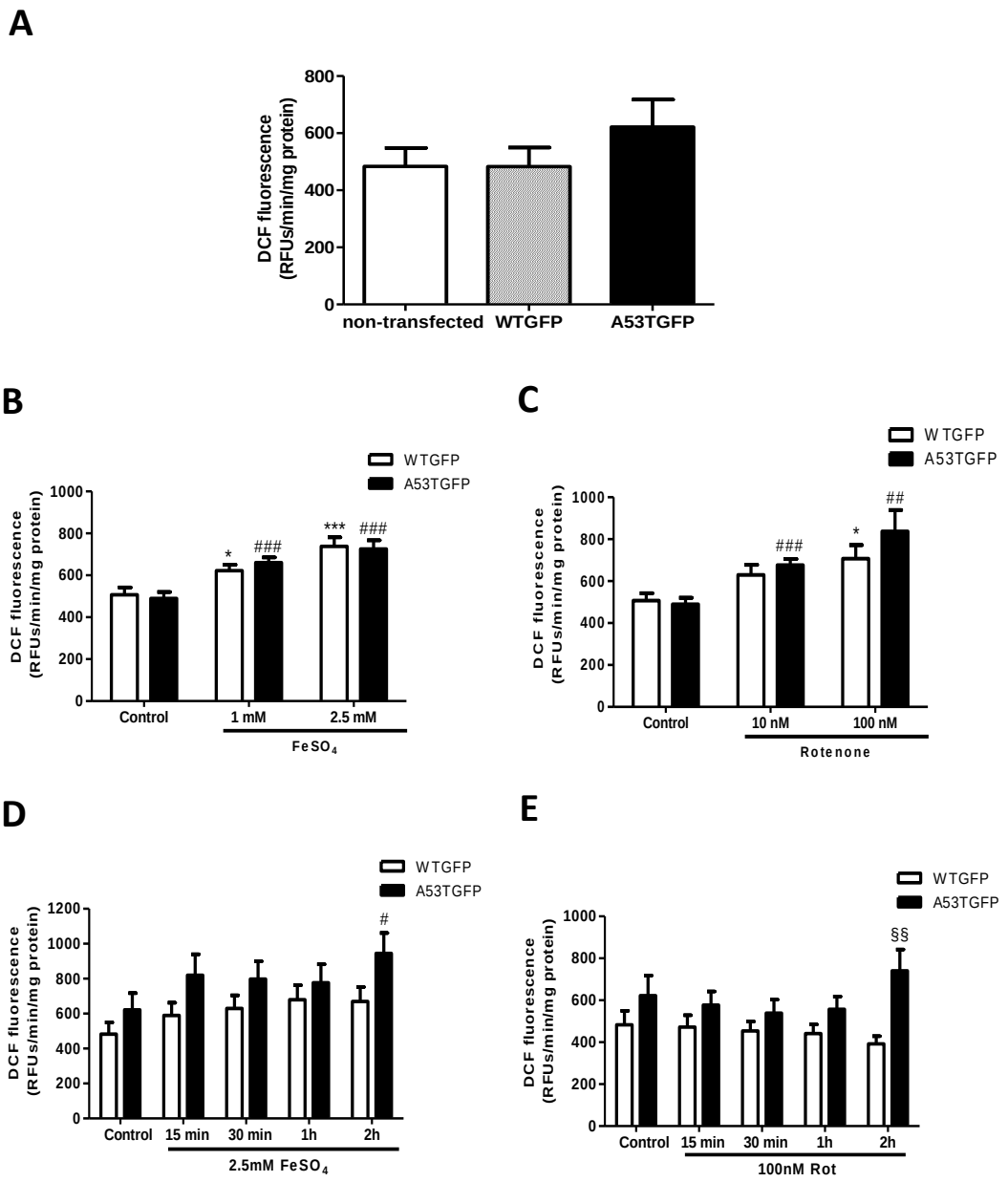


Figure 3.3. Hydroperoxide production in WT and A53T alpha-synGFP after exposure to iron or rotenone for different periods of time. Hydroperoxide generation was analysed by DCF fluorescence in non-transfected SH-SY5Y cells and transfected with WT or A53T alpha-synGFP for 24 h (**A**). SH-SY5Y cells were transfected with WT or A53T alpha-synGFP and further incubated with

$FeSO_4$ (**B, D**) or rotenone (**C, E**) during 4 days (**B, C**) and short periods of time (**D**, **E**). In all experiments, levels of hydroperoxides were measured following DCF fluorescence after 30 min of incubation with 20 μM $DCFH_2$-DA. Data in the graphs are the mean±SEM of three independent experiments performed in quadruplicates. In **B**: *p<0.05 and ***p<0.001 related to the WTGFP control; ###p<0.001 compared to A53TGFP control. In **C**: *p<0.05 related to WTGFP control; ##p<0.01 and ###p<0.001 related to A53TGFP control. In **D** and **E**: #p<0.05 related to A53TGFP control; §§p<0.01 related to WT alpha-synGFP under the same experimental conditions. $DCFH_2$DA – 2'7'-dichlorodihydrofluorescein diacetate

By measuring ethidium fluorescence, we observed that SH-SY5Y cells overexpressing A53T alpha-synGFP under basal conditions were endowed with a significantly higher amount of superoxide radical, compared to WT alpha-synGFP transfected cells (Figure 3.4 A,B). Increased ethidium fluorescence observed in A53T alpha-synGFP overexpressing cells was not largely affected following exposure to 2.5 mM $FeSO_4$ (for 4 days) (Figure 3.4A). Moreover, prolonged exposure to high rotenone concentration (100 nM) caused a significant rise in superoxide anion production in SH-SY5Y cells overexpressing either WT or A53T alpha-synGFP, when compared to the respective untreated controls (Figure 3.4B) and to cells treated with 10 nM rotenone. No significant changes in superoxide anion formation were observed in cells exposed to 1 mM iron or 10 nM rotenone for 4 days (Figure 3.4 A,B). As described for DCF fluorescence, we also tested the effect of short exposure (up to 2h) to the highest concentrations of iron and rotenone (2.5 mM and 100 nM, respectively); however, no changes in superoxide production were observed under these conditions (Figure 3.4C, D).

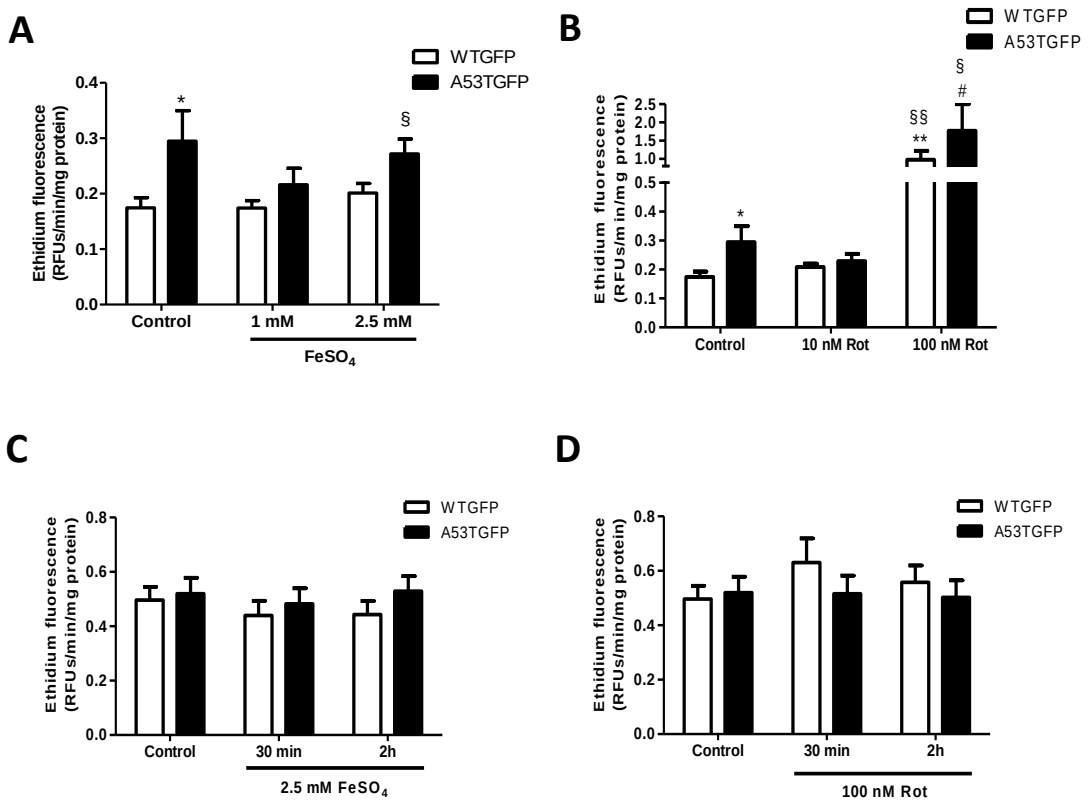


Figure 3.4. Levels of superoxide anion production in WT and A53T alpha-synGFP after exposure to iron or rotenone for different periods of time. SH-SY5Y cells were transfected with WT or A53T alpha-synGFP and further incubated with $FeSO_4$ (**A, C**) or rotenone (**B, D**) during 4 days (**A, B**) and short periods of time (**C**, **D**). The levels of superoxide anion were determined by following ethidium fluorescence after a pre-incubation with 5 µM DHE for 1 h. Data in the graphs are the mean±SEM of three independent experiments performed in quadruplicates. In **A**: *p<0.05 related to the respective control and §p<0.05 related to cells expressing WT alpha-synGFP after incubation with 2.5mM $FeSO_4$. In **B**: *p<0.05, **p<0.01 related to WTGFP control; #p<0.05 related to A53TGFP control; §p<0.05 and §§p<0.01 related to cells incubated with 10 nM rotenone.; DHE – dihydroethidine.

These results demonstrate that mutant A53T alpha-syn cells exhibit a higher production of superoxide anions and increased susceptibility to oxidative stress upon exposure to iron and rotenone.

We further analysed the free radical generated in the initial period of exposure to 2.5 mM $FeSO_4$ and 100 nM rotenone. Electron paramagnetic resonance (EPR) experiments were performed in the presence of DMPO. Interestingly, we observed a rapid generation of a radical by following immediate exposure to $FeSO_4$, which unfortunately was not possible to simulate or identify (Figure 3.5B). SH-SY5Y cells in the presence of DMPO only did not generate a radical spectrum (Figure 3.5A). As in non-transfected cells, formation of a similar radical was observed in WT and A53T alpha-syn expressing cells incubated with iron and immediately scrapped in the culture medium (Figure 3.5C, D). No radical could be detected in cells treated with rotenone in initial periods of exposure (data not shown).

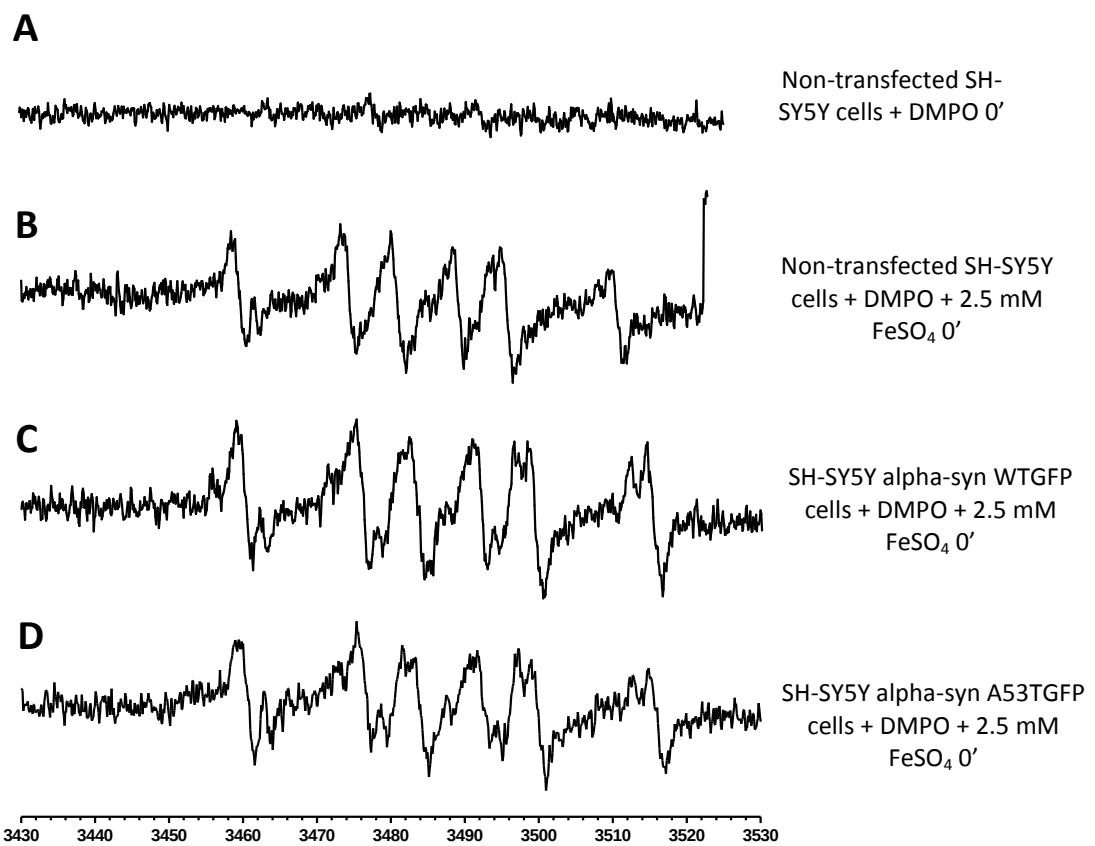


Figure 3.5. EPR spin-trapping of a radical generated from $FeSO_4$ in the presence of DMPO. **A** represents the experimental spectrum recorded in SH-SY5Y cells incubated with DMPO (100 mM) only. Spectrum represented in **B** shows non-transfected SH-SY5Y cells exposed to 2.5 mM $FeSO_4$. **C** and **D** constitute representative experimental spectra from WT and A53T alpha-syn overexpressing SH-SY5Y cells in the presence of 2.5 mM $FeSO_4$.

3.3.3. Effect of iron and rotenone on mitochondrial changes

Since SH-SY5Y cells overexpressing A53T alpha-synGFP exhibited higher oxidative stress upon exposure to iron and rotenone, and generation of superoxide anion occurred in the absence of toxic stimuli, we further analysed the relative changes in mitochondrial function and morphology using MitoTracker Red, a fluorescent compound sensitive to changes in mitochondrial membrane potential.

In untreated condition, control cells expressing WT alpha-synGFP displayed intact, well defined mitochondria, distributed throughout the cell body and with a tubular morphology (Figure 3.6A: b-c), suggesting functional organelles. On the other hand, SH-SY5Y cells expressing WT alpha-synGFP incubated with 2.5 mM iron during four days exhibited aggregated mitochondria (Figure 3.6Af-insert), possibly resulting from increased fragmentation. Moreover, a decrease in MitoTracker Red fluorescence was evident, indicating mitochondrial depolarization (Figure 3.6A: e-f). A reduction in MitoTracker Red fluorescence was also observed in cells overexpressing WT alpha-synGFP and further incubated with 100 nM rotenone, when compared to control/untreated conditions. Rotenone-treated mitochondria also lost the tubular shape and appeared fused presenting a faint MitoTracker Red fluorescence, largely evidencing mitochondrial depolarization (Figure 3.6A: h-i). Interestingly, expression of mutant A53T alpha-synGFP induced some mitochondrial aggregation (Figure 3.6Bc-insert), similarly to WT cells treated with iron, although mitochondrial potential was not largely affected. In mutant cells treated with 2.5 mM $FeSO_4$, we observed a dramatic decrease in MitoTracker Red fluorescence, suggesting a

high degree of mitochondrial depolarization, and a complete loss of normal mitochondrial tubular network (Figure 3.6B: e-f). This effect was more pronounced when compared to WT alpha-synGFP transfected cells in the same experimental condition (Figure 3.6A: e-f). A53T alpha-synGFP cells exposed to 100 nM rotenone also presented a reduction in MitoTracker Red fluorescence and some cells showed aggregated mitochondria (Figure 3.6B: h-i); however, the effect of this prolonged exposure was less dramatic when compared to mutant cells treated with 2.5 mM iron.

Although mitochondrial changes could not be specifically attributed to cells expressing alpha-synGFP, in the population of cells transfected with mutant A53T alpha-synGFP we detected the highest degree of mitochondrial depolarization, which seems to correlate with the slight increase in the number of cells undergoing necrotic cell death (Figure 3.2A, B), also exhibiting increased ROS formation (Figure 3.3 and 3.4).

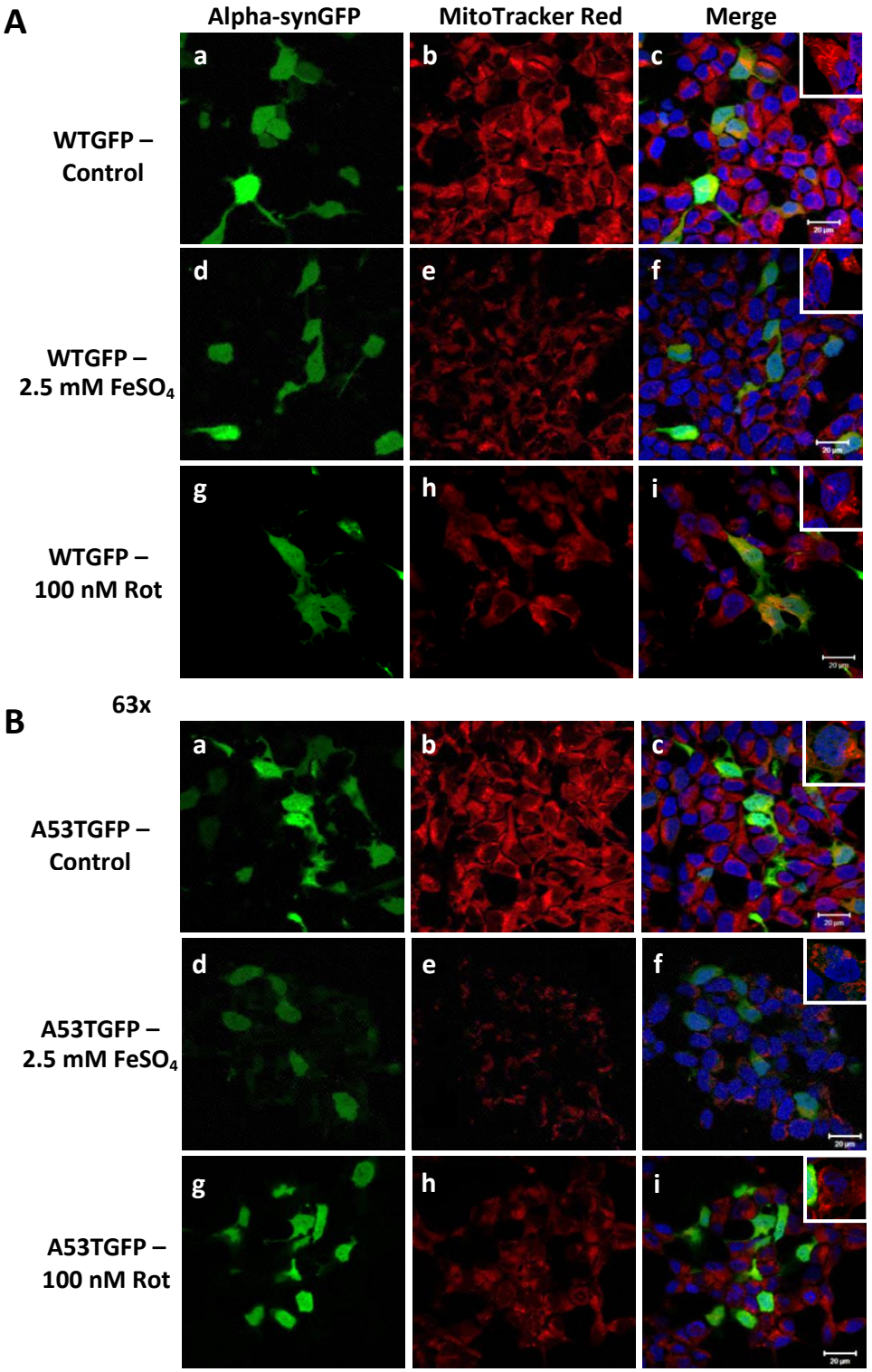

A
Alpha-synGFP
MitoTracker Red
Merge
a
b
c
WTGFP –
Control
d
e
f
WTGFP –
2.5 mM FeSO4
g
h
i
WTGFP –
100 nM Rot
63x
B
a
b
c
A53TGFP –
Control
d
e
f
A53TGFP –
2.5 mM FeSO4
g
h
i
A53TGFP –
100 nM Rot

Figure 3.6. Mitochondrial depolarization and morphological changes induced by iron or rotenone on WT or A53T alpha-synGFP overexpression cells. SH-SY5Y cells were transfected with WT (**A**) or A53T (**B**) alpha-synGFP and further exposed to 2.5 mM $FeSO_4$ (**d-f**) or 100 nM (**g-i**) rotenone during four days. Cells were incubated with MitoTracker Red, labeled with Hoechst 33342 and observed by confocal microscopy. Images represent one of five fields of a coverslip prepared for each condition and selected from three independent experiments. Insert pictures show high amplification of mitochondria in WT or A53T alpha-synGFP transfected cells submitted to iron or rotenone treatment.

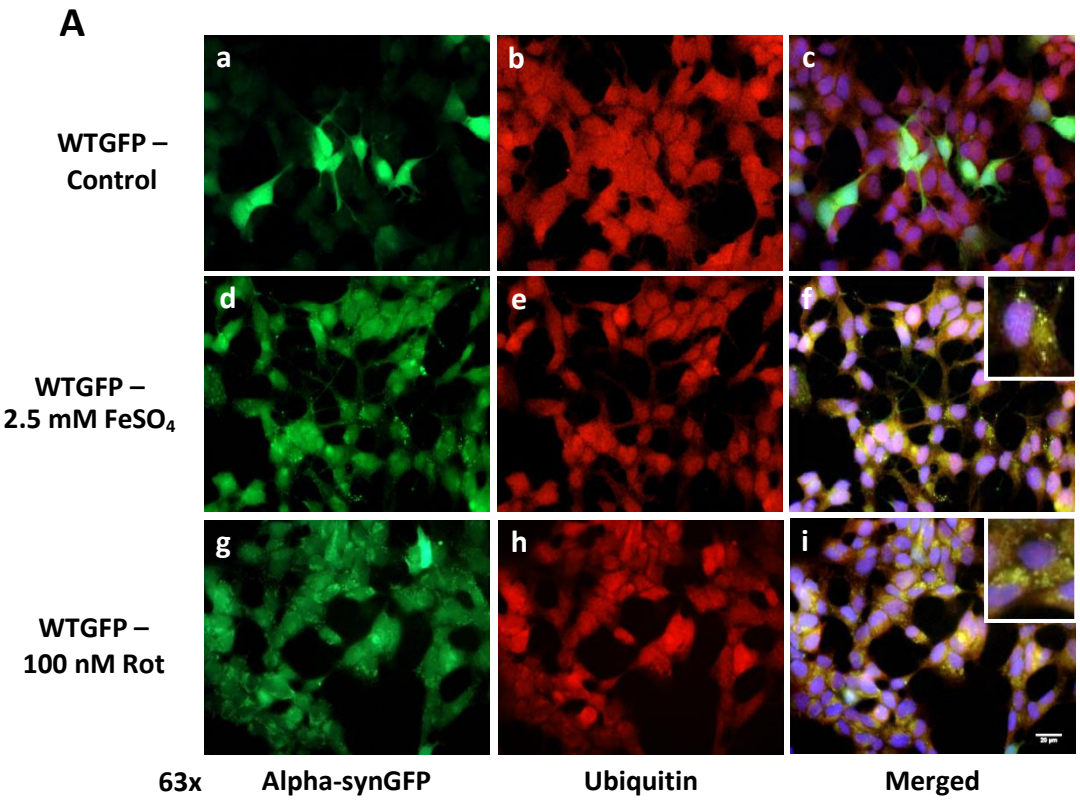
3.3.4. Alterations in alpha-syn conformation after prolonged exposure to iron or rotenone

Aggregation of alpha-syn under oxidative stress conditions has been described by several groups in different PD models (Takahashi et al., 2007; Li et al., 2011). Therefore, we investigated whether SH-SY5Y cells overexpressing WT or A53T alpha-synGFP were able to form alpha-syn aggregates that co-localized with ubiquitin (Ub, a protein highly present in LBs from PD patients) after prolonged exposure to iron or rotenone.

Immunocytofluorescence analysis showed that transiently transfected SH-SY5Y cells were able to form alpha-syn inclusions upon prolonged exposure to iron or rotenone (Figure 3.7). A homogeneous co-localized distribution of alpha-syn and Ub proteins was observed in WT alpha-synGFP cells (Figure 3.7A: a-c). After four days exposure to 2.5 mM $FeSO_4$, SH-SY5Y cells overexpressing WT alpha-synGFP showed an increase in alpha-syn inclusions that were also immunopositive for Ub (Figure 3.7A: d-f). Furthermore, treatment with 100 nM rotenone during four days induced the formation of inclusions that co-localized with Ub (Figure 3.7A: g-I).

In mutant A53T alpha-synGFP expressing cells submitted to the same conditions, we detected that non-treated cells (control) *per se* presented some alpha-syn inclusions (Figure 3.7B: a-c), when compared to those expressing WT alpha-synGFP (Figure 3.7A: a-c).

Moreover, SH-SY5Y cells overexpressing A53T alpha-synGFP exposed to 2.5 mM iron during four days evidenced the presence of alpha-syn inclusions that were immunopositive for Ub (Figure 3.7B: d-f). In cells overexpressing A53T alpha-synGFP treated with 100 nM rotenone, we also detected a marked increase in the formation inclusions (Figure 3.7B: g-i).



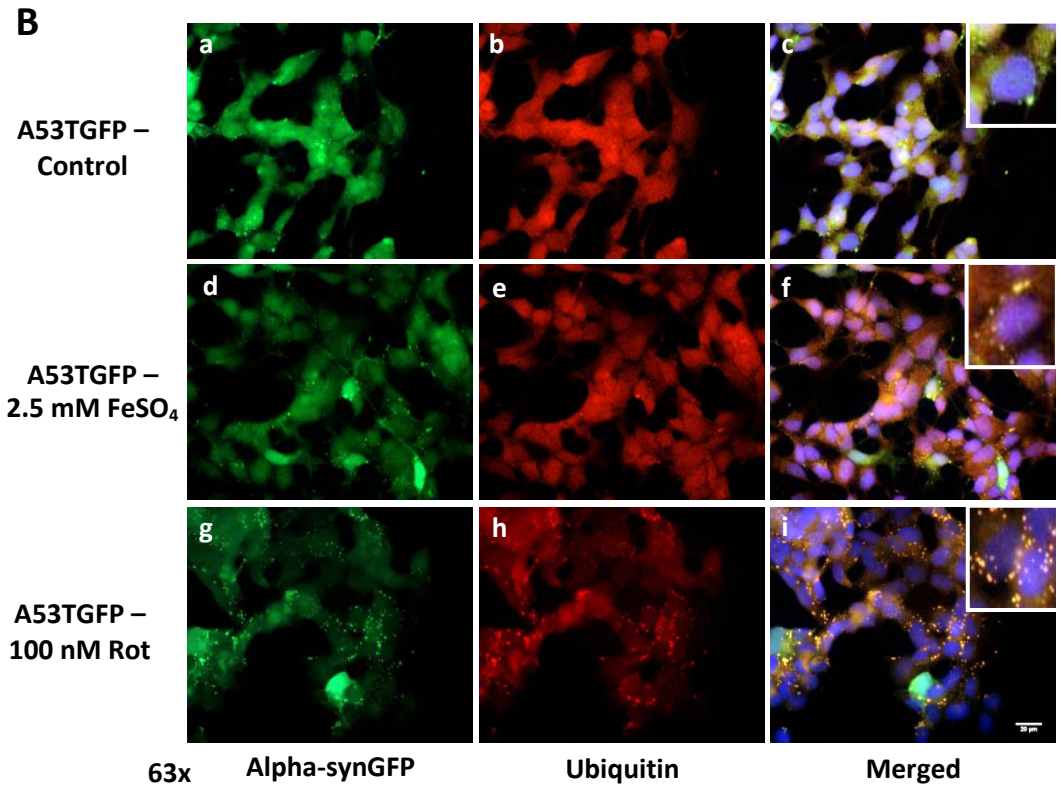


Figure 3.7. Iron or rotenone prolonged incubation promote Ub-labelled alpha-syn inclusions in SH-SY5Y cells. Cells were transfected with WT (**A**) or A53T (**B**) alpha-synGFP and subjected to a daily incubation (during 4 days) with $FeSO_4$ (2.5 mM) (**d-f**) or rotenone (100 nM) (**g-i**). SH-SY5Y cells were immunostained for ubiquitin (red) while alpha-synGFP transfected cells appear in green. Nuclei were stained with Hoechst 33342. Insert pictures show high amplification of intracellular aggregates formed after incubation with iron or rotenone in cells overexpressing WT or A53T alfa-synGFP.

These results suggest that prolonged oxidative stress stimuli and mitochondrial inhibition lead to alpha-syn inclusion formation in SH-SY5Y cells, an effect that is more pronounced in cells overexpressing A53T alpha-syn GFP.

3.3.5. Alpha-syn phosphorylation upon exposure to rotenone and iron – role of PP2A

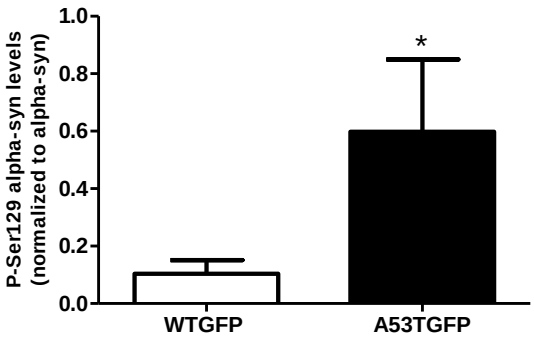
To correlate the susceptibility to oxidative stress, mitochondrial inhibition and inclusion formation in cells expressing WT or mutant A53T alpha-synGFP with alpha-syn post-translational modifications, we examined the levels of phosphorylated alpha-syn at Ser129, a common alpha-syn modification present in LBs (Fujiwara et al., 2002; Anderson et al., 2006). Following transfection of WT or A53T alpha-synGFP, SH-SY5Y cells were incubated during four consecutive days with $FeSO_4$ or rotenone in order to evaluate the effects of oxidative stress and mitochondrial dysfunction on alpha-syn phosphorylation at Ser129 (P-Ser129 alpha-syn).

Under basal conditions, A53T alpha-syn transfected cells presented significant higher levels of P-Ser129 alpha-syn when compared to WT cells (Figure 3.8A). The results depicted in Figure 3.8B show higher P-Ser129 levels in WT alpha-syn cells subjected to iron treatment, when compared to the respective non-treated condition. Furthermore, 2.5 mM $FeSO_4$ exposure largely increased P-Ser129 levels in A53T alpha-synGFP cells, compared to the untreated control, evidencing a major role for mutant alpha-syn. Indeed, this effect was also significantly detectable for 1 mM iron concentration, although it was more pronounced for the highest concentration of iron (2.5 mM). WT or A53T alpha-synGFP transfected cells were also incubated with 10 and 100 nM rotenone. Despite a great tendency for enhanced P-Ser129 of WT alpha-syn in the presence of this mitochondrial inhibitor, no significant changes were observed. However, mutant A53T transfected cells displayed a significant increase in P-Ser129 alpha-syn levels after exposure to 100 nM rotenone, when compared to untreated cells (Figure 3.8C). These data suggest that both iron and rotenone, ascribed to increased ROS formation, increase P-Ser129 levels, particularly in cells overexpressing A53T alpha-synGFP.

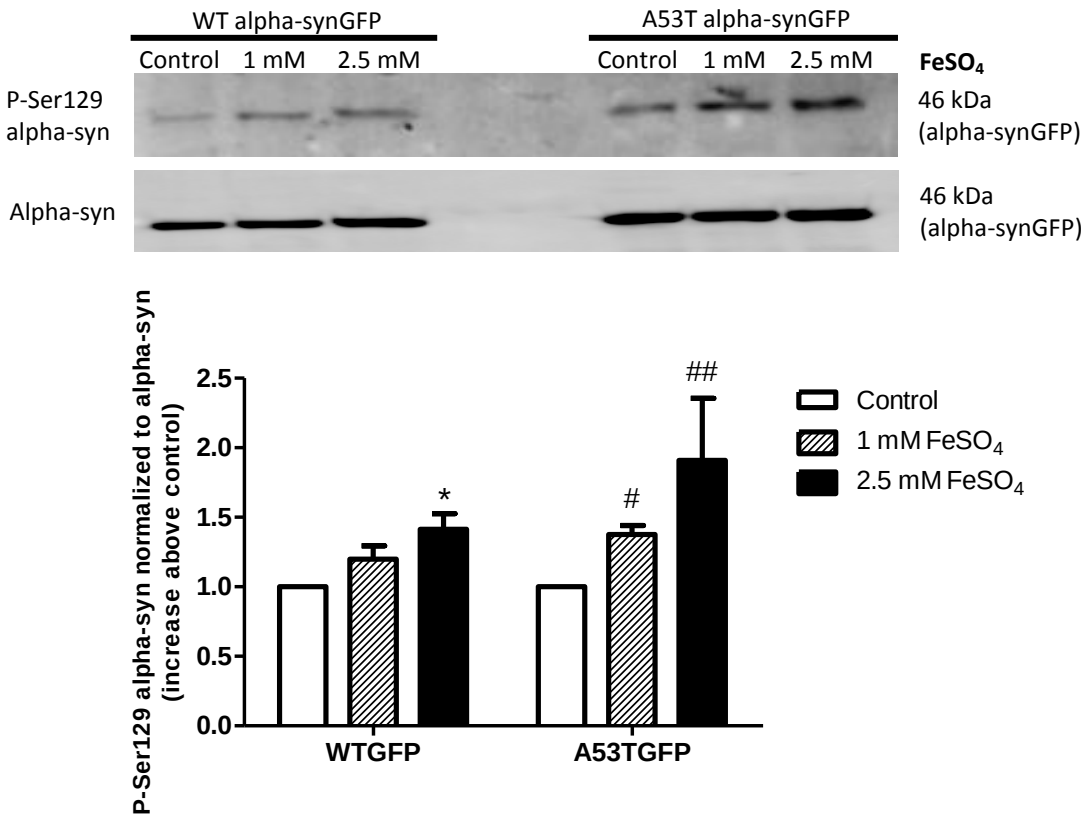
Given the evidence for higher levels of P-Ser129 after prolonged exposure to iron (2.5 mM) or rotenone (100 nM), we also analysed PP2A activity in transfected WT and A53T alpha-syn SH-SY5Y cells under the same experimental conditions, as this phosphatase was

previously shown to dephosphorylate alpha-syn at this specific residue (Lee et al., 2011). Concordantly with data shown in Figure 3.8A, PP2A activity was effectively diminished in untreated A53T alpha-synGFP transfected cells, compared to cells overexpressing the WT protein (Figure 3.8D). Furthermore, we observed a slight decrease in this phosphatase activity in cells expressing A53T alpha-synGFP following 4-day treatment with $FeSO_4$, compared to WT alpha-synGFP cells under the same experimental condition. No changes were detected after prolonged exposure to 100 nM rotenone in both transfected cells, which suggests that different toxic stimuli evoke alpha-syn phosphorylation through distinct mechanisms.

A


P-Ser129 alpha-syn levels
(normalized to alpha-syn)
1.0
0.8
0.6
0.4
0.2
0.0
*
WTGFP
A53TGFP

B


WT alpha-synGFP
A53T alpha-synGFP
Control
1 mM
2.5 mM
Control
1 mM
2.5 mM
FeSO4
P-Ser129
alpha-syn
46 kDa
(alpha-synGFP)
Alpha-syn
46 kDa
(alpha-synGFP)
P-Ser129 alpha-syn normalized to alpha-syn
(increase above control)
2.5
2.0
1.5
1.0
0.5
0.0
*
#
##
WTGFP
A53TGFP
Control
1 mM FeSO4
2.5 mM FeSO4

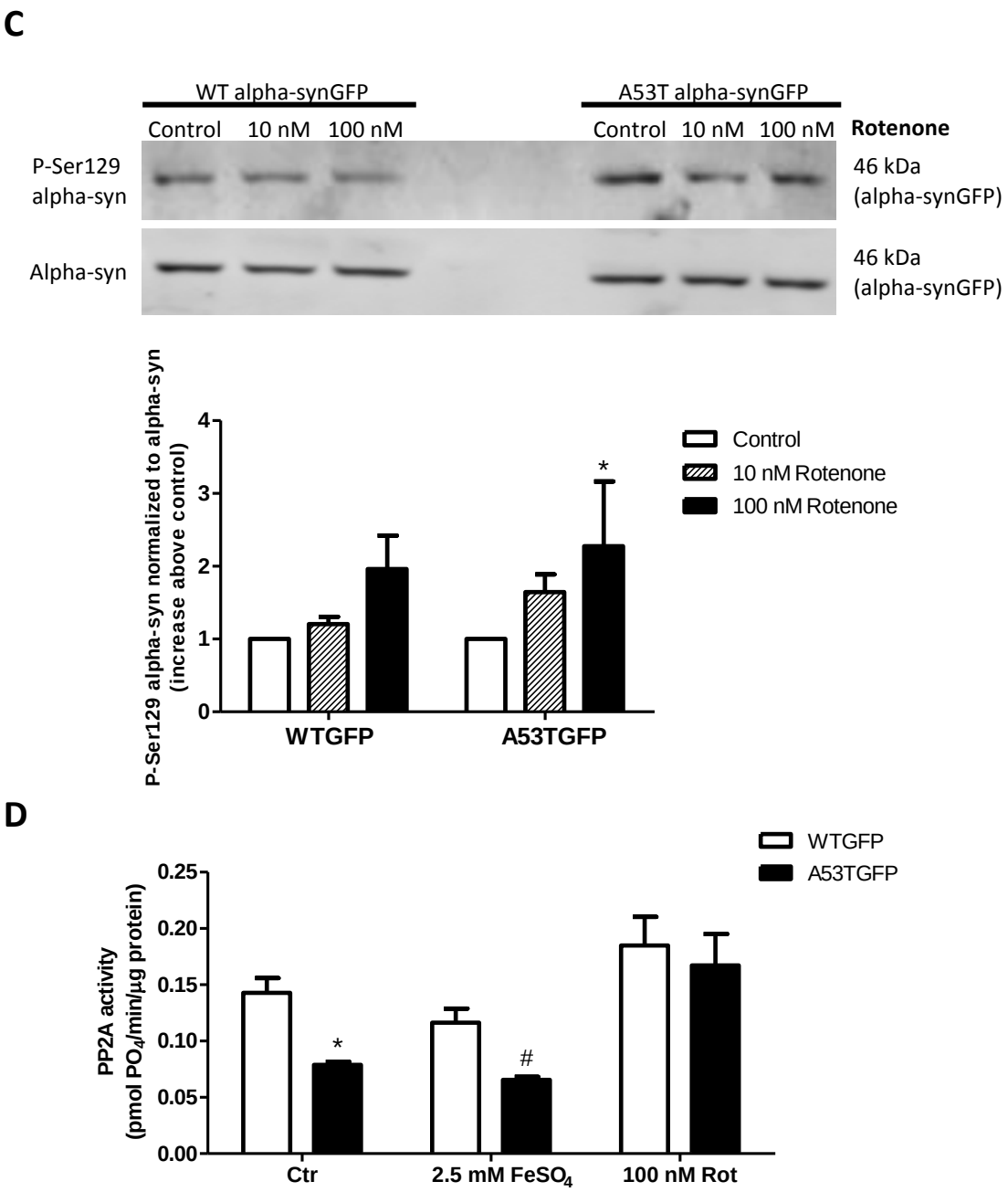


Figure 3.8. Prolonged exposure to iron or rotenone induced alpha-syn phosphorylation at Ser 129 and modified PP2A activity. Cells were transfected with WT or A53T alpha-synGFP (controls) (**A**) and further exposed to 1 mM and 2.5 mM $FeSO_4$ (**B, D**), or 10 nM and 100 nM

rotenone (**C, D**) during 4 days. Levels of P-Ser129 alpha-syn were analysed by western blotting (**A-C**). In **D** is represented PP2A activity of total extracts in cells transfected with WT or A53T alpha-synGFP, following treatment with 2.5 mM iron and 100 nM rotenone. Data in graphs (**A-C**) represent the mean±SEM of WT and A53T alpha-synGFP phosphorylated at Ser 129 (normalized to alpha-syn) from three to six independent experiments. In **D**, data in the graph are the mean±SEM of 3 independent experiments performed in triplicates. In **A**, *p<0.05 compared to WT alpha-synGFP. In **B**, *p<0.05 compared to control WTGFP; #p<0.05 and ##p<0.01 compared to the A53TGFP control. In **C**, *p<0.05 compared to A53TGFP control. In **D**, *p<0.05 compared to WTGFP control and #p<0.05 compared to WTGFP under the same experimental condition.

Overall, these results hint that early ROS formation and pronounced mitochondrial impairment induced by iron may be important to define enhanced phosphorylation at Ser129 of mutant alpha-syn, correlated with alpha-syn inclusion formation and increased susceptibility for necrosis.

3.4. Discussion

Although the etiology of PD is complex, there is strong evidence that mitochondrial dysfunction and oxidative stress play key roles in the disease pathogenesis. Furthermore, many studies have implicated alpha-syn as a molecular marker in sporadic and familial forms of PD. Particularly, the presence of phosphorylated alpha-syn at Ser129 in LBs of PD brains suggests that it may play an important role in alpha-syn metabolism and in the pathological pathways that occur in PD (Anderson et al., 2006; Fujiwara et al., 2002). Under normal conditions, alpha-syn was shown to be partially phosphorylated at Ser129 (Chau et al., 2009). Indeed, SH-SY5Y cells overexpressing WT alpha-syn demonstrated basal levels of Ser129 phosphorylation. Interestingly, mutant A53T alpha-syn overexpressing cells revealed a significant increase in levels of phosphorylated alpha-syn at Ser129 under basal/unstimulated conditions, when compared to the overexpressed WT protein. Still in alpha-syn mutant cells, increased superoxide anion was generated, along with some morphological mitochondrial changes, revealing a high correlation between mitochondrial-driven ROS formation and increased P-Ser129 alpha-syn.

Under basal conditions, we also demonstrated that cells overexpressing mutant alpha-syn showed, *per se,* alterations in alpha-syn conformation with aggregated morphology. A53T alpha-syn was previously reported to accelerate fibril formation compared with WT alpha-syn *in vitro* (Conway et al., 1998). Thus, in transgenic mice, A53T mutant alpha-syn also caused greater neurotoxicity than the WT protein (Lee et al., 2002; Maingay et al., 2005; Dawson et al., 2010). Sato et al. (2011) expressed A53T mutant alpha-syn in the SN of rats and concluded that the group expressing the mutant A53T protein developed degeneration of dopaminergic neurons, with 50% of neurons progressively lost by 4 weeks (Sato et al., 2011). Furthermore, results from *in vivo* and *in vitro* studies suggest that mitochondrial accumulation of mutant familial A53T alpha-syn occurred at faster rates than WT and exhibited mitochondrial abnormalities at an earlier time point (Cookson, 2005; Gasser, 2001; Devi et al., 2008).

Our data showed that prolonged exposure to ferrous iron (particularly at 2.5 mM) significantly augmented P-Ser129 alpha-syn levels, an event preceded by increased radical formation (detected by EPR) and raised intracellular hydroperoxides, in cells overexpressing WT or mutant A53T alpha-syn. Furthermore, under these experimental conditions, we observed a decrease in mitochondrial membrane potential in both types of cells, concomitant with the formation of alpha-syn inclusions immunopositive for Ub, with little cell death. Besides these effects, the results evidenced a greater susceptibility for SH-SY5Y cells expressing mutant (A53T) alpha-syn when exposed to the toxic stimuli, which correlated with higher levels of P-Ser129 alpha-syn. Excessive oxidative stress and decreased mitochondrial complex I activity found in nigral dopaminergic neurons in PD and the pathological finding that iron co-localizes with alpha-syn in LBs (Galvin et al., 2000; He et al., 2011) led to the hypothesis of a close relationship between iron, oxidative stress and alpha-syn. Previous studies by Takahashi et al. reported that a four-day exposure to $FeCl_2$ was sufficient to promote assembly of WT alpha-syn oligomers mainly composed of C-terminally truncated fragments in 3D5 cells, without causing cell death. The authors also described a time-dependent significant increase in ROS production after treatment with $FeCl_2$, which was exacerbated in cells overexpressing WT alpha-syn, along with increased P-Ser129 alpha-syn (Takahashi et al., 2007). Enhancement of alpha-syn phosphorylation by $FeCl_2$ was inhibited in cultures treated with casein kinase 2 (CK2) inhibitor, suggesting CK2 could be a major kinase responsible for alpha-syn phosphorylation. Our work goes a step further by defining a role for A53T mutant alpha-syn in exacerbating ROS production and P-Ser129 alpha-syn, concomitantly with inclusion formation and a decrease in PP2A activity. Recent studies from Lee et al. (2011) demonstrated that activation of PP2A stimulated the dephosphorylation of alpha-syn at Ser129 *in vitro* and *in vivo* and the resulting reduced levels of phosphorylated alpha-syn ameliorated the neuropathology and behavioural deficits of a transgenic mouse model of alpha-synucleinopathy. In another recent study (Wu et al., 2012) demonstrated a direct effect of alpha-syn aggregation on PP2A activity impairment. Using brains from DLB patients and with alpha-syn triplication, the authors

observed a loss of PP2A activity in association with alpha-syn aggregation, supporting the hypothesis that PP2A activity appeared to be attenuated in response to alpha-syn aggregation. Our data revealed a correlation between enhanced levels of phosphorylated mutant A53T alpha-syn and diminished activity of PP2A. Under the presence of mutant alpha-syn and following prolonged exposure to iron, SH-SY5Y cells also showed a higher number of inclusions, suggesting that alpha-syn protein modifications could be interfering with PP2A signalling pathway, by lowering its activity as a phosphatase. Therefore, it is possible that decreased PP2A activity could dephosphorylate less mutant alpha-syn and thus contribute to its toxic role in PD pathogenesis.

Also concordantly with our data, He and co-workers (2011) previously showed that overexpression of WT alpha-syn in SK-N-SH cells exposed to ferrous iron resulted in a significant decrease in mitochondrial membrane potential and increased ROS production. The results suggested that WT alpha-syn augmented the vulnerability of neurons to iron-induced toxicity through intracellular mitochondrial impairment and ROS production. The mechanism for the involvement of alpha-syn in iron-induced toxicity might be explained by the direct binding of iron to alpha-syn causing a conformational change in this protein, leading to the formation of toxic intermediate oligomers during this process (Kostka et al., 2008; Outeiro et al., 2008). Another possible explanation could be the oxidative damage and mitochondrial dysfunction generated by the interaction between iron and alpha-syn. As shown by these authors, oxidation caused by ferrous iron can promote alpha-syn aggregation; in turn, alpha-syn aggregation may exacerbate oxidative damage. The findings provided a link between alpha-syn and the toxicity induced by iron, by lowering the threshold of the cells to oxidative lesion. Importantly, Li et al. (2011) demonstrated that iron-induced intracellular alpha-syn aggregation was partially dependent on oxidative stress, also suggesting an interaction between iron and alpha-syn. Indeed, we demonstrated that under oxidative stress conditions, cells overexpressing mutant A53T alpha-syn were more susceptible to the effect of ROS formation, accompanied by

mitochondrial changes (depolarization and fragmentation), leading to alterations in alpha-syn conformation and Ser129 phosphorylation.

Mitochondrial dysfunction has been largely implicated in PD pathogenesis. Studies in an animal model with chronic intravascular infusion of the complex I irreversible inhibitor rotenone showed a parkinsonian phenotype and nigral degeneration pathology with cytoplasmic inclusions immunopositive for alpha-syn and Ub (Betarbet et al., 2000; Sherer et al., 2003). In the present work, we combined a cellular model of PD by overexpressing WT or mutant A53T alpha-syn with the exposure to rotenone. As described above for the effects produced by iron, we show that prolonged treatment with rotenone (at 100 nM) significantly increased hydroperoxide and superoxide anion production, reduced mitochondrial membrane potential and clearly induced the formation of alpha-syn inclusions, particularly in mutant A53T alpha-syn SH-SY5Y cells. Furthermore, under conditions of mitochondrial impairment, the levels of P-Ser129 alpha-syn were significantly enhanced in cells overexpressing the mutant form of this protein, however in a pathway distinct from that involving PP2A activity. Sugeno et al. (2008) previously showed that incubation with high doses of rotenone (100 nM) for 24 h induced 2-3% of aggregate-positive cells, whereas low doses (10 nM) and long-time exposure (120 h) produced about 10% of inclusion-positive cells. These aggregates were immunoreactive for alpha-syn, phosphorylated alpha-syn and Ub, indicating that the inclusions shared a number of pathological features with LBs (Fujiwara et al., 2002; Goldman et al., 1983; Kuzuhara et al., 1988; Spillantini et al., 1997). Furthermore, an increase in intracellular ROS levels measured by $DCFH_2$-DA after 72 h of treatment with low doses of rotenone was reported in cells overexpressing WT alpha-syn. In the same study, the authors observed that expression of mutant S129A alpha-syn, in which the Ser129 phosphorylation was blocked, induced fewer aggregates in SH-SY5Y cells compared to those expressing WT alpha-syn. In addition, blocking of Ser129 phosphorylation decreased apoptotic neuronal cell death caused by mitochondrial toxin exposure. Indeed, in a mammalian cellular model it was reported that the expression of the mutant S129D mimicking phosphorylated alpha-syn increased protein

aggregates compared to WT cells (Liu et al., 2007). The results suggested that overexpressing WT alpha-syn in cells exposed to a low dose of rotenone increased alpha-syn phosphorylation at Ser129, promoting intracellular aggregate formation, mitochondrial damage and apoptosis. After rotenone exposure, we observed enhanced superoxide anions in WT and particularly in A53T alpha-syn overexpressing cells, corroborating a primary role for rotenone as an inducer of mitochondrial superoxide formation. Cells overexpressing A53T alpha-syn also favoured the formation of intracellular hydroperoxides after exposure to rotenone, independently of necrotic cell death features. Is has been shown that alpha-syn undergoes a number of post-translational modifications, including phosphorylation and nitration under normal and disease status (Recchia et al., 2004; Okochi et al., 2000). It is likely that these modifications contribute to increased mitochondrial alpha-syn accumulation by altering the rate of mitochondrial targeting, as previously described by Devi and colleagues (Devi et al., 2008).

In the present study we demonstrate a clear correlation between ROS formation, mitochondrial depolarization, Ub-positive alpha-syn inclusions and alpha-syn Ser129 phosphorylation. These cellular and proteins changes are noticed following exposure to the oxidative stress inducer $FeSO_4$ and the mitochondrial complex I inhibitor rotenone, for a relatively long period of time (4 days), mimicking the modifications triggered by prolonged exposure to toxic stimuli, in cells overexpressing WT or mutant A53T alpha-syn, the latter linked to pathological changes occurring in some familial forms of PD. Furthermore, we show that mutant A53T alpha-syn expressing cells are more susceptible to the toxic stimuli, when compared to WT alpha-syn expressing cells. Overall, our data gives strength to the possibility that mitochondrial impairment and ROS production caused by prolonged exposure to rotenone and iron might induce alpha-syn phosphorylation at Ser129 and the formation of alpha-syn inclusions, particularly after expression of mutant alpha-syn. Alpha-syn A53T mutation could then be associated with more earlier forms of the disease due to the greater susceptibility to pathological events, namely those inducing oxidative stress.

The data suggest that intracellular ROS formation induced by increased levels of oxidative stress or mitochondrial dysfunction may be important for defining subsequent alpha-syn aggregation and phosphorylation, which may precede massive cell death in sporadic and familial forms of PD. Finally, we add evidence for a possible role of PP2A in alpha-syn phosphorylation, suggesting that targeting PP2A could be a promising therapeutic approach to mitigate the neuropathology of alpha-syn, particularly in autosomal dominant familial cases caused by A53T mutation.

CHAPTER 4

Oxidative stress in cells overexpressing wild-type alpha-synuclein – role of antioxidants and nuclear transcription

4.1. Summary

Alpha-synuclein (alpha-syn) is an abundant neuronal protein that has been linked both to normal synaptic function and neurodegeneration. Mutations in the gene encoding alpha-syn as well as duplications and triplications of wild-type (WT) alpha-syn gene have been associated to Parkinson's disease (PD). Moreover, aggregated alpha-syn is a major component of Lewy bodies found in sporadic and some inherited forms of PD. A number of processes have been implicated in the degeneration of dopaminergic neurons characteristic of PD, including oxidative stress. Here, we defined the occurrence of oxidative stress in SH-SY5Y cells overexpressing WT alpha-syn in a doxycycline (Dox) regulated manner in a Tet Off system, before and after exposure to iron and further determined the changes in the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2). Data evidenced an increase in hydroperoxide levels under basal conditions in cells overexpressing alpha-syn (-Dox), compared to cells expressing the endogenous protein (+Dox). Exposure to iron largely increased ROS production in both + Dox and -Dox cells. Analysis of superoxide dismutase 1 (SOD1) protein levels and activity revealed a decrease in this enzyme in untreated –Dox cells. SOD2 levels were also diminished in –Dox control cells, although its activity remained unaltered under these conditions. Moreover, glutathione (GSH) and oxidized glutathione (GSSG) levels also diminished in –Dox cells under basal conditions, but no changes were observed in the activity of enzymes of the glutathione redox cycle, glutathione peroxidase (GPx) and glutathione reductase (GRed), suggesting deficient GSH synthesis. Accordingly, the levels of glutamate-cysteine ligase, catalytic subunit (GCL_C) were decreased in –Dox cells and following exposure to iron. In addition, the levels of Nrf2 were diminished in nuclear fractions of –Dox cells and after iron treatment in –Dox cells. Data suggest that overexpression of alpha-syn modifies the antioxidant response and thus the susceptibility to oxidative stress, in agreement with the pathological events described in PD.

4.2. Introduction

Degeneration of dopaminergic neurons and the accumulation of alpha-syn in the form of Lewy bodies (LBs) and Lewy neurites (LNs) are major hallmarks of PD (Dauer and Przedborski, 2003). Although the exact function of alpha-syn is unknown, evidence strongly suggests that it has a crucial role in the pathogenesis of PD (Vekrellis et al., 2011; Kim et al., 2012). Familial autosomal dominant forms of the disease have been documented in families with SNCA (alpha-syn) mutations or gene duplication/triplication. Importantly, genome-wide association studies (GWASs) have clearly shown that SNCA is also linked to sporadic PD. Diffuse accumulation of alpha-syn protein occurs with aging in the *substantia nigra pars compacta* (SN)(Li et al., 2004; Chu and Kordower, 2007) and prior to inclusion formation in PD patients (Chu et al., 2006). Therefore, one can predict that an increase in the levels of the WT protein may be sufficient to cause neurodegeneration and may underlie the pathogenesis of sporadic PD. However, no singular pathological mechanism initiating PD has been identified so far. Indeed, the current hypotheses include oxidative stress, which may be linked to modified support of antioxidant defenses, mitochondrial dysfunction, accumulation of transition metals, impairment of protein folding quality control, inflammation and decreased neurotrophic support (Thomas and Beal, 2007).

To maintain a physiological redox balance, cells are provided with a wide variety of endogenous antioxidant enzymes. Production of these cytoprotective enzymes is induced upon exposure to ROS via a mechanism regulated at the transcriptional level (Kensler et al., 2007; de Vries et al., 2008). Genes that code for proteins involved in reactive oxygen species (ROS) detoxification share a common promoter element, the antioxidant response element (ARE), which activation is coordinated by Nrf2. Nrf2 translocates to the nucleus upon exposure to electrophiles or ROS, where it binds ARE and activates the transcription of antioxidant and detoxifying genes (Itoh et al., 2003; Kobayashi and Yamamoto, 2006; Motohashi and Yamamoto, 2004; Itoh et al., 2004), including SOD1 (McCord and Edeas,

2005), heme oxygenase-1 (HO-1) (Favreau and Pickett, 1995; Prestera et al., 1995), nicotinamide adenine dinucleotide phosphate (NADPH):quinone oxidoreductase-1 (NQO1) (Favreau and Pickett, 1995; Wang and Williamson, 1994), glutathione-S-transferases (GSTs) (Rushmore and Pickett, 1990), the glutathione synthetic enzyme glutamate-cysteine ligase (GCL), glutathione peroxidase (GPx), glutathione reductase (GRed) (Galloway et al., 1999; Galloway et al., 1997; Mulcahy and Gipp, 1995) and the thioredoxin (Trx) (Hintze et al., 2003) and peroxiredoxin (Prx) families (Ishii et al., 1999). Some of these enzymes represent a powerful antioxidant defense mechanism to the cells. Moreover, data concerning the functional status of the Nrf2-ARE system in PD are scarce. Investigation of the localization of Nrf2 in the SN of PD brains demonstrated a strong nuclear immunoreactivity in neurons (Ramsey et al., 2007; de Vries et al., 2008), suggesting that although the Nrf2-ARE signaling pathway is activated there may be factors counteracting the effective Nrf2-activated gene transcription. A recent study from Cook and colleagues (2011) showed that PD-patient derived cells (from the olfactory mucosa) have decreased levels of GSH and higher levels of oxidative stress, compared to healthy controls. The authors demonstrated that deactivation of Nrf2 by siRNA in control donor cells recapitulated the PD-associated changes in metabolic function and provided evidence for a restorative role for Nrf2 in regulating disease-associated metabolic deficiencies, including elevation of GSH content (Cook et al., 2011).

Depletion of GSH has been described in several studies to occur specifically in the SN of PD patients (Pearce et al., 1997; Perry et al., 1982; Riederer et al., 1989). Although patients with multiple system atrophy or progressive supranuclear palsy present degeneration of DA neurons, GSH depletion is not detected (Perry and Yong, 1986; Sian et al., 1994a). However, in incidental LB disease, which may be considered as a pre-symptomatic form of PD (Jellinger, 2008) and is present in up to 10% of individuals over the age of 60, GSH depletion is already present in the SN (Jenner et al., 1992) and appears to precede complex I dysfunction, iron accumulation and striatal dopamine loss (Zeevalk et al., 2008). These findings suggest that decreased GSH levels appear as an early event in PD etiology, possibly

facilitating or leading to a cascade of oxidative stress lesions. This view is supported by *in vitro* studies demonstrating that prolonged GSH depletion induces inhibition of complex I activity (Chinta et al., 2007).

Nuclear alpha-syn localization has been reported to increase under conditions of oxidative stress both *in vitro* and *in vivo* (Monti et al., 2010; Siddiqui et al., 2012). Although its function is unknown, alpha-syn was demonstrated to colocalize with histones together with reduced levels of histone acetylation (Goers et al., 2003; Kontopoulos et al., 2006). Transcriptional deregulation has been described as part of the pathogenic mechanisms leading to neuronal dysfunction in neurodegenerative disorders such as Huntington's disease (HD) (Cha, 2000). One of the transcription pathways that is affected in HD is the cAMP-responsive element (CRE)-mediated pathway which regulates diverse sets of genes involved in plasticity, growth and cell survival (Zou and Crews, 2006). So far, very few studies have focused their attention in transcription deregulation in PD and the possible role of alpha-syn in this process.

Thus, in this part of the work, we analysed the effects caused by WT alpha-syn overexpression on the susceptibility to oxidative stress induced by iron and further determined the changes in nuclear transcription factors, namely Nrf2, using the neuroblastoma cell line SH-SY5Y overexpressing WT alpha-syn in a doxycycline (Dox) regulated manner (Tet-Off system) as a PD cellular model. Our results indicate that cells overexpressing WT alpha-syn exhibit increased ROS levels under basal conditions, which are exacerbated by iron exposure. Furthermore, we give evidence for diminished levels of proteins involved in the cellular antioxidant defense mechanisms upon overexpression of WT alpha-syn, whereas treatment with iron induces regulation of detoxification pathways apparently independently of alpha-syn overexpression.

4.3. Results

4.3.1. Inducible cells overexpressing WT alpha-syn display increased levels of ROS

Taking into account the increased ROS production verified in SH-SY5Y cells transiently transfected with WT or A53T mutant alpha-syn upon treatment with iron or rotenone, in this part of the study we analysed the generation of ROS in SH-SY5Y cells conditionally expressing WT alpha-syn for 5 days. Cells were initially exposed to the same toxic stimuli, iron and rotenone, using different concentrations and shorter time exposure, in the presence or in the absence of Dox, to evoke modified expression of alpha-syn, in a Tet-Off system. Results demonstrated that under basal conditions, cells overexpressing WT alpha-syn (-Dox) showed an increase in ROS levels measured by DCF fluorescence. This effect was significantly exacerbated upon exposure to iron (500 μM) during 2 hours (h) in both cells (Figure 4.1A). In retinoic acid (RA)-differentiated SH-SY5Y cells during 10 days, we defined that the difference in the levels of ROS between +Dox and –Dox cells in basal conditions and upon iron treatment (500 μM, for 2 h) was similar to that detected in proliferative cells (Figure 4.1B). Therefore, the following experiments were performed in undifferentiated cells.

Treatment with rotenone (10 μM) for 2 hours did not significantly affect hydroperoxide levels in +Dox or -Dox cells, when compared to untreated conditions (Figure 4.1C).

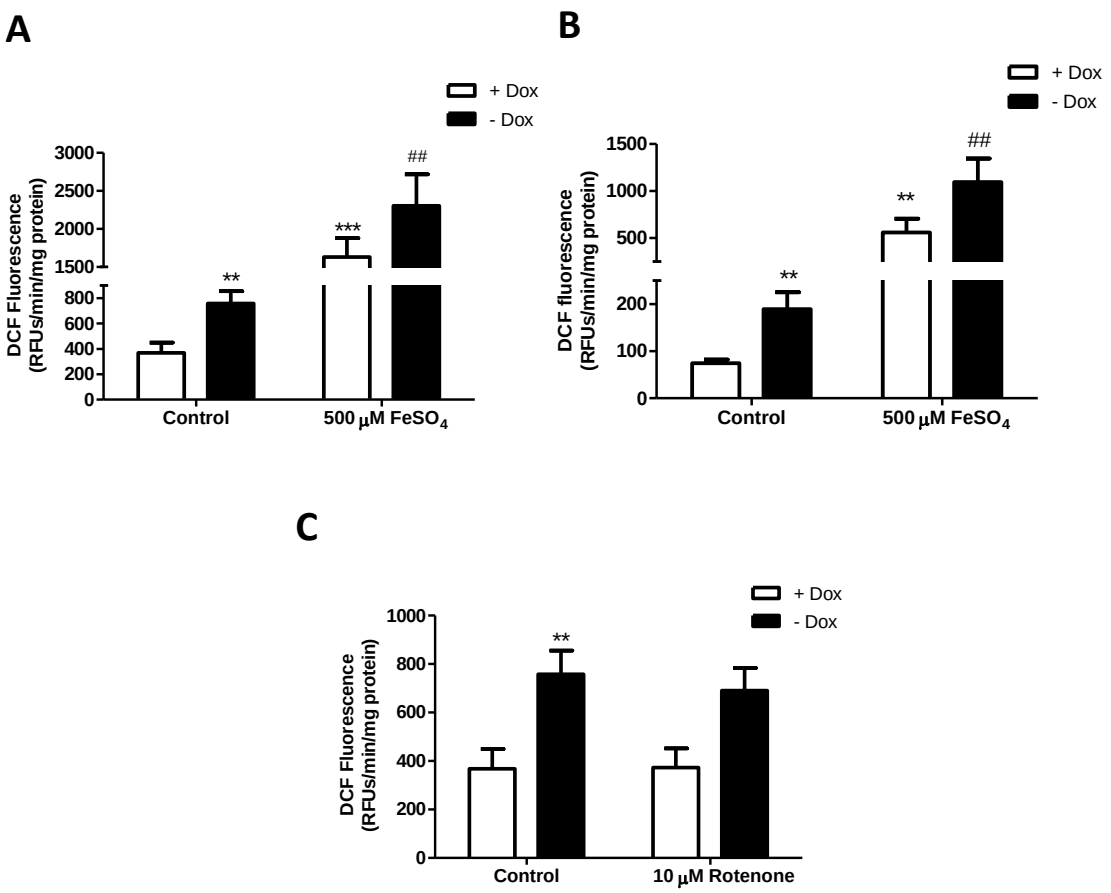


Figure 4.1 – Hydroperoxide production in induced SH-SY5Y WT alpha-syn cells after exposure to iron or rotenone. Cells were kept in the presence or in the absence of Dox during 5 days. Differentiated cells were kept in the presence of RA (20 μg/ml) for 10 days (**B**). At the day of the experiment, cells were incubated with $FeSO_4$ (500 μM) (**A**, **B**) or rotenone (10 μM) (**C**), for 2 h. Levels of hydroperoxides were measured following DCF fluorescence after 30 min incubation with 20 μM $DCFH_2$-DA. Data are the mean±SEM of three independent experiments performed in triplicates. Statistical analysis: **p<0.01 and ***p<0.001 compared to +Dox control; ##p<0.01 compared to – Dox control. **RA**: retinoic acid

4.3.2. No changes in SH-SY5Y cell viability following alpha-syn overexpression

In the previous section (Chapter 3), we showed that transient overexpression of WT or mutant A53T alpha-syn followed by iron or rotenone prolonged exposure slightly affected SH-SY5Y cell viability, with the percentage of cell death occurring by necrosis never exceeding 1.5% following incubation with iron. Since hydroperoxide levels were consistently elevated in untreated –Dox cells, next we determined whether alpha-syn overexpression was sufficient to trigger modified levels of proteins involved in apoptosis. We analysed the levels of both pro- and anti-apoptotic proteins, namely mitochondrial and cytosolic cytochrome c (cyt c) and apoptosis inducing factor (AIF) and mitochondrial B-cell leukaemia/lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist-killer protein (Bak) by western blotting. As depicted in Table 4.1, no significant alterations were observed in the levels of any of these proteins, suggesting that none of the apoptotic pathways (caspase-dependent and caspase-independent) was activated.

Table 4.1 – Mitochondrial and cytosolic levels of apoptotic proteins before and after overexpression of WT alpha-synuclein.

	Mitochondria		**Cytosol**	
Proteins	+ Dox	- Dox	+ Dox	- Dox
Bax	1.00 ± 0.49	1.14 ± 0.49	n.d.	n.d.
Bcl-2	1.00 ± 0.07	0.96 ± 0.09	n.d.	n.d.
Bak	1.00 ± 0.004	1.00 ± 0.09	n.d.	n.d.
cyt c	1.00 ± 0.27	1.76 ± 0.65	1.00 ± 0.15	1.03 ± 0.04
AIF	1.00 ± 0.14	1.32 ± 0.11	1.00 ± 0.02	0.97 ± 0.06

Levels of mitochondrial and cytosolic proteins involved in the apoptotic pathways were analysed by western blotting, in the presence or in the absence of Dox, during 5 days. Mitochondrial and

cytosolic fractions prepared from inducible SH-SY5Y cells overexpressing alpha-syn were analysed by SDS-PAGE and immunoblotted for Bax, Bcl-2, Bak, cyt c and AIF. Note that the levels of mitochondrial proteins were normalized to heat shock protein 60 (HSP60), while the cytosolic proteins were normalized to α-tubulin. Data are the mean ± SEM of 3 independent experiments. n.d., not determined.

In order to corroborate data shown in Table 4.1, we also analysed caspase-3-like activity in both +Dox and –Dox cells in the absence or in the presence of iron (500 μM). Although we observed a tendency for an increase in caspase-3 activation in –Dox cells under basal conditions, this was not statistically significant (Figure 4.2), in agreement with Vekrellis and colleagues (2009) and with data depicted in Table 4.1. Treatment with iron did not trigger caspase-3 activation either, showing that despite iron-induced increased in ROS levels, no evidence for apoptosis could be detected in cells exposed to this oxidative stress inducer.

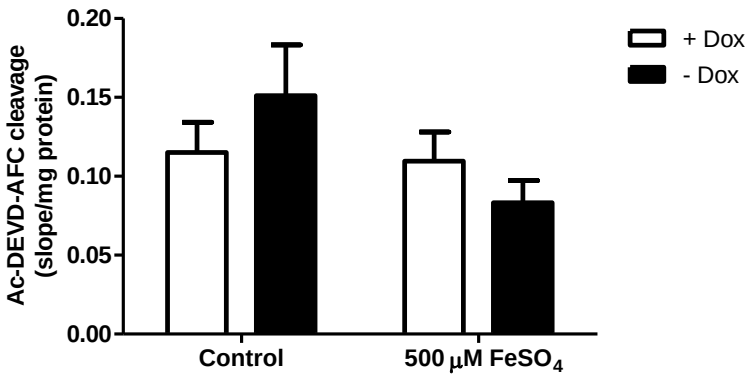


Figure 4.2 – Caspase-3 activation following alpha-syn overexpression and iron exposure in SH-SY5Y cells. Cells were cultured in the presence or in the absence of Dox for 5 days. At the day of the experiment, cells were incubated with iron (500 μM) during 2 h. Caspase-3-like activity was measured by following the cleavage of the fluorimetric substrate Ac-DEVD-AFC. The results are expressed as the mean±SEM of 4 distinct experiments, performed in triplicates.

Although we did not observe changes in the levels of apoptotic proteins induced by alpha-syn overexpression, we further examined the reducing capacity of SH-SY5Y cells under basal conditions using the Alamar blue assay, previously described as an indicator of cell viability (Al-Nasiry et al., 2007). As shown in Figure 4.3 and concordantly with the lack of differences obtained in Table 4.1 and Figure 4.2, no changes were observed between +Dox and –Dox cells, indicating that overexpression of WT alpha-syn during 5 days did not interfere with the reducing capacity of SH-SY5Y cells.

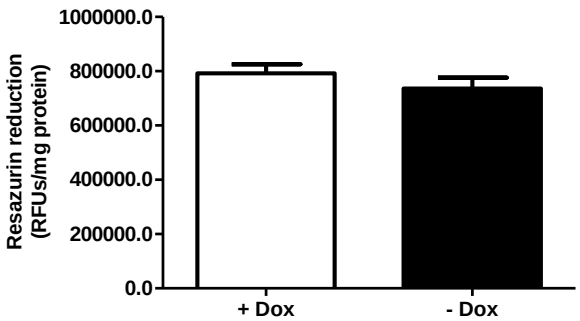


Figure 4.3 – Effect of WT alpha-syn overexpression on the reducing capacity of SH-SY5Y cells. Cells were maintained in the presence or in the absence of Dox during 5 days. At the day of the experiment, resazurin (1 mg/ml) was diluted in RPMI 1640 culture medium and incubated during 2 h at 37ºC. Cells reducing capacity was measured by fluorescence at 530 nm excitation and 590 nm emission. Data are the mean±SEM of three independent experiments performed in triplicates.

4.3.3. Activity and expression levels of SOD enzymes in inducible SH-SY5Y cells overexpressing alpha-syn – influence of iron exposure

Taking into account that oxidative stress may occur due to an overwhelming production of ROS, namely superoxide ($O_2^{\bullet -}$) and H_2O_2, and/or decreased enzymatic activity of important defense systems, we analysed whether changes in the levels or activity of antioxidants

could modulate ROS generation in inducible SH-SY5Y cells overexpressing WT alpha-syn (–Dox) and after exposure to iron, under experimental conditions not affecting cell viability.

Therefore, we started by measuring the activity of total SOD, i.e. the sum of SOD1 and SOD2 activities, in total cell homogenates. Under basal conditions, the activity of total SOD was diminished in –Dox cells, compared to +Dox cells (Figure 4.4A), which may help to explain the elevated levels of hydroperoxides observed in these cells. As shown in Figure 4.1A, 500 µM $FeSO_4$ significantly enhanced the generation of ROS in +Dox and –Dox cells. The activity of total SOD was also augmented in the presence of 500 µM $FeSO_4$, when compared to control cells overexpressing alpha-syn (Figure 4.4A), suggesting that although this antioxidant system was active, it might not be involved or be effective in modulating ROS levels induced by this iron concentration. As a means to evaluate which intracellular SOD could be involved in this process, we further analysed the activity of Cu/Zn-SOD (or SOD1) in the same experimental conditions. We verified that SOD1 activity was diminished in –Dox control cells, compared to +Dox cells; however, no alterations were observed in the presence of 500 µM iron (Figure 4.4B), suggesting that SOD1 was not the main contributor for the increase in total SOD activity under these circumstances. Therefore, we also investigated the activity of Mn-SOD (or SOD2) in total cell fractions. As shown in Figure 4.4C although there were no significant changes under basal conditions, SOD2 activity was moderately increased in +Dox and –Dox cells exposed to 500 µM iron, apparently contributing for the increase in total SOD.

In addition, and taking into account the changes in SOD1 activity observed under basal/control conditions, in cells subjected to WT alpha-syn overexpression, we examined SOD1 protein levels by western blotting. Interestingly, SOD1 levels were decreased in alpha-syn overexpressing cells. Moreover, a slight but significant increase in SOD1 levels was observed in –Dox cells exposed to 500 µM iron (Figure 4.4D). We also analysed the levels of SOD2 under the same experimental conditions. Although we did not detect significant changes in the activity of cellular SOD2 between +Dox and –Dox control cells, a decrease in this protein levels was observed in cells overexpressing WT alpha-syn (Figure

4.4E). Moreover, SOD2 levels remained decreased after iron exposure in +Dox cells (Figure 4.4E), suggesting that iron may upregulate SOD2 activity, without affecting its protein levels.

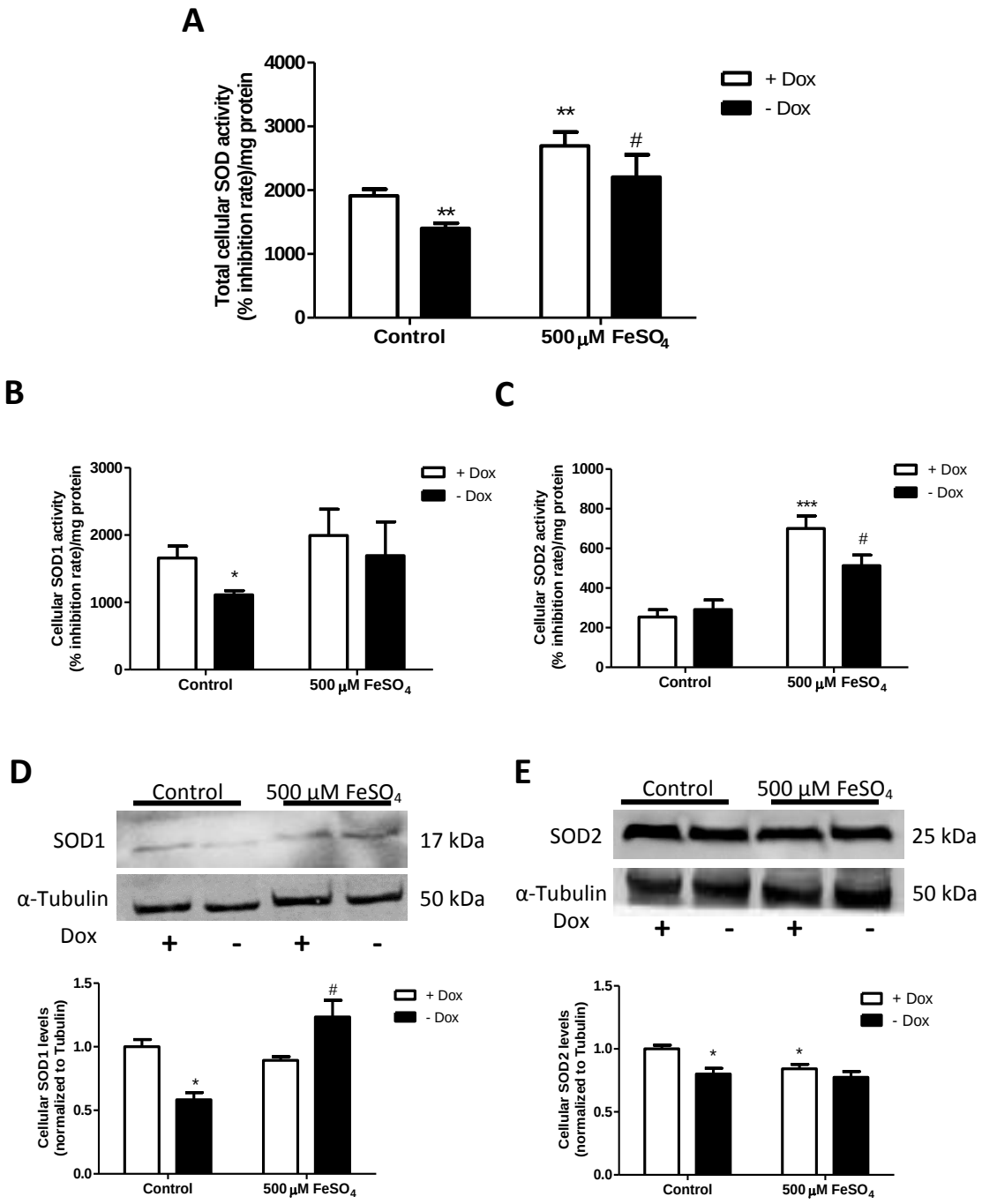


Figure 4.4 – Analysis of SOD activities and SOD1 and SOD2 protein levels upon exposure to iron. Cells were kept in the presence or in the absence of Dox during 5 days. Incubation with $FeSO_4$ (500 µM) was performed during 2 h. Total SOD (**A**), SOD1 (**B**) and SOD2 (**C**) activities were measured in cellular fractions and the results are expressed as % inhibition rate per mg of protein. % inhibition rate corresponds to the % of WST-1 formazan formation inhibition by SOD. In **D** and **E**, total cellular extracts were immunoblotted for SOD1 and SOD2, respectively, and normalized to tubulin. Data in the graphs are the mean±SEM of 3-4 independent experiments. Statistical analysis: *p<0.05, **p<0.01 and ***p<0.001 compared to +Dox control condition; #p<0.05 compared to –Dox control cells.

4.3.4. Involvement of glutathione antioxidant defense system on modulation of intracellular ROS levels in cells overexpressing alpha-syn following iron exposure

Glutathione is a major non-enzymatic antioxidant that maintains the cellular redox equilibrium and protects cells against exogenous and endogenous toxins, including ROS. Previous studies reported cases of depletion in GSH in the SN of PD patients (Pearce et al., 1997; Perry et al., 1982; Riederer et al., 1989). In agreement with these studies and with the data described above, our results revealed that the total levels of GSH were significantly decreased in cells overexpressing WT alpha-syn, when compared to +Dox control cells (Figure 4.5A). This decrease was accompanied by a similar alteration in the levels of GSSG in non-treated –Dox cells (Figure 4.5B). In cells exposed to 500 µM iron the levels of GSH were augmented in +Dox and –Dox cells (Figure 4.5A). Oxidized GSSG levels were also significantly higher after iron treatment in +Dox cells only (Figure 4.5B). Furthermore, we measured the activities of components of the antioxidant glutathione cycle in total cell fractions, namely cellular GPx, which catalyses the oxidation of GSH to GSSG, thus reducing H_2O_2 into H_2O,and GRed, which recycles GSSG back to GSH. Although there was a tendency for a decrease in GPx activity in –Dox control cells, this was not significant. Notably, we

could not detect significant changes in the activities of these enzymes in both +Dox and –Dox cells treated with 500 µM $FeSO_4$ either (Figure 4.5C,D).

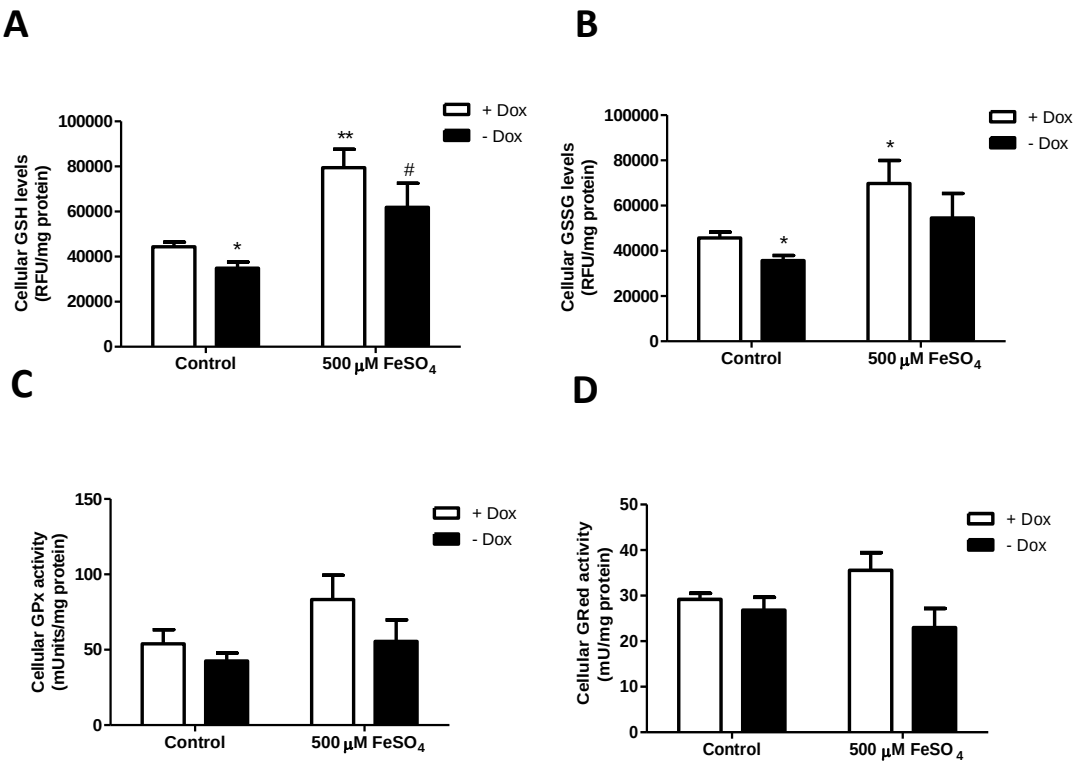


Figure 4.5 – Analysis of levels and activities of glutathione cycle-related proteins. Inducible SH-SY5Y cells were cultured in the presence or in the absence of Dox for 5 days. Treatment with $FeSO_4$ (500 µM) was performed during 2 h. Reduced glutathione (GSH) and oxidized glutathione (GSSG) levels (**A, B**) were determined in total cell homogenates. Glutathione peroxidase (GPx) and glutathione reductase (GRed) activities (**C, D**) were also measured in total cell extracts. Data are the mean±SEM of 3-4 independent experiments. Statistical analysis: *p<0.05 and **p<0.01 compared to +Dox control condition. #p<0.05 compared to –Dox control cells.

Unaltered glutathione redox cycle enzyme activities along with similar changes in both GSH and GSSG levels largely suggested modified GSH synthesis. Glutamate-cysteine ligase (GCL) is the rate-limiting enzyme in GSH synthesis and is considered a major determinant of cellular GSH levels (Franklin et al., 2009). Therefore, we next investigated GCL activity and

the levels of the catalytic subunit of GCL (GCLc). Cells overexpressing alpha-syn (-Dox) revealed lower levels of GCLc compared to cells expressing the endogenous protein (+Dox) (Figure 4.6A). Concordantly, GCL activity was decreased in –Dox cells under basal conditions, when compared to +Dox cells (Figure 4.6B), thus supporting the decrease in reduced and oxidized glutathione (Figure 4.5A, B) and the elevated levels of ROS detected in these cells (Figure 4.1A, B). On the other hand, after 2 h incubation with iron (500 μM) we observed inverse effects on protein levels and activity of GCL. While GCLc levels were significantly diminished after treatment with $FeSO_4$ (Figure 4.6A) in both +Dox and –Dox cells, GCL was highly active under these experimental conditions (Figure 4.6B), suggesting that increased GCL catalytic efficiency did not depend on GCLc protein levels. Nevertheless, enhanced GCL activity was concordant to the rise in GSH and GSSG levels evoked by iron treatment (Figure 4.5A).

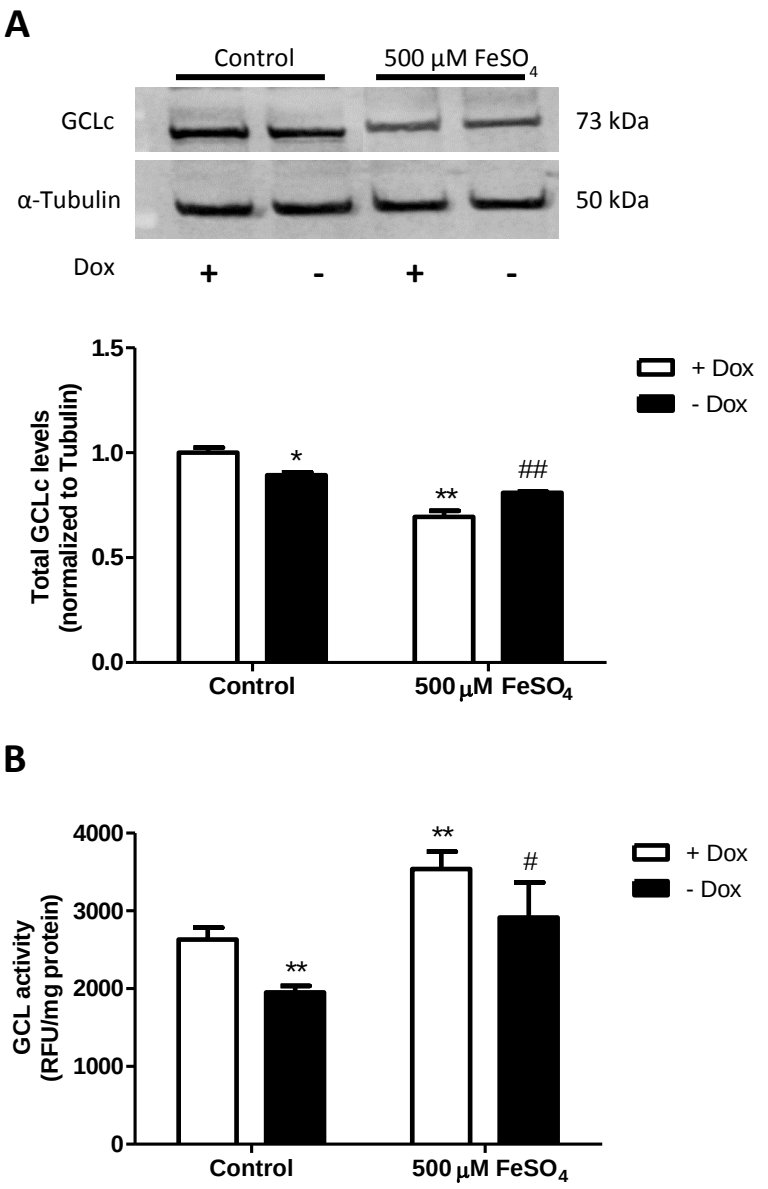


Figure 4.6 – GCLc levels and activity in SH-SY5Y cells overexpressing alpha-syn - influence of iron. Inducible SH-SY5Y cells were maintained in culture in the presence or in the absence of Dox for 5 days. Treatment with iron (500 μM) was performed during 2 h. Total cellular extracts were immunoblotted for GCLc (catalytic subunit) and normalized to tubulin (**A**). Graph in **B** shows GCL

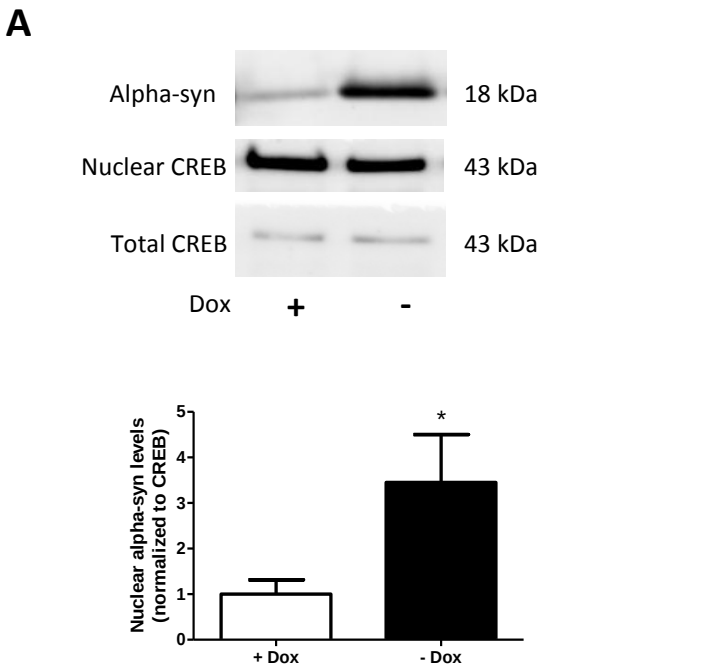
activity under the same conditions described above. Data plotted in the graphs are the mean±SEM of 3 independent experiments. Statistical analysis: *p<0.05 and **p<0.01 related to +Dox control condition. #p<0.05 and ##p<0.01 related to control –Dox.

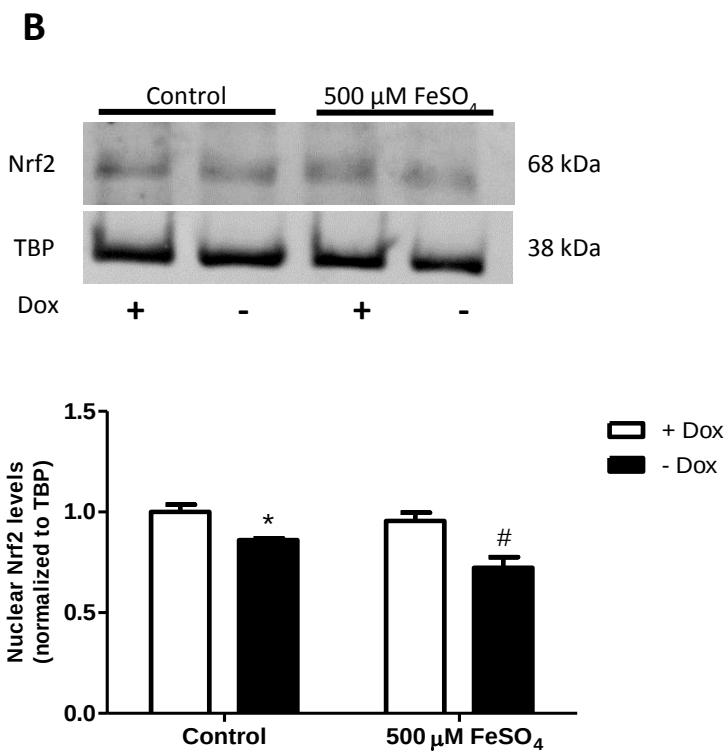
4.3.5. Lower nuclear Nrf2 levels in SH-SY5Y cells overexpressing WT alpha-syn

Localization of alpha-syn in the nucleus has been demonstrated in a variety of experimental systems (Monti et al., 2010); within the nucleus, alpha-syn was reported to affect the expression of genes involved in various cellular or neuronal functions including transcription (Crews et al., 2008; Winner et al., 2008). Thus, we analysed the protein levels of alpha-syn in nuclear fractions isolated from SH-SY5Y cells overexpressing WT alpha-syn in an inducible manner. As shown in figure 4.7A, alpha-syn was highly localized in the nuclei of –Dox cells (3.4-fold increase above +Dox cells). The purity of the subcellular fractions was verified by western blotting analysis, as can be observed through CREB staining in nuclear and total extracts (Figure 4.7A).

Due to the differences observed in the levels and activities of proteins involved in the antioxidant defense system, namely SOD1 and GClc, and given the fact that the expression of these proteins can be activated at a transcription level by Nrf2 (Clark and Simon, 2009), we also determined the nuclear levels of Nrf2, which activation is induced by exposure to ROS, and other transcription factors. Our results showed a reduction in Nrf2 levels in untreated –Dox cells (Figure 4.7B), corroborating the effects described above concerning the augmented levels of ROS and the reduced antioxidant defense-associated proteins in alpha-syn overexpressing cells. After 2 h exposure to iron (500 μM), we verified a slight decrease in Nrf2 levels in –Dox cells, when compared to the respective control (Figure 4.7B). These data appear to be concordant with the slight decrease in GCLc levels, but do not explain the increase in SOD1 levels in –Dox cells exposed to iron, largely suggesting that other transcription factors apart from Nrf2 or an extremely oxidized cell environment may regulate the protein levels of these enzymes.

Based on previous results indicating that nuclear alpha-syn could contribute to neurotoxicity in part *via* its ability to impact on transcription (Ren et al., 2000; Voutsinas et al., 2010; Outeiro et al., 2007; Duce et al., 2006; Leng and Chuang, 2006; Siddiqui et al., 2012) and the fact that Nrf2 levels were diminished in cells overexpressing alpha-syn we also investigated whether alpha-syn could alter other nuclear transcription factors. Results depicted in Figure 4.7C – F, revealed that alpha-syn overexpression did not interfere with the levels of p-CREB, CREB, CBP and NFkBp65, suggesting a closer and more direct link between alpha-syn and oxidative stress pathways.


A
Alpha-syn
18 kDa
Nuclear CREB
43 kDa
Total CREB
43 kDa
Dox
+
-
Nuclear alpha-syn levels (normalized to CREB)
5
4
3
2
1
0
*
+ Dox
- Dox


B
Control
500 µM FeSO4
Nrf2
68 kDa
TBP
38 kDa
Dox
+
-
+
-
Nuclear Nrf2 levels (normalized to TBP)
1.5
1.0
0.5
0.0
*
#
Control
500 µM FeSO4
+ Dox
- Dox

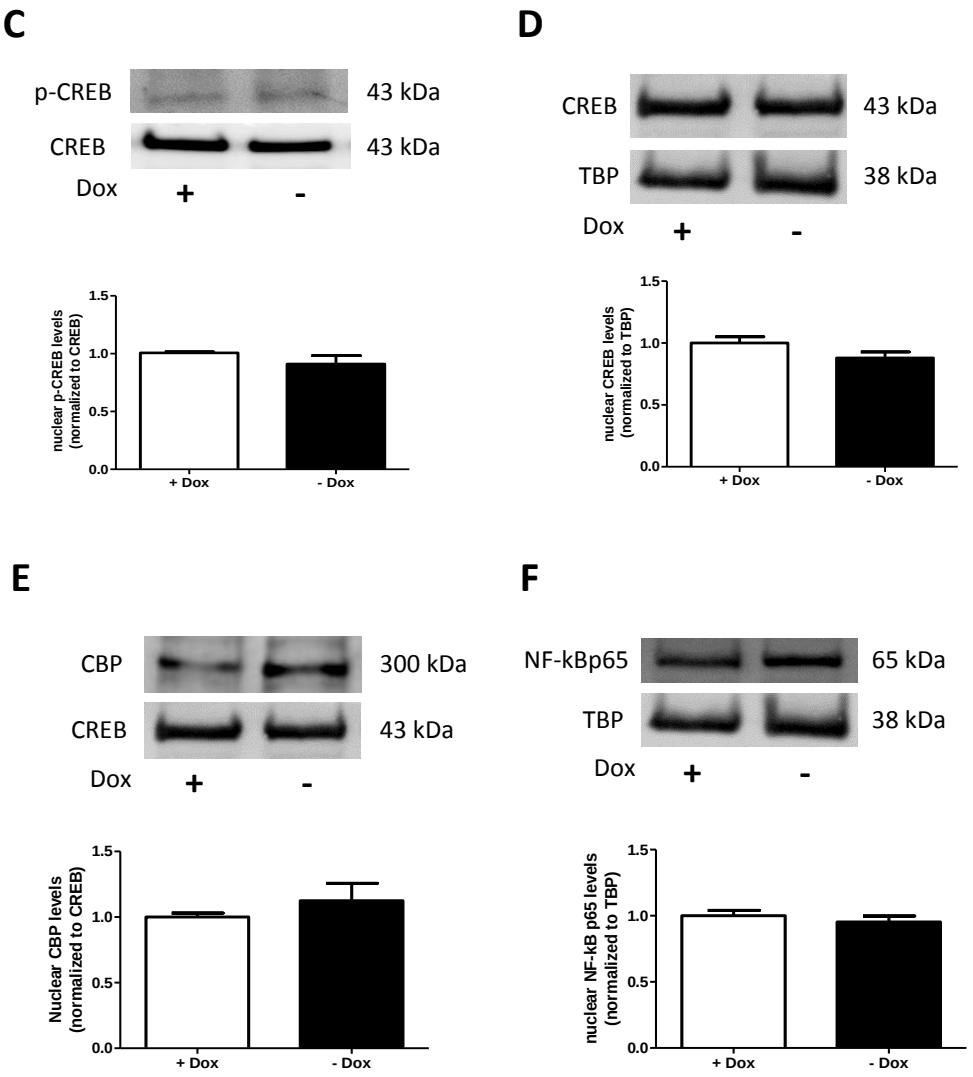


Figure 4.7 - Changes in nuclear alpha-syn and transcription factors in cells overexpressing WT alpha-syn and exposed to iron. Cells were cultured in the presence or in the absence of Dox during five days. At the day of the experiment, cells were incubated with iron (500 µM) during 2 h. (**A**) Nuclear lysates were immunoblotted for alpha-syn and normalized to CREB. Total extracts were also immunoblotted for CREB in order to confirm the purity of subcellular fractions. (**B**) Nuclear extracts prepared from inducible SH-SY5Y cells were analysed by western blotting for Nrf2 and normalized to TBP. (**C, D, E, F**) Nuclear fractions from +Dox and –Dox cells were immunoblotted for p-CREB, CREB, CBP and NF-kBp65 and normalized to CREB or TBP. Data plotted in the graphs are the

mean±SEM of 3 independent experiments. *p<0.05 related to +Dox control condition. Statistical analysis: #p<0.05 related to control –Dox.

CREB – c-AMP response element-binding; TBP – TATA box binding protein; CBP – CREB-binding protein; NF-kB – nuclear factor kappa B.

4.4. Discussion

Several studies have demonstrated the correlation between alpha-syn and oxidative stress. In this study, we used a cellular model based on the overexpression of WT alpha-syn, a protein widely described to underlie the pathogenesis of sporadic PD and the forms linked to SNCA genetic triplication, and used iron as a toxic stimulus in order to exacerbate the oxidative stress condition commonly linked to PD. Interestingly, SH-SY5Y cells exhibited different results under basal conditions and in the presence of the toxic stimulus, since iron-induced oxidative stress did not exacerbate the effects observed in cells overexpressing WT alpha-syn (-Dox), when compared to cells expressing the endogenous protein. Overall, we reported two distinct observations: i) a clear relationship between alpha-syn overexpression, increased ROS levels and a deficient antioxidant defense system linked to decreased nuclear Nrf2 in untreated –Dox cells; and ii) the effects caused by iron, which were independent from alpha-syn and triggered different cell responses, apparently to counterbalance the higher increase in free radical generation.

In many reported cases, overexpression of WT and mutant alpha-syn caused no differences in baseline viability, but increased cell susceptibility to a variety of insults, including oxidative stress (Lee et al., 2001; Zhou et al., 2000; Ko et al., 2000; Junn and Mouradian, 2002; Tabrizi et al., 2000). SNCA induced pluripotent stem (iPSC)-derived DA neurons from a PD patient with a naturally-occurring genomic triplication of SNCA gene showed an inherent expression of markers of oxidative stress and sensitivity to oxidative stress induced by peroxide, suggesting that the dominantly-acting PD mutation is intrinsically able to change normal cell function (Byers et al., 2011). Our results demonstrated that untreated SH-SY5Y cells overexpressing WT alpha-syn (-Dox) displayed elevated levels of intracellular ROS, under conditions not affecting cell viability or the susceptibility for apoptotic cell death. These data are in agreement with Vekrellis and colleagues (2009), who showed that proliferative neuroblastoma SH-SY5Y cells, in which alpha-syn was induced as long as 10

days did not present signs of cell death (Vekrellis et al., 2009). The authors provided evidence for a death pathway activated in retinoic acid-differentiated –Dox cells only, which combined both apoptotic and non-apoptotic features. Although they observed the release of cyt c from mitochondria, which was prevented by Bcl-xL expression, and activation of caspase 9, suggesting a classical intrinsic apoptotic pathway, they failed to find evidence for caspase 3 activation and to show cellular apoptotic morphology by electron microscopy. The factors accounting for the differential death consequences in the undifferentiated *versus* differentiated states are unknown, but may include subtle differences in clearance mechanisms, or the involvement of cell cycle molecules and/or other proteins differentially expressed in the two states.

Post-mortem analysis of brains from parkinsonian patients has revealed a profound loss of the antioxidant GSH, increased SOD activity and elevated free iron in the SN (Sofic et al., 1992; Sofic et al., 2006). In erythrocytes from sporadic PD patients, Baillet et al. (2010) showed that SOD activity was enhanced compared to control individuals, suggesting an upregulation of this antioxidant enzyme in order to maintain the balance of the redox status in these cells (Baillet et al., 2010). In our model, we demonstrated a reduction in SOD1 activity in –Dox cells, which was accompanied by a decrease in cellular SOD1 levels. SOD2 levels were also diminished in –Dox cells, without alterations in the activity of this enzyme. While counteracting previous evidences, these results are in agreement with the elevated ROS production observed in these cells and with deficient nuclear levels of Nrf2, possibly reflecting decreased Nrf2-dependent transcription.

Moreover, diminished cellular antioxidant GSH and GSSG levels in cells overexpressing alpha-syn was largely correlated with a significant decrease in the levels of GCLc subunit and GCL activity in –Dox cells. These findings highly suggested that diminished GSH levels may therefore be derived from depletion in GCL levels, resulting in reduced activity of GCL. Importantly, decreased GCLc was correlated with reduced levels of Nrf2 in the nucleus. Studies from Sian and colleagues (1994) revealed that the activity of GCL is unaffected in

post-mortem tissue from brains of PD patients, thus not explaining the low levels of GSH found in the SN of PD patients (Sian et al., 1994b). One hypothesis could be based on the alterations in cysteine supply that is critical for GSH synthesis (Zeevalk et al., 2008). Circulating levels of cysteine were shown to be decreased with age (Droge and Schipper, 2007), suggesting that such decrease may contribute to the GSH depletion in PD, which incidence increases with age (Dauer and Przedborski, 2003). Reduction of GSSG back to GSH, catalysed by GRed (Dickinson and Forman, 2002), in addition to the *de novo* synthesis, is important in maintaining cellular levels of GSH. Levels of GRed are reported to be increased in PD patients (Dickinson and Forman, 2002; Ilic et al., 1999), suggesting that the levels of GSH should be increased. Thus, GSSG levels were reported to be non-significantly altered in *post-mortem* brains of PD patients (Sian et al., 1994a). Nevertheless, and as stated above, the later studies did not clarify whether PD patients presented alpha-syn mutations or aberrant expression, which in our model seems to be modulating the antioxidant defense mechanisms, namely SOD and GSH synthesis.

Antioxidants, such as SOD and GSH, are required to restore the imbalance in cellular homeostasis caused by oxidative stress (Hybertson et al., 2011). SOD1 and GCLc, apart from other detoxifying enzymes, are modulated by the Nrf2-ARE system, which activation has been investigated in PD models. Nevertheless, the extent and nature of cellular responses to Nrf2 activation have not been established for any PD patient-derived model. Our results indicated a slight, but significant decrease in nuclear Nrf2 levels in SH-SY5Y cells overexpressing WT alpha-syn, which may explain the reduced levels in intracellular SOD1, GCLc and thus GSH and GSSG. Analysis of the localization of Nrf2 in the SN of PD brains demonstrated that, in addition to the cytoplasm, a strong nuclear immunoreactivity was observed in neurons (Ramsey et al., 2007). Furthermore, gene expression studies in human olfactory neurosphere-derived (hONS) cell lines from sporadic PD patients revealed a significant deregulation of the Nrf2-mediated oxidative stress response pathway, compared to hONS cells from healthy controls (Matigian et al., 2010). *Post-mortem* brain studies are also consistent with a role for Nrf2 in PD, as it was detected in the nucleus of remaining

neurons within the SN (Ramsey et al., 2007), suggesting that the remaining cells were able to induce downstream detoxification mechanisms. Lee et al. (2003) revealed that neuronal cultures from Nrf2-null mice presented an approximate 15% decrease in GSH levels compared to control neurons (Lee et al., 2003), and depletion of GSH specifically in cathecolaminergic neurons resulted in age-related neurodegeneration in mice (Chinta et al., 2007). In addition, Suh and colleagues (2004) provided evidence for a reduced Nrf2-mediated gene expression in the aging rat liver, which could be a potential mechanism underlying the loss of GSH synthesis in these old animals (Suh et al., 2004). Although we detected elevated levels of hydroperoxides in –Dox cells, we observed a reduction in Nrf2 nuclear levels, which translocation and activation pathway is promoted by low levels of cytosolic ROS. It seemed that WT alpha-syn overexpression while turning these cells more susceptible to ROS, may be interfering with Nrf2-ARE signaling pathway thus downregulating the antioxidant defense mechanisms. Indeed, alpha-syn localization in the nucleus has been associated with conditions of oxidative stress in a variety of experimental systems (Monti et al., 2010; Siddiqui et al., 2012). Due to the depletion in nuclear Nrf2 levels observed in cells overexpressing WT alpha-syn, we hypothesized whether this protein could be also interfering with the levels of other transcription factors, namely those related with CREB and NF-kB pathways. Very few studies have directed their investigation to transcription deregulation in PD. In contrast with Nrf2, we did not find changes in the nuclear levels of p-CREB, CREB, CBP or NK-kBp65 in cells overexpressing alpha-syn. Indeed, our data support a possible selective interaction between WT alpha-syn and Nrf2, thus interfering in the pathways related to oxidative stress by downregulating the antioxidant defense mechanisms in –Dox cells.

It is well known that nigral dopaminergic neurons are particularly susceptible to oxidative stress. Iron appears to play a key role in increasing the susceptibility of pigmented, neuromelanin-containing neurons of SN (Double et al., 2010; Alberio et al., 2012). In the previous chapter, we gave evidence for the effects induced by prolonged exposure to iron

in cells transiently expressing WT or A53T mutant alpha-syn. Here, we used iron as a short stimulus (2 h) in SH-SY5Y cells conditionally overexpressing alpha-syn, which provides the advantage of an entire cell population expressing identical amounts of protein in a Dox regulated manner. As described before, under basal (control) conditions, cells overexpressing WT alpha-syn presented higher levels of ROS and depletion in SOD1, GSH and GCLc levels, which was accompanied by lower activities of related enzymes and by a decrease in nuclear Nrf2 levels, compared to +Dox cells. Upon treatment with iron, we observed expected exacerbated levels of hydroperoxides in both +Dox and –Dox cells and enhanced activity/levels of antioxidants responsible for maintaining the intracellular redox balance. Kim and colleagues (2012) showed that lymphoblastoid cell lines derived from a PD patient with SNCA gene duplication were more resistant to H_2O_2 and rotenone stimuli, compared to the control (Kim et al., 2012). While this may be compared with the results obtained upon iron exposure, we should take into account that iron effects were independent from alpha-syn overexpression, since we observed almost the same alterations in +Dox cells. In fact, iron not only did not trigger caspase-3 activation, but also seemed to activate the major intracellular antioxidants to protect against iron-induced ROS production. This was confirmed by an increased activity of total SOD observed in +Dox and –Dox cells treated with iron, when compared to control conditions. This effect was mainly due to the rise in the activity of SOD2, even though lower levels of this protein were detected in +Dox cells exposed to iron; conversely, SOD1 activity remained unchanged, although a slight increase was observed in its levels upon treatment with 500 µM iron in –Dox cells. This may be accounted for by decreased degradation of SOD1 as a result of enhanced oxidized cell environment. In the case of SOD2, it is possible that short iron stimulus could be upregulating its activity without interfering with protein levels. Seymen and co-workers (1997) studied the effects of iron supplementation on GSH levels, GPx and SOD activities in erythrocytes in experimental hyperthyroidism and verified significant rises in these protein levels upon iron treatment as compared with the control condition (Seymen et al., 1997). According to these data, we also observed substantial elevated levels

of GSH and GSSG upon exposure to iron in both +Dox and –Dox cells; however, significantly unchanged GPx and GRed activities were observed under the same experimental conditions. GSH synthesis is dependent on its amino acid precursors L-glutamate, L-glycine and L-cysteine. As stated before, L-cysteine has been identified as rate limiting in GSH synthesis (Armstrong et al., 2004; Bender et al., 2000; Dun et al., 2006). System Xc^-, a cysteine-glutamate antiporter, is a heterodimeric protein that mediates the exchange of intracellular L-glutamate for extracellular L-cystine (REDDY et al., 1961; Rimaniol et al., 2001; Tomi et al., 2003). In the presence of high extracellular concentrations of L-glutamate, the antiporter functions in reverse, exchanging extracellular L-glutamate for intracellular L-cystine (Rimaniol et al., 2001). Once L-cystine enters the cell, it is likely reduced to two cysteine-residues that are available for incorporation into protein or GSH synthesis (Lim et al., 2005). A study from Lall et al. (2008) demonstrated in two mammalian cell types that iron-induced increase in L-glutamate availability augmented L-cystine uptake, with subsequent increases in GSH levels (Lall et al., 2008). Therefore, it is possible that an increased availability of cysteine could explain increased activity of GCL, the rate-limiting enzyme in GSH synthesis, and thus increased levels of GSH and GSSG in cells overexpressing WT alpha-syn. Therefore, intracellular antioxidants induced by iron could serve as a cytoprotective mechanism. Our results also showed a slight decrease in nuclear Nrf2 levels in cells overexpressing alpha-syn after iron treatment, which in part may help to explain the decrease in GCLc protein levels. The GCL enzyme is a dimeric, predominantly inactive protein composed by two separately encoded proteins, a catalytic subunit (GCLc) and a modulatory subunit (GCLm) (Martin and Teismann, 2009). Both subunits are controlled at a transcriptional level by a variety of cellular stimuli, including oxidative stress. Krejsa et al. (2010) showed that activation of GCL occurred rapidly (10 – 90 min) after oxidative stress induction without changes in GCL protein levels (Krejsa et al., 2010). The authors also demonstrated that part of GCLc and GCLm subunits existed as monomers in untreated Jurkat cells and shifted to higher molecular weight forms following treatment with H_2O_2 and glutathione depletion, used as oxidative models, suggesting that post-

translational regulation of GCL activity may involve direct modifications of the GCL subunits. Thus, altered redox balance, i.e., oxidizing conditions, appear to favour the formation of the heterodimeric complexes and/or to decrease the dissociation rate of the binding subunits in the GCL holoenzyme, stimulating GCL activity, while reducing conditions inhibit GCL activity *via* dissociation of the GCL subunits. In addition, studies in other cell models, including primary human T cells, lymphoblasts and human pulmonary fibroblasts demonstrated similar effects on GCL activation after glutathione depletion (Krejsa et al., 2010). This suggested that rapid post-translational activation of GCL after perturbation of the intracellular redox state can be pointed as a general phenomenon. Therefore, it is possible that short iron exposure could promote the formation of high activity heterodimeric complexes, which resulted in a shift to more efficient GSH production following intracellular oxidative stress.

In general, we propose that WT alpha-syn overexpression may be involved in modifying SH-SY5Y cells susceptibility to oxidative stress, by downregulating the activities and levels of components of the antioxidant defense pathways, namely at a transcriptional level, involving decreased Nrf2 transcriptional regulation, which may contribute to explain the oxidative pathological events described in PD. Furthermore, we give evidence for a compensatory antioxidant defense mechanism activated by short oxidative stress stimulus; despite this, we observed increased ROS formation under these conditions, which may precede and eventually trigger subsequent events that ultimately lead to the neurodegeneration process observed in PD.

CHAPTER 5

Influence of wild-type alpha-synuclein overexpression on mitochondrial activity and related oxidative stress

5.1. Summary

Alpha-synuclein (alpha-syn), implicated in the pathogenesis of sporadic and familial forms of Parkinson's disease (PD), is thought to affect mitochondrial function, although the exact mechanisms still remain unclear. Indeed, several evidences suggest a role for mitochondrial dysfunction in the pathophysiology of PD, in particular an impairment of complex I, which contributes to elevated levels of oxidative stress. Thus, in this part of the work we aimed to establish a relationship between enhanced mitochondrial WT alpha-syn and mitochondrial ROS linked to mitochondrial impairment in doxycycline (Dox)-inducible SH-SY5Y cells. Indeed, cells overexpressing WT alpha-syn (-Dox) possess large amounts of alpha-syn in mitochondria. Mitochondria from –Dox cells revealed a decrease in complex I activity, while the levels of the nuclear encoded 30 kDa subunit of complex I were not altered. Under these conditions, no significant alterations in the levels of transcription factors implicated in mitochondrial biogenesis, namely mitochondrial transcription factor A (TFAM) and nuclear peroxisome proliferator activated receptor (PPAR) γ co-activator 1 alpha (PGC-1alpha) were observed in –Dox cells. Results also revealed that -Dox cells displayed higher levels of superoxide radical, as compared to +Dox cells, which were not promoted by iron exposure. Concordantly, we observed a reduction in superoxide dismutase 1 (SOD1) activity in mitochondrial fractions of –Dox cells, which was exacerbated by iron. In contrast, mitochondrial SOD2 activity was not affected under basal conditions in –Dox cells, but was highly increased upon iron treatment. Reduced and oxidized glutathione (GSH and GSSG, respectively) remained decreased in mitochondrial fractions from –Dox cells in untreated conditions, while iron did not affect the levels of this endogenous antioxidant. These data show that WT alpha-syn overexpression enhances localized alpha-syn in the mitochondria, leading to decreased activity of mitochondrial complex I and increased mitochondrial ROS, which may be boosted due to low antioxidant defenses in the organelle.

5.2. Introduction

Mitochondrial dysfunction has long been implicated in the pathogenesis of PD. The most direct evidence for disrupted mitochondrial metabolism came from studies of autopsy tissue and *in vitro* cell cultures derived from patients with PD. One important finding in *post-mortem* studies was that the activity of complex I (also referred as NADH-ubiquinone oxidoreductase), a major component of the electron transport chain, was decreased in the *substantia nigra pars compacta* (SN) (Henchcliffe and Beal, 2008) and frontal cortex (Parker, Jr. et al., 2008) in patients with PD. Furthermore, impairment of electron transport chain was also detected in PD human platelets (Haas et al., 1995) and in skeletal muscle (Penn et al., 1995). Similarly, reduced complex I activity and increased susceptibility to mitochondrial dysfunction was demonstrated in cybrids containing mitochondrial DNA (mtDNA) from PD patients, suggesting that mtDNA encoded defects may play a role in PD (e.g. Swerdlow et al., 1996). An additional piece of evidence suggesting that mtDNA plays a role in the pathogenesis of PD is the increased proportion of mtDNA deletions observed in PD patients as compared to age-matched control subjects (Bender et al., 2006). There is also increasing evidence that a number of genetic mutations associated with PD have deleterious effects on mitochondrial function. These include alpha-syn, parkin, PINK1, DJ1 and LRRK2 genes, which have been suggested to have a direct or indirect role on mitochondrial dysfunction and disease pathogenesis. Missense mutations in alpha-syn as well as genomic duplications or triplications of the alpha-syn gene were associated with autosomal-dominant PD (reviewed in Lesage and Brice, 2009) that is thought to contribute to the disease through a toxic gain of function. Many studies have suggested that alpha-syn can be localized in mitochondria (Thomas and Beal, 2007; Devi et al., 2008). Indeed, an association between neurodegeneration and mitochondrial damage was shown in mice harbouring the human A53T alpha-syn mutation (Hsu et al., 2000). These mice not only presented mitochondrial accumulation of human alpha-syn and mitochondrial degeneration associated with increased mtDNA damage, but also impaired activity of cytochrome c (cyt c) oxidase or

complex IV. Furthermore, overexpression of human alpha-syn in mice increased susceptibility to neurodegeneration following administration of mitochondrial toxins, namely 1-methyl-4-phenyl-1,2,3,6-tetrapyridine (MPTP) and paraquat (Klivenyi et al., 2006). Devi and colleagues (2008) identified a cryptic mitochondrial targeting sequence in the N-terminal 32 amino acids of human apha-syn, which is crucial for mitochondrial targeting of alpha-syn. Thus, the authors gave evidence for an association between alpha-syn and the inner mitochondrial membrane, which led to reduced mitochondrial complex I activity and increased reactive oxygen species (ROS) production (Devi et al., 2008).

Most of the mitochondrial proteins are encoded by nuclear genes and imported from the cytoplasm to the mitochondria. Among these is TFAM, which encodes the mitochondrial transcription factor A (Ekstrand et al., 2004; Kang and Hamasaki, 2005; Kang et al., 2007; Reyes et al., 2002) and shows high affinity to mtDNA, controlling its transcription and playing a major role in replication, damage, sensing and DNA repair (Bonawitz et al., 2006; Garstka et al., 2003; Kaufman et al., 2007; Pohjoismaki et al., 2006). TFAM is also of great importance for maintaining ATP production (Bonawitz et al., 2006; Ekstrand et al., 2004). A conditional TFAM knock-out mouse line (the MitoPark mouse) showed Parkinson-like motor disabilities, which were ameliorated by Levo-3,4-dihydroxyphenylalanine (L-DOPA) administration (Ekstrand et al., 2007), suggesting that TFAM dysfunction may be involved in PD.

Another target that has been extensively investigated in PD subjects is PGC-1alpha, a transcriptional co-activator that enhances mitochondrial biogenesis, fatty acid oxidation and oxidative metabolism, besides controlling the expression of enzymes involved in gluconeogenesis pathways in the liver (Yoon et al., 2001; Tsunemi and La Spada, 2012). As a co-activator, PGC-1alpha also interacts with a variety of other transcription factors, including nuclear respiratory factors 1 and 2 (NRF1 and NRF2), which are responsible for regulating the expression of many nuclear-encoded mitochondrial genes, including cyt c, the components of complexes I-V and TFAM (Kelly and Scarpulla, 2004). Recent microarray studies from *post-mortem* PD SN suggest that impairment of PGC-1alpha could also be

involved in PD pathogenesis leading to downregulation of PGC-1alpha-regulated genes (Zheng et al., 2010). These and other findings largely implicated the defects in mitochondrial electron transport, glucose utilization and glucose sensing in PD pathogenesis. Furthermore, activation of PGC-1alpha could rescue dopaminergic neuron loss induced by mutant alpha-syn or the pesticide rotenone in primary neuronal models (Zheng et al., 2010).

Numerous studies have demonstrated that oxidative stress due to mitochondrial dysfunction have deleterious effects on neurons. Under physiological conditions, mitochondria are the primary source of ROS in the cell (Lenaz, 2001; Murphy, 2009; Holley et al., 2010), since superoxide anion ($O_2^{\cdot-}$) is produced by mitochondrial respiration. Superoxide dismutases (SODs) are the major ROS detoxifiers in the cell (Fridovich, 1989; Holley et al., 2010), catalysing the dismutation of superoxide radicals into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (Fridovich, 1995; Holley et al., 2010). From the three types of SODs existing in cells, SOD1 and SOD2 are described to be localized within mitochondria. Copper- and zinc-containing SOD (CuZnSOD or SOD1) is found largely in the cytoplasm (Slot et al., 1986), with lower amounts localized in the mitochondrial intermembrane space (Okado-Matsumoto and Fridovich, 2001). SOD2 (or MnSOD) is localized in the mitochondrial matrix only (Weisiger and Fridovich, 1973; Holley et al., 2010). Furthermore, there are two forms of mitochondrial glutathione peroxidases (GPx): GPx1 and phospholipid-hydroperoxide GPx, localized in the mitochondrial matrix and at the inner mitochondrial membrane, respectively (Esworthy et al., 1997; Maiorino et al., 2003), which act by reducing H_2O_2 to water using GSH, and consequently producing GSSG.

In this section, we aimed to establish a relationship between increased levels of WT alpha-syn in mitochondria, possible changes in transcription factors involved in mitochondrial biogenesis and mitochondrial impairment linked to ROS generation. Furthermore, we examined the levels and activities of relevant antioxidants in mitochondrial fractions, under basal conditions and following exposure to iron, used as an oxidative stress inducer.

5.3. Results

5.3.1. Decreased activity of mitochondrial complex I but unaltered mitochondrial-related transcription factors in SH-SY5Y cells overexpressing WT alpha-syn

Due to the strong evidence supporting a role for aberrant mitochondrial function in PD pathology, namely reduced activity of complex I, we first evaluated whether WT alpha-syn overexpression could be modifying the activity of this respiratory chain mitochondrial complex. As depicted in Figure 5.1A, complex I activity revealed a significant decrease in –Dox cells, suggesting that WT alpha-syn overexpression could interfere with mitochondrial respiratory chain function in this PD cell model. Under these conditions, no significant differences in citrate synthase (CS) activity were observed between –Dox and +Dox cells (Figure 5.1B). We also analysed the levels of the nuclear-encoded complex I subunit of 30 kDa in order to evaluate whether decreased complex I activity induced by alpha-syn overexpression could be related with this enzyme subunit. However, we found no changes in the levels of this protein between +Dox and –Dox lysates (Figure 5.1C).

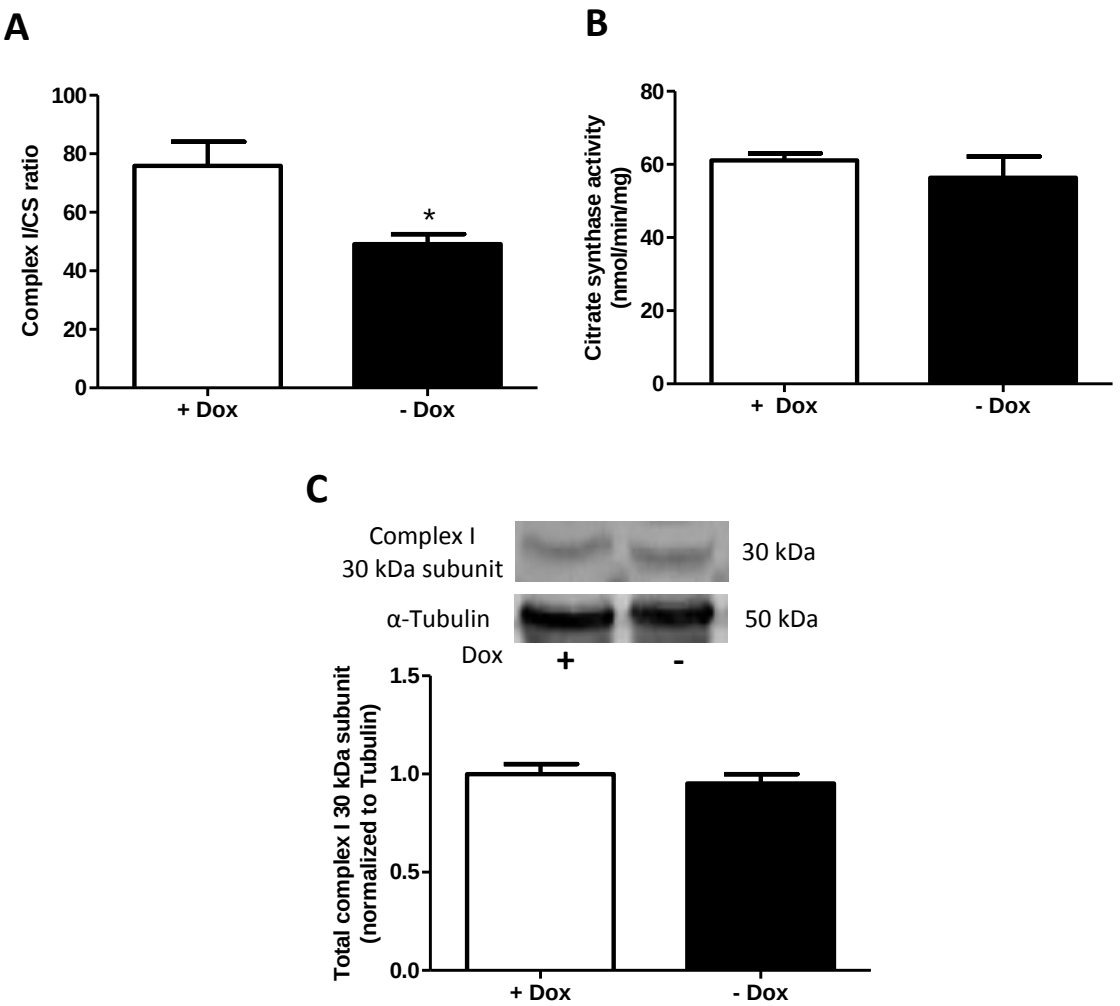


Figure 5.1 – Mitochondrial complex I activity and analysis of complex I 30 kDa subunit levels in inducible SH-SY5Y cells. Cells were cultured in the presence or in the absence of Dox during 5 days. The activities of complex I of the mitochondrial respiratory chain and citrate synthase (CS) were determined spectrophotometrically in mitochondrial fractions (**A, B**). In **C**, total extracts were immunoblotted for the 30 kDa subunit of complex I. Graphs in **A** and **B** show the mean±SEM of 5 independent measurements of the mitochondrial complex I and CS activities. In **A**, the activity of mitochondrial complex I was normalized for CS activity. In **C**, data in the graph are the mean±SEM of 30 kDa subunit of complex I levels normalized to α-tubulin from 3 independent experiments. Statistical analysis: *$p<0.05$, compared to +Dox condition.

Our previous results (Chapter 4) demonstrated enhanced alpha-syn levels in the nuclei of SH-SY5Y cells overexpressing WT alpha-syn (–Dox cells) and decreased levels of Nrf2 under basal conditions. Therefore, here we investigated the presence of alpha-syn in the mitochondria of these cells and whether it could alter the expression of the transcriptional co-activator PGC-1alpha and TFAM, involved in mitochondrial biogenesis. Importantly, we observed significant raised levels of alpha-syn in the mitochondria of SH-SY5Y cells overexpressing this protein, when compared to +Dox cells (Figure 5.2A). However, the same mitochondrial fractions revealed no differences in TFAM levels in inducible SH-SY5Y cells, in comparison to +Dox cells (Figure 5.2B), suggesting unchanged transcription of complex subunits encoded by mtDNA. PGC-1α nuclear levels were also not changed following overexpression of WT alpha-syn (Figure 5.2C), when compared to cells expressing the endogenous protein. Moreover, mtDNA copy number was not significantly different between +Dox and –Dox cells (data not shown). These results indicated that although alpha-syn was highly localized in mitochondrial fractions of –Dox cells, it did not affect mitochondrial transcriptional regulation of important genes involved in the maintenance of oxygen consumption and ATP production.

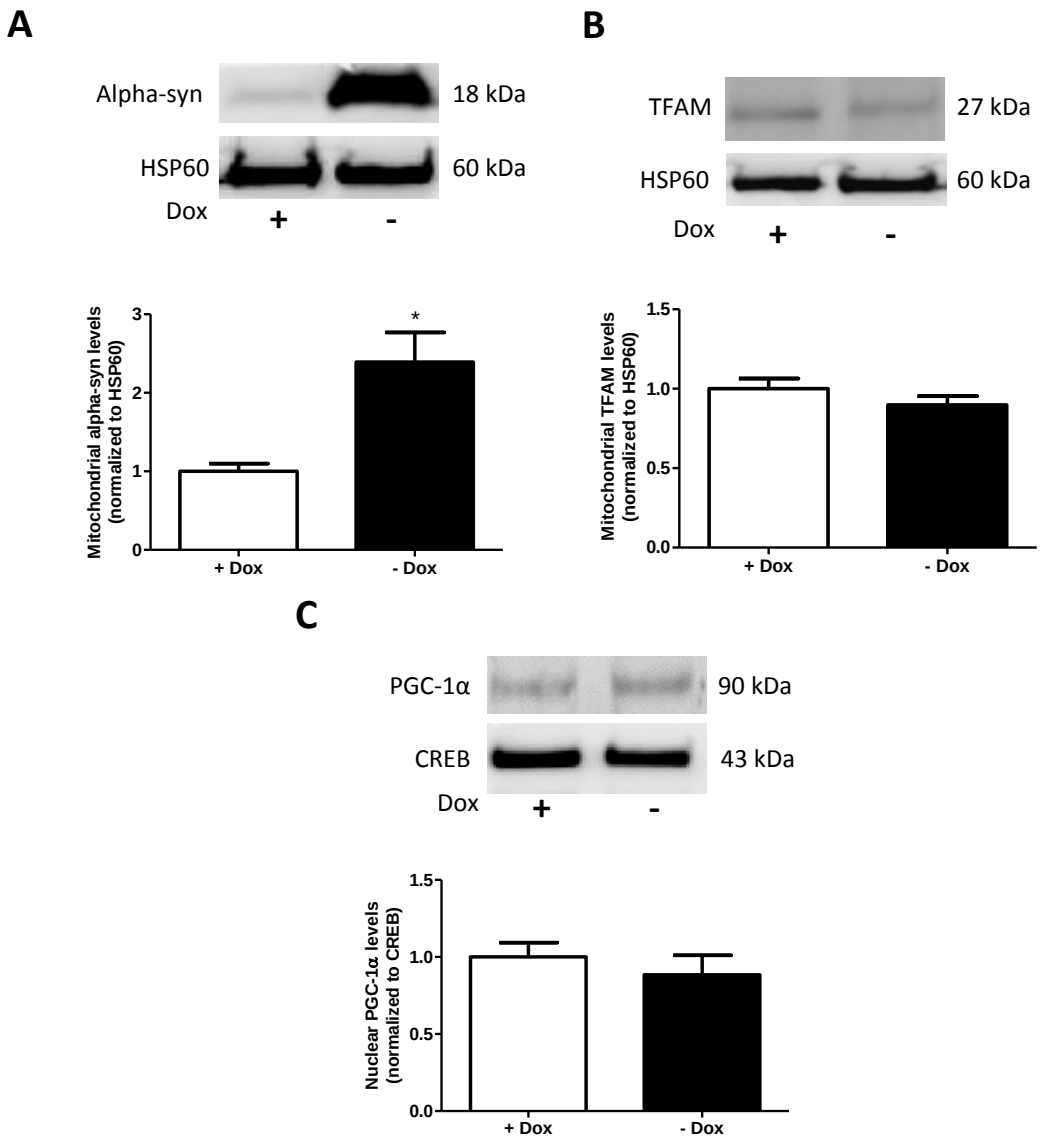


Figure 5.2 – Mitochondrial alpha-syn does not impact on TFAM and PGC-1α levels in inducible SH-SY5Y cells. Cells were cultured in the presence or in the absence of Dox during 5 days. Mitochondrial extracts were immunoblotted for alpha-syn (**A**) and TFAM (**B**), whereas nuclear fractions were immunoblotted for PGC-1α (**C**). Graphs in **A** and **B** represent the mean±SEM of alpha-syn and TFAM levels, respectively, normalized to HSP60, from 3-4 independent experiments. In **C**,

data in the graph are the mean±SEM of PGC-1α levels normalized to CREB from 3 independent experiments. In **A**: *p<0.05 compared to +Dox condition.

5.3.2. Enhanced mitochondrial levels of superoxide anions in inducible cells overexpressing WT alpha-syn

Mitochondria are one of the main sources of superoxide radicals in the cells. Taking into account the results obtained in the previous chapter, which revealed a significant increase in hydroperoxide levels in cells overexpressing WT alpha-syn, we measured the levels of intracellular and mitochondrial superoxide anions under the same experimental conditions. We observed significant higher levels of superoxide anions measured by ethidium fluorescence in –Dox cells, when compared to cells expressing endogenous alpha-syn (Figure 5.3A). Using the fluorescent probe MitoSox Red, which selectively detects mitochondrial superoxide radicals, we also found elevated levels of this ROS in SH-SY5Y cells overexpressing WT alpha-syn, indicating that mitochondria effectively contributes for ROS generation in these cells (Figure 5.3B). These results are in agreement with the decreased activity of complex I observed in –Dox cells. Since we observed a great exacerbation of hydroperoxides production in both +Dox and –Dox SH-SY5Y cells after a 2 h treatment with 500 μM $FeSO_4$ (Chapter 4), we hypothesized whether this oxidative stress inducer could be involved in mitochondrial superoxide anion generation in these cells. However, and as shown in Figure 5.3B, iron did not significantly potentiate the increase in mitochondrial superoxide radical in these cells.

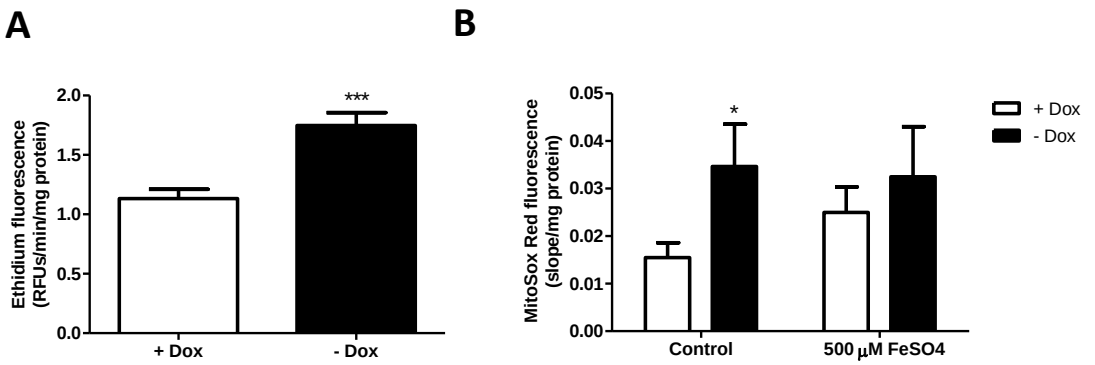


Figure 5.3 – Intracellular and mitochondrial superoxide anion production in induced SH-SY5Y WT alpha-syn cells. Cells were kept in the presence or in the absence of Dox during 5 days. At the day of the experiment, cells were incubated with iron (500 µM for 2 h). Levels of superoxide anion were measured following DHE fluorescence after 60 min of incubation with 5 µM DHE at 37ºC (**A**). Levels of mitochondrial superoxide anion were evaluated by following MitoSox Red fluorescence after 10 min of incubation with 5 µM MitoSox Red (**B**). Data in the graphs are the mean±SEM of three independent experiments performed in triplicates. Statistical analysis: *$p<0.05$ and ***$p<0.001$ compared to +Dox control.

5.3.3. Modified mitochondrial antioxidant capacity of SH-SY5Y cells overexpressing WT alpha-syn

To address a possible cause for the elevated mitochondrial ROS levels observed in –Dox cells, we analysed the levels and activities of proteins involved in the antioxidant defense pathways in mitochondrial fractions, as performed for total lysates (Chapter 4). As shown in Figure 5.4A, total mitochondrial SOD activity, i.e. the sum of SOD1 and SOD2 activities, was greatly diminished in the mitochondria of –Dox cells under untreated conditions. Measurements of SOD1 and SOD2 activities further demonstrated that this decrease in mitochondrial total SOD was mainly due to reduced activity of SOD1 in –Dox cells (Figure 5.4B), whereas SOD2 activity remained unaltered (Figure 5.4C). Similarly to experiments shown in Figure 5.3B, in this part of the work we analysed the effect of 2 h exposure to iron.

We observed an increase in mitochondrial total SOD after treatment with 500 µM $FeSO_4$ in inducible cells (Figure 5.4A), which may help to explain the absence of elevated mitochondrial superoxide anion determined by Mitosox Red fluorescence (Figure 5.3B). In this particular case, mitochondrial SOD2 seemed to be the main contributor, since incubation with iron greatly enhanced the activity of this enzyme (Figure 5.4C). In contrast, mitochondrial SOD1 activity was considerably diminished in both +Dox and –Dox cells after iron exposure.

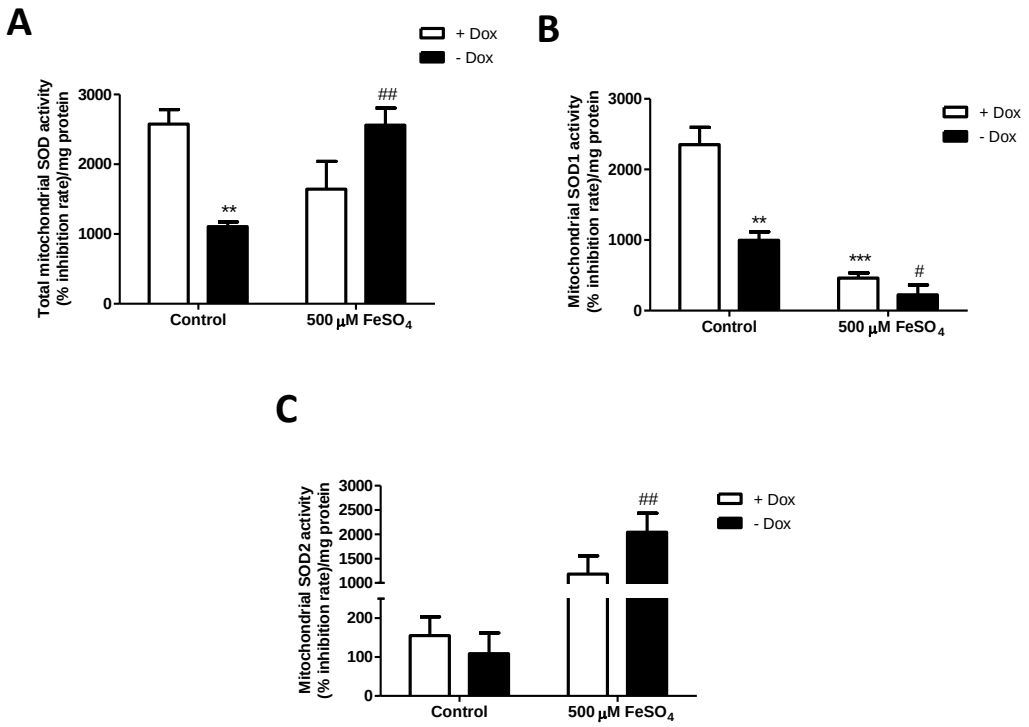


Figure 5.4 – Analysis of mitochondrial SOD activities in inducible SH-SY5Y cells upon exposure to iron. Cells were maintained in the presence or in the absence of Dox during 5 days. Incubation with iron (500 µM) was performed during 2 h. Total SOD (**A**), SOD1 (**B**) and SOD2 (**C**) activities were measured in mitochondrial fractions and the results are expressed as % inhibition rate per mg of protein. % inhibition rate corresponds to the % of WST-1 formazan formation inhibition by SOD. Data in the graphs are the mean±SEM of 4 independent experiments. Statistical analysis:

**p<0.01 and ***p<0.001 compared to + Dox control condition. #p<0.05 and ##p<0.01 compared to –Dox control condition.

We also evaluated the levels of glutathione cycle proteins, namely GSH and GSSG in mitochondrial fractions of inducible SH-SY5Y cells. According to the diminished activity of SOD1 enzyme in basal conditions, levels of GSH and GSSG also decreased in the mitochondrial fractions of alpha-syn overexpressing cells (Figure 5.5A, B). Furthermore, incubation with iron during 2 h diminished GSH levels in +Dox cells only (Figure 5.5A).

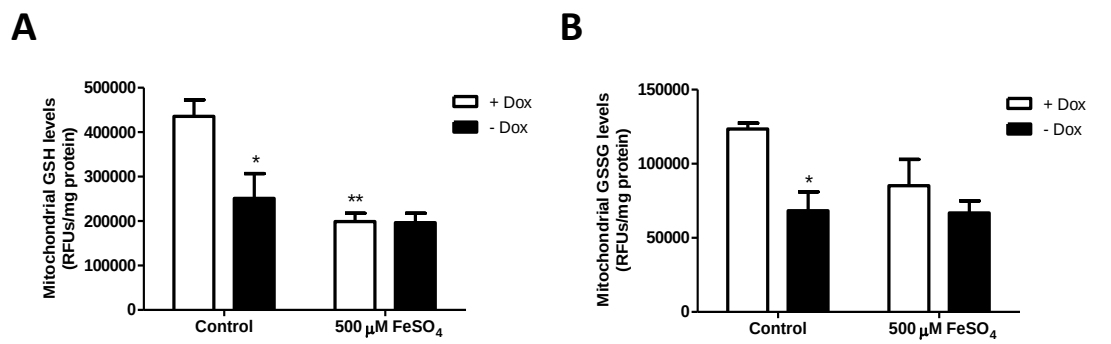


Figure 5.5 – Analysis of WT alpha-syn overexpression on mitochondrial glutathione cycle-related protein levels – effect of iron. Inducible SH-SY5Y cells were kept in the presence or in the absence of Dox during 5 days. Treatment with 500 μM $FeSO_4$ was performed for 2 h. Reduced glutathione (GSH) (**A**) and oxidized glutathione (GSSG) (**B**) levels were determined in mitochondrial fractions. Data in the graphs are the mean±SEM of 4 independent experiments. Statistical analysis: *p<0.05 and **p<0.01 compared to +Dox untreated condition.

These results suggest that alpha-syn overexpression seems to modify mitochondrial proteins involved in antioxidant mechanisms, by lowering SOD activities and GSH levels. Treatment with iron for 2 h hinted that a short exposure to this oxidative stress inducer can

trigger the activity of mitochondrial antioxidant enzymes, namely SOD2, which may act as an initial defense mechanism against toxic stimuli.

5.4. Discussion

Data shown in this Chapter revealed enhanced mitochondrial superoxide anions along with decreased SOD and GSH antioxidant capacity in SH-SY5Y cells overexpressing WT alpha-syn retaining increased levels of alpha-syn in mitochondria. Moreover, overexpression of WT alpha-syn led to decreased activity of mitochondrial complex I and unchanged levels of PGC-1alpha and TFAM transcription factors.

Superoxide anions are produced as byproducts of oxidative phosphorylation, making mitochondria the main source of ROS generation within the cell. While this is a normal process, with basal levels of ROS being limited by a range of antioxidants, pathological situations accounting for by dysfunctional mitochondrial respiratory chain activity can lead to elevated amounts of ROS produced by the electron transport chain, and to ineffective antioxidant protection mechanisms. It has long been recognized that mitochondrial dysfunction and oxidative stress are associated with the pathogenesis of PD (Abou-Sleiman et al., 2006; Fiskum et al., 2003; Greenamyre et al., 2003; Lin and Beal, 2006). Furthermore, data on overexpression of WT and familial mutant forms of alpha-syn in cell culture models and transgenic mouse models have reported cellular changes, including mitochondrial abnormalities (Hsu et al., 2000; Martin et al., 2006; Devi et al., 2008). Devi and colleagues (2008) showed that the N-terminal 32 amino acids of human alpha-syn contain a mitochondrial targeting signalling sequence, which is important for mitochondrial targeting of alpha-syn. These authors demonstrated through *in vitro* and *in vivo* studies that alpha-syn is constitutively localized to mitochondria of dopaminergic neurons and human *post-mortem* brains, providing evidence through biochemical experiments and immunoelectron microscopy that mitochondria-localized alpha-syn was predominantly associated with the inner membrane in the human system; however, the physiological functions of alpha-syn present in the mitochondrial compartment are not clear (Devi et al., 2008). Results from knockdown of alpha-syn in fetal dopaminergic primary neuronal cells (Devi et al., 2008) and

alpha-syn knockout mice (Ellis et al., 2005) showed impaired connectivity between complexes I and III. This may be linked to a possible role for constitutive alpha-syn acting as a chaperone (Ahn et al., 2006; Kim et al., 2000), which localization in mitochondria could be required for the maintenance of the physiological functions of mitochondrial electron transport chain. Observations by Ellis et al. (2005) and Devi et al. (2008) suggest that endogenous alpha-syn could be of great importance in maintaining the normal function of mitochondrial complexes involved in the oxidative phosphorylation. Interestingly, *in vivo* data using *post-mortem* PD brains and results from fetal dopaminergic primary neuronal cells hinted that progressive accumulation of alpha-syn in the mitochondria impaired complex I function and caused increased oxidative stress. This conclusion was supported by experiments showing that overexpression of alpha-syn lacking the mitochondrial targeting signal not only failed to localize to mitochondria, but also did not cause mitochondrial abnormalities. According to the results from Devi and collaborators (2008), revealing a significant correlation between mitochondrial alpha-syn accumulation and decreased complex I activity in human PD basal ganglia, our data demonstrated decreased complex I activity in SH-SY5Y cells exhibiting high levels of alpha-syn in mitochondria. Similarly to Devi et al. (2008), mitochondrial alpha-syn was linked to increased ROS generation by the organelle. This is also in agreement with previous data showing that increased generation of superoxide anions was accompanied by a reduction in the activity of mitochondrial respiratory chain complexes (Abou-Sleiman et al., 2006; Gu et al., 1997; Swerdlow et al., 1998) and also corroborates associated respiratory complex I deficits in mitochondria of PD patients

PGC-1alpha expression has been reported to be compromised in the sporadic PD brain (Zheng et al., 2010). Binding of alpha-syn to the PGC-1alpha promoter was also found to occur in brain tissues from PD patients (Zheng et al., 2010). Despite high levels of alpha-syn in the nuclei of inducible SH-SY5Y cells (Chapter 4), no changes were observed in the levels of the master regulator of mitochondrial gene expression, PGC-1alpha. Siddiqui et al. (2012) showed in an *in vitro* cell model that WT alpha-syn specifically binds to a subset of

promoter sequences including PGC-1alpha. The authors also revealed that the degree of alpha-syn-chromatin binding following oxidative induction correlated with reduced PGC-1alpha promoter activity, mRNA and protein levels and decreased expression of PGC-1alpha target genes. Live cell imaging and electron microscopy analysis demonstrated that repression of PGC-1alpha induced morphological mitochondrial abnormalities and mitochondrial dysfunction, including alterations in complex I activity and scarce metabolic capacity, factors that may impact on neuronal survival, particularly under stress conditions (Chinta et al., 2010). Another mitochondrial transcriptional factor that has been extensively studied is TFAM, which is necessary for mitochondrial transcription and is also required for mtDNA maintenance in mammals (Larsson et al., 1998; Galter et al., 2010). Neurons lacking TFAM develop progressive respiratory chain deficiency followed by cell death (Sorensen et al., 2001). Analysis of alpha-syn-enriched mitochondrial fractions showed that similarly to PGC-1alpha, TFAM levels were not changed in this cell model. Therefore, we may anticipate that decreased activity of mitochondrial complex I is apparently not due to modified expression of mitochondrial complex subunits; indeed, no differences in the levels of the nuclear-encoded complex I 30 kDa subunit were observed between +Dox and –Dox cells.

Taking into account the results of the previous Chapter, where we gave evidence for decreased intracellular capacity of antioxidants in –Dox cells, here we showed that mitochondria also presented reduced SOD1 activity. Interestingly, SOD2, which is localized to the matrix of mitochondria only, was not altered in –Dox cells. Nevertheless, the presence of iron during 2 h seemed to trigger the activity of this enzyme in –Dox cells, which may explain the unraised mitochondrial superoxide radical levels in the presence of this toxic stimulus, as compared to control untreated conditions. Strengthening our idea that augmented ROS could be due to downregulated antioxidant pathways, we also found decreased GSH and GSSG levels in the mitochondria of –Dox cells. Because GSH synthesis enzymes are not found in mitochondria (Griffith and Meister, 1985), transport of cytosolic GSH into the mitochondrial matrix is an important determinant of GSH status in the organelle (Lash, 2006). Therefore, it is plausible to assume that if GSH synthesis is

diminished in –Dox cells (as observed in Chapter 4), mitochondria also present decreased GSH. The same hypothesis would be applied for the levels of mitochondrial GSH in the presence of 500 µM iron, which were highly increased in total extracts but not in mitochondrial fractions of both +Dox and –Dox cells. These data suggest that iron exposure may be blocking GSH transport to mitochondria. It is possible that iron-induced ROS may affect the activity or lead to the inactivation of GSH carriers. Glutathione transport into the mitochondria was shown to be mediated by the oxoglutarate carrier and the dicarboxylate carrier (DIC), both localized at the inner mitochondrial membrane (Chen and Lash, 1998; Martensson et al., 1990). Kamga et al. (2010) found that these transporters are expressed in rat brain and suggested that DIC was the main GSH transporter in cortical mitochondria. Thus, the authors claimed that DIC-mediated GSH transport was essential to maintain ROS homeostasis and normal respiratory function (Kamga et al., 2010).

Establishing a link between the high iron levels and mitochondrial dysfunction observed in the SN of PD patients, one may predict that during disease development a diminishment in the transport of reduced GSH into the mitochondria may occur, hence contributing to augment ROS generated by this organelle. In this part of the work, we found that increased levels of alpha-syn in mitochondria correlated with decreased activity of mitochondrial complex I, according with previous studies concerning PD pathogenesis. Moreover, increased mitochondrial alpha-syn was correlated with enhanced superoxide anion generation, which may be accounted for by depleted antioxidant activity. Iron exposure (for 2 h) seemed to be insufficient to induce mitochondrial ROS generation, although it triggered SOD2 activity in cells overexpressing alpha-syn, which may constitute an early defense response against short stimulus. Thus, we may predict that boosting antioxidant expression and thus mitochondrial antioxidant levels and activity may constitute a possible therapeutic target in halting the progression of PD pathology.

CHAPTER 6

Conclusions

Parkinson's disease (PD) is still mostly known as an incurable, fatal, motor disease. It affects now 7-10 millions of people worldwide but its incidence is increasing as the world population age. Initially considered a single clinical entity, involving selective degeneration of the nigrostriatal pathway and a concomitant reduction in the striatal levels of DA, research advances from the last two decades led to a different concept of the disease. PD´s etiology is complex, probably involving several factors that lead to the pathogenic processes underlying the typical motor features and nonmotor symptoms. Thus, basic questions have been raised concerning the mechanisms that contribute to its onset and progression. Is there a unifying mechanism accounting for neurodegeneration? How do mutations help to understand the disease? Why are neurons from the SN especially vulnerable? Where and why does neurodegeneration begin? Which mechanisms are the basis for progressive SN cell loss? These and other questions remain unanswered, however many efforts have been employed in this field and information about the mechanisms underlying cell death in PD is growing.

One of the major causes of the disease is linked to alpha-syn, a protein apparently involved in neuronal function. Alpha-syn has been associated to PD due to various reasons: 1) It is the most abundant protein present in LBs; 2) Genetic mutations occurring in SNCA gene lead to autosomal dominant PD; 3) Duplications and triplications of alpha-syn gene have been also related to familial forms of PD and 4) Sporadic PD forms are clearly linked to alpha-syn. Other mechanisms have been proposed to impact on neurodegeneration observed in the SN of PD brains, namely mitochondrial dysfunction through complex I inhibition and increased levels of oxidative stress.

In this thesis, we gain new insights into the correlation between alpha-syn and the previous described damaging cellular mechanisms, namely oxidative stress and mitochondrial dysfunction, by studying the: 1) susceptibility to mutant A53T *versus* WT alpha-syn toxicity in the presence of iron or rotenone in SH-SY5Y cell line, and 2) effects of WT alpha-syn

overexpression on antioxidant defense protein levels, transcriptional regulation and mitochondrial activity in a Dox-inducible SH-SY5Y cell line.

Iron is a classic oxidative stress inducer, which levels have been described to be augmented in the SN of PD patients. Rotenone targets mitochondria by inhibiting complex I activity of the mitochondrial respiratory chain and has been widely used in animal models to reproduce features of PD. In Chapter 3 we demonstrated a strong correlation between ROS formation, mitochondrial depolarization, Ub-positive alpha-syn inclusions and alpha-syn Ser129 phosphorylation. These cellular and protein changes are observed following prolonged (4 days) treatment with iron ($FeSO_4$) and rotenone, mimicking the modifications triggered by prolonged exposure to toxic stimuli, in cells overexpressing WT or mutant A53T alpha-syn. Furthermore, we show that mutant A53T alpha-syn expressing cells are more susceptible to the toxic stimuli, when compared to WT alpha-syn expressing cells. The data gives strength to the possibility that mitochondrial impairment and ROS production caused by prolonged exposure to rotenone and iron might induce alpha-syn phosphorylation at Ser129 and the formation of alpha-syn inclusions, particularly after expression of mutant alpha-syn. By covering the inheritable pattern of the disease, results led us to predict that alpha-syn A53T mutation could then be associated with earlier forms of PD due to the greater susceptibility to pathological events, namely those inducing oxidative stress. We also add evidence for a possible role of PP2A in alpha-syn phosphorylation, suggesting that targeting PP2A could be a promising therapeutic approach to mitigate the neuropathology of alpha-syn, particularly in autosomal dominant familial cases caused by A53T mutation.

The work described in Chapters 4 and 5 of this thesis took advantage of a Dox regulated cell model, in which the total cell population expressed WT alpha-syn protein, in contrast to the transient transfection model used in Chapter 3, in which only approximately 30% of the cells were alpha-synGFP positive.

The effects caused by WT alpha-syn overexpression on the susceptibility to oxidative stress in the absence or in the presence of iron ($FeSO_4$) were analysed and changes in nuclear transcription factors, namely Nrf2, were further determined. Data indicate that cells overexpressing WT alpha-syn exhibit increased ROS levels, which are exacerbated by iron exposure. Furthermore, we report a decrease in the levels of proteins involved in the cellular antioxidant defense mechanisms upon overexpression of WT alpha-syn, whereas treatment with iron induces regulation of detoxification pathways apparently independently from alpha-syn overexpression. We propose that WT alpha-syn overexpression may be involved in modifying SH-SY5Y cells susceptibility to oxidative stress, by downregulating the activities and levels of components of the antioxidant defense pathways due to modified Nrf2 transcriptional levels, which can contribute to explain the oxidative pathological events described in PD. Furthermore, we give evidence for a compensatory antioxidant defense mechanism activated by these cells after short oxidative stress stimulus, which may precede and eventually trigger subsequent events that ultimately lead to the neurodegeneration process observed in PD.

The last part of this thesis focused on the effects of WT alpha-syn overexpression and consequent enhanced levels of WT alpha-syn in mitochondria on mitochondrial activity and possible alterations of transcriptional factors involved in mitochondrial biogenesis. The results revealed an increase in mitochondrial ROS in cells overexpressing WT alpha-syn, accompanied by a reduction in the activity of mitochondrial complex I and unaltered levels of PGC-1α and TFAM. According to what was observed for total fractions, mitochondria of SH-SY5Y cells overexpressing alpha-syn revealed a reduction in the antioxidant capacities, thus conducing to the elevated levels of ROS consistently observed in these cells.

Overall, our findings highlight the importance of investigating the basic molecular mechanisms underlying alpha-syn modifications associated to WT and mutant forms of this protein. Herein we show that mitochondrial dysfunction and increased levels of ROS, which seem to be interconnected through common links in dying dopaminergic neurons in PD,

may be exacerbated by alpha-syn, but also contribute to modify the phosphorylation and aggregation status of alpha-syn, thus potentially contributing for accelerating PD-related pathology. Thus, understanding these pathways will give new perspectives to develop neuroprotective strategies directed to prevent neurodegeneration in PD.

As a final remark, one should consider that PD is a complex, multifactorial disease in which different factors lead to the pathogenic process. In this regard, *in vitro* models, such as SH-SY5Y cell line used in this work as a PD model, although providing the advantage of a controlled environment, lack the complexity and the cellular microenvironment critical for disease development. Nevertheless, cellular models are particularly useful for exploring biochemical mechanisms of disease progression and are essential to investigate the molecular action of new drugs or potential toxins, search for single genetic factors in the pathogenic process at the cellular level and at least can provide insights for validation of new targets in animal models and/or humans. Although we are far from finding an integrative mechanism underlying PD pathogenesis, each new discovery is an input to better consolidate the knowledge of the disease.

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