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ROLE OF SELECTIVE KINASES AND GDNF ON IRON-MEDIATED ALPHA-SYNUCLEIN PHOSPHORYLATION-RELEVANCE TO PARKINSON'S DISEASE

Paulo André Ribeiro dos Santos

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Professora Doutora Ana Cristina Rego (Universidade de Coimbra).

Paulo André Ribeiro dos Santos

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RESUMO

A doença de Parkinson (PD, do inglês "*Parkinson's disease*") é uma patologia neurodegenerativa crónica e progressiva, caracterizada pela perda seletiva dos neurónios dopaminérgicos nigroestriatais. As manifestações clínicas desta doença neurodegenerativa incluem dificuldades motoras, instabilidade postural, bradicinésia, tremor de repouso, dificuldades na marcha e rigidez muscular. A evidência neuropatológica da doença é a intensa e progressiva perda dos neurónios dopaminérgicos que contêm neuromelanina na *substantia nigra pars compacta* (SNpc) e a presença de corpos de Lewy (LB), maioritariamente constituídos por α-sinucleína (α-syn, do inglês "*α-synuclein*"). Vários mecanismos têm sido propostos para explicar o processo neurodegenerativo nos neurónios nigroestriatais, incluindo a disfunção mitocondrial e o stresse oxidativo. Estudos anteriores demonstraram um aumento dos níveis de ferro (Fe) na SN de cérebros de doentes de Parkinson, implicando a ocorrência de stresse oxidativo no processo neurodegenerativo. Contudo, atualmente não existe um tratamento neuroprotetor efetivo para a PD.

De acordo com a literatura, a agregação e o estado de fosforilação da proteína α-syn desempenham um papel importante na patogénese da PD. Alguns estudos demonstraram que a α-syn depositada nos LBs está altamente fosforilada no resíduo de serina (Ser)129 (quase 90% da α-syn), atribuindo um papel importante à fosforilação na agregação da α-syn e na formação dos LBs; contudo o seu papel na neurotoxicidade ainda permanece controverso. Um grupo de cinases parece ser responsável pela fosforilação da α-syn. Neste grupo de cinases estão incluídas as *polo-like kinases* (PLK1 e PLK2), *casein kinases* (CK-1 e CK-2) e a *leucine-rich repeat kinase 2* (LRRK2). Por outro lado, evidências anteriores sugerem que o GDNF (do inglês "*glial-derived neurotrophic factor*") pode oferecer uma potencial proteção terapêutica contra esta doença. Assim, o objetivo deste estudo foi avaliar o papel de tais cinases (através da sua inibição com compostos potencialmente seletivos) e do GDNF na regulação da fosforilação da Ser129 da α-syn em células expostas a Fe (um indutor do stresse oxidativo). Além disso, avaliámos o efeito dos inibidores, Fe e GDNF na toxicidade celular. Para tal, usámos células humanas derivadas de neuroblastoma, as células SH-SY5Y, que sobre-expressam de forma condicionada a α-syn *wild-type* (WT), através de um sistema de expressão *Tet-Off* regulado por doxiciclina (Dox).

Os nossos resultados demonstraram que a sobre-expressão da α-syn despoletada na ausência da Dox, induziu um decréscimo na viabilidade celular, quando comparado com as células incubadas com Dox, avaliada pelo método de Alamar Blue. Estes resultados sugeriram que a produção anormal de α-syn é tóxica. Também mostrámos que, com a exceção dos

inibidores das PLKs, concentrações nanomolares dos inibidores de cinases conduziram a uma percentagem aceitável de células viáveis. Além disso, a avaliação da integridade da membrana plasmática e da atividade da caspase-3 em células expostas aos inibidores seletivos, a $FeSO_4$ (Fe, 500μM) e GDNF (20 ng/ml), não revelou efeitos citotóxicos significativos sobre as células, quando comparado com o respetivo controlo. Demonstrámos ainda que o GDNF induziu a ativação precoce da via de sinalização PI-3K/Akt, tal como demonstrado pelo aumento dos níveis de fosforilação da Akt 10 min após a incubação com GDNF. É interessante notar que, a exposição ao Fe ou GDNF durante 2 h aumentou os níveis de fosforilação da α-syn na Ser129. Contudo, a pré-incubação de GDNF preveniu a fosforilação da α-syn induzida por Fe. Esta observação sugere que o GDNF é importante para reduzir a fosforilação da α-syn induzida pelo stresse oxidativo. Por fim, a pré-incubação das células com os inibidores da LRRK2 ou CK-2 (em particular), seguido da exposição a Fe, impediu o aumento da fosforilação da α-syn induzida pelo Fe.

Os resultados apresentados neste trabalho parecem indicar que a CK-2 está entre as potenciais cinases responsáveis pela fosforilação do resíduo de Ser129 da α-syn. Do mesmo modo, o GDNF parece ser capaz de contrariar o aumento da fosforilação da α-syn despoletado pelo Fe, o que pode depender da ativação da via de sinalização intracelular PI-3K/Akt. Tendo em conta que a fosforilação da α-syn parece desempenhar um papel importante na patogénese da doença, estas estratégias podem representar uma solução terapêutica promissora na PD.

ABSTRACT

Parkinson's disease (PD) is a chronic progressive neurodegenerative disorder which pathology is characterized by a profound and selective loss of nigrostriatal dopaminergic neurons. Clinical manifestations of this neurodegenerative disease include motor impairments, postural instability, bradykinesia, resting tremor, gait difficulties and rigidity. The neuropathological evidence of the disease are the progressive and intense loss of neuromelanin-containing dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) and the presence of Lewy bodies (LB), mainly composed of α-synuclein (α-syn). Several mechanisms have been proposed to be implicated in the neurodegeneration of SNpc neurons, including mitochondrial dysfunction and oxidative stress. Previous studies have reported an increase in iron (Fe) levels in the SN of PD brains, implicating oxidative stress as an important process of cell fate. However, currently there is no neuroprotective effective treatment for PD.

Previous evidences have shown that α-syn aggregation and phosphorylation states play an important role in PD pathogenesis. Some reports demonstrated that α-syn deposited in LBs is highly phosphorylated at serine (Ser)129 (almost 90% of α-syn), suggesting an important role for phosphorylation in α-syn aggregation and LB formation, although it is still debatable its role in neurotoxicity. A group of kinases have been suggested to be responsible for α-syn phosphorylation. These include polo-like kinases (PLK1 and PLK2), casein kinases (CK-1 and CK-2) and leucine-rich repeat kinase 2 (LRRK2). Moreover, previous evidences suggest that glial-derived neurotrophic factor (GDNF) may offer potential therapeutic protection in PD. Thus, the aim of this study was to evaluate the role of such kinases (through inhibition by potential selective compounds) and GDNF in regulating the phosphorylation of α-syn at Ser129 in cells subjected to Fe, an inducer of oxidative stress. In addition, we evaluated the effects of kinase inhibitors, Fe and GDNF on cell toxicity. For this purpose, we used SH-SY5Y human neuroblastoma cells conditionally overexpressing wild-type (WT) α-syn in a Tet-Off system regulated by doxycycline (Dox).

Our results demonstrated that α-syn overexpression triggered by the absence of Dox led to a decrease in cell viability when compared to cells incubated with Dox, as assessed by Alamar Blue assay. This may suggest that an abnormal production of α-syn is toxic. We also showed that, with the exception of PLKs inhibitors, nanomolar concentrations of selected kinase inhibitors led to an acceptable percentage of viable cells. Moreover, assessment of plasma membrane integrity and caspase-3-like activity in cells exposed to the selected inhibitors, as well as $FeSO_4$ (Fe, 500μM) and GDNF (20 ng/ml) did not reveal significant cytotoxic effects upon the cells, compared with respective control. We also were able to demonstrate that GDNF induced the early activation of PI-3K/Akt signaling pathway, as highlighted by increased levels

of phosphorylated Akt 10 min after incubation with GDNF. Interestingly, exposure for 2 h with Fe or GDNF increased α-syn (Ser129) phosphorylation. However, pre-incubation of GDNF prevented Fe-induced α-syn phosphorylation state. This observation may suggest that GDNF is important for reducing α-syn phosphorylation induced by oxidative stress. Finally, pre-incubation of cells with inhibitors of LRRK2 or CK-2 (in particular) followed by Fe exposure largely precluded Fe-induced stimulation of α-syn phosphorylation.

Data presented in this work seem to indicate that CK-2 is among potential kinases involved in α-syn phosphorylation at Ser129. Likewise, GDNF seems to be able to counteract increased α-syn phosphorylation evoked by Fe, which may largely depend on the activation of intracellular PI-3K/Akt signaling pathway. Because α-syn phosphorylation has been shown to play an important role in disease pathogenesis, these strategies may represent a promising therapeutic solution in PD.

Key words: Parkinson's disease, α-synuclein phosphorylation, oxidative stress, kinase inhibitors, GDNF.

ABBREVIATIONS

AD - Alzheimer's disease

Akt - V-akt murine thymoma viral oncogene homolog

ARE - Antioxidant response element

ARTN - Artemin

ASK1 - Apoptosis signal regulating kinase 1

ATF3 - Activating transcription factor 3

ATP - Adenosine triphosphate

ATP13A2 - ATPase type 13A2

bARK - Beta-adrenergic receptor kinase

Bax - Bcl-2-associated X protein

Bcl-X_L - B-cell lymphoma-extra large

Bcl-2 - B-cell lymphoma 2

BSA - Albumin from bovine serum

CAPS - 3-(Cyclohexylamino)-1-propanesulfonic acid

CDK1 - Cyclin-dependent kinase 1

Cib - Calcium- and integrin-binding protein

CK - Casein kinase

CNS - Central nervous system

COR - Carboxy-terminal of Roc

COX-2 - Cycloxygenase-2

Ctr - Control

DARPP-32 - Dopamine- and cyclic AMP-regulated phosphoprotein of 32 kDa

Daxx - Death domain-associated protein 6

DAT - dopamine transporter

DMT1 - Divalent metal transporter 1

DNA - Deoxyribonucleic acid

Dox - Doxycycline

DTT - Dithiothreitol

4E-BP1 - Eukaryotic translation initiation factor 4E-binding protein 1

E2F-1 - E2F transcription factor 1

ECF - Enhanced chemifluorescence reagent

EGFR - Epidermal growth factor receptor

Elk-1 - ETS domain-containing protein Elk-1

ER - Endoplasmatic reticulum

ERK - Extracellular signal-regulated kinase

FADD - Fas-associated protein with death domain

FBS - Fetal bovine serum

FBXO7 - F-box protein 7

Fe - Iron II

$FeSO_4$ - Iron (II) sulphate

Fnk - Fibroblast growth factor-inducible kinase

Fpn - Ferroportin

GCL - Glutamate-cysteine ligase

GDNF - Glial-derived neurotrophic factor

GFL - GDNF family ligand

GFR - GDNF family receptor

GIGYF2 - Grb10 interacting GYF protein 2

GPCR - G protein-coupled receptor

GRK - G protein-coupled receptor kinase

GSH - Glutathione (L-γ-glutamyl-L-cysteinyl glycine)

GST - Glutathione S-transferase

GTP - Guanosine triphosphate

HEK 293 cells - Human embryonic kidney 293 cells

HEPES - 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid

HO-1 - Heme oxygenase 1

Hsp90 - Heat shock protein 90

HTRA2/OMI - Htr serine peptidase 2 / known also as OMI

IgG - Immunoglobulin G

IgM - Immunoglobulin M

JNK - c-Jun N-terminal kinase

iNOS - Inducible nitric oxide synthase

IRE - Iron-responsive element

IRP - Iron-regulatory proteins

Keap1 - Kelch-like ECH-associated protein 1

LBs - Lewy bodies

LBD - Lewy Body Dementia

LDH - Lactate dehydrogenase

LfR - Lactoferrin receptors

LNs - Lewy neurites

LRRK2 - Leucine-rich repeat kinase 2

MAO - Monoamine oxidase

MAPK - Mitogen-activated protein kinase

MAPKKK - Mitogen-activated protein kinase kinase kinase

MAP-1B - Microtubule-associated protein 1B

MDC1 - Mediator of DNA Damage Checkpoint protein 1

MDM2 - Murine double minute 2

MKLP1 - Mitotic kinesin-like protein 1

MPP^+ - 1-methyl-4-phenyl pyridinium ion

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

mtDNA - Mitochondrial DNA

NADH - Nicotinamide adenine dinucleotide hydrate

NADPH - Reduced form of nicotinamide adenine dinucleotide phosphate

NaF - Sodium Fluoride

NF-κB - Nuclear factor kappa-light-chain-enhancer of activated B cells

NQO1 - NADPH quinone oxidoreductase 1

Nrf2 - Nuclear factor erythroid 2-related factor

NRTN - Neurturin

6-OHDA - 6-hydroxydopamine

PARP - Poly[ADP-ribose] polymerase-1

PBD - Polo box domain

PBS - Phosphate buffered solution

PD - Parkinson's disease

PFA - paraformaldehyde

PGC-1α - PPARgamma coactivator 1alpha

PINK1 - Phosphatase and tensin (PTEN) homolog-induced putative kinase 1

PI3K - Phosphatidylinositol 3-kinase

PLA2G6 - Phospholipase A2, group VI

PLD - Phospholipase D

PLK - Polo-like kinase

PMSF - Phenylmethanesulfonyl fluoride

Prk - Proliferation-related kinase

PSPN - Persephin

PTEN - phosphatase and tensin homolog

PVDF - Polyvinylidene Difluoride

p53 - Protein 53

p70S6K - 70 kDa ribosomal protein S6 kinase 1

Rab - Family of proteins belonging to Ras superfamily

RBD - Rapid eye movement sleep behavior disorder

RFU - relative fluorescence units

RING - Really Interesting New Gene

ROC - Ras of complex proteins

ROS - Reactive oxygen species

RPMI 1640 - Roswell Park Memorial Institute's 1640 medium

Sak - Snk akin kinase

SAPK - Stress-activated protein kinase

Sept4 - Septin 4

Ser87 - Serine87

Ser129 - Serine129

siRNA - Small interference ribonucleic acid

Snk - Serum-inducible kinase

SNP - Single-nucleotide polymorphism

SN - *Substantia nigra*

SNpc - *Substantia nigra pars compacta*

SOD 1 - Superoxide dismutase 1

SOD 2 - Superoxide dismutase 2

SV-2 - Synaptic vesicle protein 2

α-syn - α –Synuclein

β-syn - β –Synuclein

TEMED - Tetramethylethylenediamine

Tf - Transferrin

TFAM - Mitochondrial transcription factor A

TfR - Transferrin receptors

TH - tyrosine hydroxylase

TK - tyrosine kinase

UBL - Ubiquitin-like

UCHL1 - Ubiquitin carboxyl-terminal esterase L1

UIM - Ubiquitin interacting motifs

UPS - Ubiquitin-proteasome system

UTR - untranslated region

VAMP - Vesicle-associated membrane protein

WT - Wild-type

XRCC1 - X-ray repair cross-complementing protein 1

CHAPTER 1 – INTRODUCTION

1.1. PARKINSON´S DISEASE - AN OVERVIEW

1.1.1. Historical milestones

In 1817 “An Essay of the Shaking Palsy” was the first report in which, his author, James Parkinson, described the clinical syndrome that was later to bear his name (Parkinson, 2002).

He identified six cases, three of whom he personally examined the other three he observed on the London streets. Anteriorly referred as ‘‘paralysis agitans’’, Charcot later in the 19th century gave credit to Parkinson denominating the disease as ‘‘maladie de Parkinson’’ or Parkinson’s disease (PD). Charcot also recognized non-tremulous forms of PD and correctly distinguished that slowness of movement should be differentiated from weakness or ‘‘lessened muscular power’’, a term originally used by Parkinson (Kempster *et al.*, 2007). More than a century passed (1919) after the first reference to the disease by Parkinson before it was recognized that patients with PD are characterized by a profound and selective loss of nigrostriatal dopaminergic neurons, and 140 years passed (1957) before dopamine was discovered as a neurotransmitter by Carlsson and colleagues in Lund, Sweden (Bjorklund and Dunnett, 2007). In 1960 Ehringer and Hornykiewicz discovered that dopamine concentrations are deeply decreased in the striatum of patients with PD (Hornykiewicz, 2006; Bjorklund and Dunnett, 2007) enabling in the following year, the first trials of levodopa (precursor of catecholamines) in PD patients (Birkmayer and Hornykiewicz, 1961). The improvement of akinesia by the injected levodopa in PD patients was first demonstrated in 1961 and was followed by the development of oral levodopa later in the decade (Cotzias *at al.*, 1969; Birkmayer and Hornykiewicz, 1998).

1.1.2. Prevalence

Alzheimer's disease (AD) is the first most common neurodegenerative disorder followed by PD (de Lau and Breteler, 2006). In industrialized countries, the prevalence (percentage in a population at a certain moment) of PD is about 0.3% of the entire population. Such a disease is more prevalent in the elderly and its incidence gradually rises with age. The prevalence of 1% in population over 60 years of age increases to 4% in those over 80 (de Lau and Breteler, 2006). Regarding the onset of disease the mean age is about 60 years, however 5-10% of cases, designated as young onset, begin between 20 and 50 years of age (Samii et al., 2004). There is some controversy regarding the incidence of the disease either in men or in women, some studies reported that is more common in men, others however failed to found differences

between the sexes. PD incidence per year is 8-18 cases per 100,000 persons (de Lau and Breteler, 2006).

1.1.3. Pathology spreading

PD is neuropathologically characterized by a progressive and profound loss of dopaminergic neurons within the *substantia nigra* (SN), particularly affecting the ventral region of the *pars compacta*, and by the presence of intracytoplasmic, proteinaceous inclusions termed as Lewy bodies (LBs) (Forno *et al*., 1996). Analysis of *post-mortem* brains of individuals displaying PD, showed a loss of 50–70% of nigral neurons in this region compared to the same region in healthy individuals (Marsden, 1990; Lang and Lozano, 1998; Ross *et al*., 2004; Dauer and Przedborski, 2003).

During the progress of disease, the severity of the neuropathological changes continuously increases, occurring a known distribution pattern through which it is possible to predict alterations. Even in the brains of individuals, in which there is no clinical evidence for motor dysfunctions associated to the disease, this kind of alterations can be developed at some extension, and therefore is convenient to subdivide the course of disease into presymptomatic and symptomatic phases (Wolters *et al*. 2000; Del Tredici *et al*. 2002; Braak *et al*. 2003).

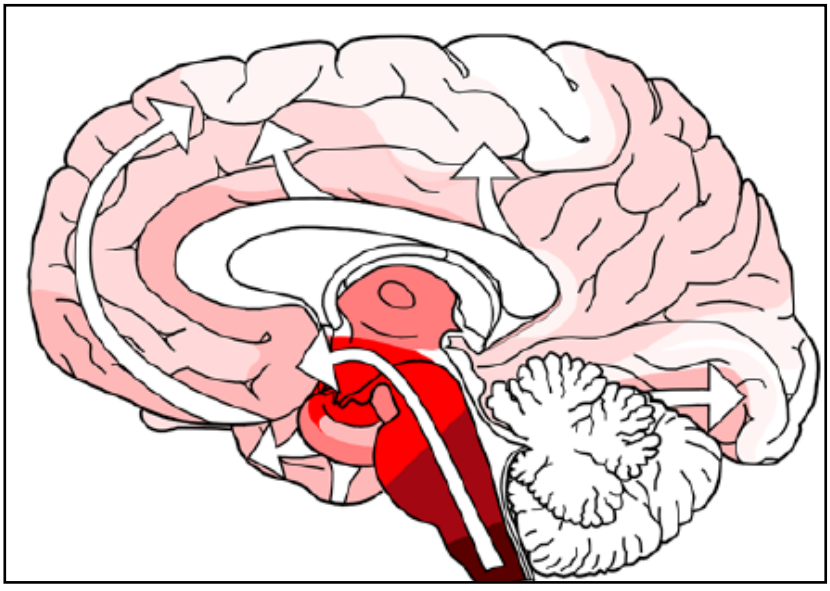


Figure 1.1 - Diagram showing the ascending pathological process (white arrows). Progression of LB deposits would initially begin in the medulla oblongata and in the olfactory bulb and progress in a caudorostral pattern into neocortex regions. The shading intensity of the colored areas corresponds to the severity of the disease. Dark red regions correspond to the initial stages (presymptomatic phase), light red regions correspond to the later stages (symptomatic phase). Based on Braak *et al*., 2004.

Some post-mortem studies based on the analysis of Lewy neurites (LNs) and LBs accumulation, have shown that some brain structures become damaged prior to SN (Del Tredici *et al*. 2002; Braak *et al*. 2003). The generation of abnormal inclusion bodies in the brain begins at the dorsal motor nucleus of the vagal nerve plus adjoining intermediate reticular zone and the olfactory bulb, and spreads out accordingly to a caudorostral predictable pattern, from the lower brain stem to the neocortex. The proposed model for PD progression is based on the identification of six neuropathological stages (Figure 1.1), each stage is characterized by the continual development of LNs in the interior of cellular processes and LBs in the cell bodies of the affected neurons (Braak *et al*., 2004 for review). During the progression of the disease, parts of the limbic, autonomic and somatomotor systems become seriously damaged. In the presymptomatic phase (stages 1-2), inclusion bodies are restricted to the medulla oblongata/pontine tegmentum and olfactory bulb/anterior olfactory nucleus. In the third and fourth stages, SN and other components of midbrain and forebrain become the centre of pathological alterations. Individuals who achieve this level, probably moves into the symptomatic phase of the disease, this corresponds to the onset of the motor symptoms. In the final stages 5-6, the neurodegenerative process reaches its greatest topographic extent entering in the mature neocortex, and the fullest manifestation of the disease is observed (Braak *et al*., 2004 for review), accompanied by neuronal cell loss.

1.1.4. Clinical features

There are four main features of PD, grouped under the acronym TRAP: Tremor at rest, rigidity, akinesia (or bradykinesia) and postural instability. Moreover, flexed posture and freezing (motor blocks) have been included among classic PD features.

1.1.4.1. Motor features

The most characteristic clinical feature of PD is bradykinesia or slowness of movement. Basal ganglia disorders have bradykinesia as a hallmark, and it encompasses difficulties with planning, initiating and executing movement and with performing simultaneous and sequential tasks (Berardelli *et al.*, 2001). The initial manifestation is often slowness in performing daily tasks and also slowness of movements and reaction times (Cooper *et al.*, 1994; Giovannoni *et al.*, 1999).

Rest tremor is also one of the most common and easily recognized symptoms of PD. Tremors are unilateral and almost always are potentiated in the distal part of an extremity. Rest tremor in PD patients can also affect the lips, chin, jaw and legs but, unlike essential tremor, rarely involves the neck/head or voice. Typically, rest tremor disappears with action and during

sleep (Jankovic, 2008 for review). Many lines of evidence indicate that essential tremor is a risk factor for PD (Shahed and Jankovic, 2007). Many PD patients, beyond rest tremor also develop postural tremor that is more prominent and disabling than rest tremor and may be the first manifestation of the disease (Jankovic *et al.*, 1999; Jankovic, 2002).

One of the most disabling symptoms of PD known as Freezing is a form of akinesia (loss of movement) (Giladi *et al.*, 2001). Although freezing is a characteristic feature of PD, it does not occur universally (Bloem *et al.*, 2004). Freezing most commonly affects the legs during walking, but the arms and eyelids can also be involved (Boghen, 1997). It characteristically manifests as a sudden and transient inability to move. This may include hesitation in the beginning of walk (start hesitation) or a sudden incapacity to move the feet during specific situations (e.g., turning or walking through a narrow passage, crossing busy streets, approaching a destination) (Jankovic, 2008 for review).

Usually accompanied by the "cogwheel" phenomenon, rigidity is characterized by increased resistance, particularly when associated with an underlying tremor, present throughout the range of passive limb movement (flexion, extension or rotation about a joint). It occurs proximally (e.g., neck, shoulders, hips) and distally (e.g., wrists, ankles) (Jankovic, 2008 for review). Moreover, neck and trunk rigidity (axial rigidity) may occur, resulting in abnormal axial postures (e.g., anterocollis the anterior flexion of the neck and scoliosis an abnormal curving of the spine.). Rigidity is often associated with postural deformities resulting in flexed neck and trunk posture and flexed elbows and knees (Jankovic, 2008 for review). Other skeletal abnormalities comprehend extreme neck flexion (''dropped head'' or ''bent spine'') and forward truncal flexion (camptocormia) and scoliosis (Askmark *et al.*, 2001; Azher *et al.*, 2005; Ashour and Jankovic, 2006; Djaldetti and Melamed, 2006).

Postural instability is generally a manifestation of the late stages of PD and usually occurs after the onset of other clinical features. The increased risk of hip fractures comes from postural instability (along with freezing of gait) that is the most common cause of falls (Williams *et al.*, 2006).

PD patients may exhibit a number of secondary motor symptoms that may have impact on their functioning at home, at work and while driving (Singh *et al.*, 2007). In some cases, voluntary movements are accompanied by unintended movements in homologous muscles on the opposite side of the body. These so-called mirror movements may be observed in early asymmetric PD (Li *et al.*, 2007). Speech disorders in patients with PD, referred to as ''tip-of-the-tongue phenomenon'', are characterized by monotonous, soft and breathy speech with variable rate and frequent word finding difficulties (Critchley, 1981; Matison *et al.*, 1982).

Respiratory abnormalities in patients with PD was also reported and can be restrictive or obstructive (Sabate *et al.*, 1996).

1.1.4.2. Non-motor features

Non-motor symptoms are a common and depreciated feature of PD (Zesiewicz *et al.*, 2006). These include autonomic dysfunction, cognitive/neurobehavioral disorders, sleep and sensory abnormalities. The clinical manifestation of non-motor symptoms and the subjacent mechanisms involving non-dopaminergic nerve cells are still intensively discussed. Affected non-dopaminergic nerve cells embrace monoaminergic cells in the raphe nuclei, cholinergic cells in the nucleus basalis of Meynert (related with cognitive deficits) (Hilker *et al*., 2005), the monoaminergic cells in the locus coeruleus (Zarow *et al*., 2003), pedunculopontine tegmental nucleus (probably associated to gait problems) (Rinne *et al*., 2008), and hypocretin cells in the hypothalamus (which may be related to the sleep disorders) (Thannickal *et al*., 2007). About 30-50% of these nondopaminergic neurons are lost in the final-stage of PD (Obeso *et al*., 2010).

1.1.5. Familial and sporadic forms

PD cases have been divided in two forms: familial and sporadic. The sporadic form is the most common and represents 90-95% of total cases. The familial/inherited form covers the remaining 5-10% of PD cases and is caused by mutations on identified critical genes (Dauer and Przedborski, 2003). Both sporadic and inherited forms of PD share the degeneration of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc), which accumulate misfolded and aggregated α-synuclein (α-syn) (Spillantini *et al.*, 1997).

Identification of genes involved in familial forms of PD, provided great advances in understanding the molecular mechanisms underlying the disease pathology, and allowed the first identification of a definitive cause for the disease (Jain *et al*., 2005). Among the most studied genes associated with familial PD, there are two autosomal-dominant genes, α-syn and leucine-rich repeat kinase 2 (LRRK2) and three autosomal-recessive genes *parkin*, phosphatase and tensin (PTEN) homolog-induced putative kinase 1 (PINK1) and DJ-1 (Jain *et al*., 2005).

Concerning sporadic PD, some efforts were made in order to find its causes and several hypotheses have been proposed including, mitochondrial dysfunction, oxidative stress, proteasomal dysfunction and pesticides. Many of these causes are also associated with familial form of PD, and is not well understood whether they are the cause or consequence of the disease. Lewy bodies are composed of α-syn modified by oxidation, resulting in an increased capacity of this protein to misfold and aggregate (Giasson *et al*., 2000). The link between

misfolded α-syn and oxidative stress is shrouded in controversy, however, oxidative stress has been shown to lead to α-syn aggregation, thus it may contribute to sporadic PD (Zhou *et al*., 2004). Rotenone, maneb, and paraquat (pesticides and herbicide, respectively) may also have a role in sporadic PD as well. Rotenone and paraquat inhibit mitochondrial complex I (Sherer *et al*., 2003; Yang *et al*., 2007) and maneb preferentially inhibits mitochondrial complex III (Cicchetti *et al*., 2009). These agents have been reported to induce specific symptoms when administered to mice (Uversky *et al*., 2004). Furthermore, mitochondrial dysfunction may also contribute to sporadic PD since rotenone, paraquat, and MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) inhibition of mitochondrial complex I, lead to cell damage (Abou- Sleiman *et al*., 2006) and increased degeneration of dopaminergic neurons (Mizuno *et al*., 1989; Kweon *et al*., 2004).

1.2. PARKINSON´S DISEASE PATHOGENESIS

1.2.1. Pathogenic mutations

Although PD was mostly considered a sporadic rather than a genetic disorder, genetic analysis of PD families, demonstrated that approximately 5-10% of individuals with clinical manifestations have an evident genetic-related cause, showing a classical dominant or recessive mode of inheritance in accordance with Mendelian laws. Over the last 10 years, were identified 15 genetic loci for PD and 11 genes (Table 1.1) for PARK loci, this includes PARK1 and PARK4/α-syn, PARK2/parkin, PARK5/ubiquitin carboxyl-terminal esterase L1 (UCHL1), PARK6/PINK1, PARK7/DJ-1, PARK8/LRRK2, PARK9/ATPase type 13A2 (ATP13A2), PARK11/Grb10 interacting GYF protein 2(GIGYF2), PARK13/Htr serine peptidase 2 (HTRA2/OMI), PARK14/phospholipase A2, group VI (PLA2G6) and PARK15/F-box protein 7 (FBXO7) (Lesage and Brice, 2009; Hatano *et al*., 2009 for review). From all of these proteins, the most well studied and more frequently associated to the disease will be further described, namely α-syn, parkin, DJ-1, PINK1 and LRRK2.

Table 1.1 - Summary of genes associated with PD

PARK loci	Map position	Gene	Forms of PD	Functions	Mutations	Onset
PD-associated loci and genes with conclusive relevance						
PARK1/4	4q21-q23	SNCA	EOPD AD and sporadic	Membrane trafficking	A30P, E46K, A53T, Genomic duplications/triplications	~40
PARK8	12q12	LRRK2	LOPD AD and sporadic	Membrane trafficking, Kinase	>40 missense variants, >7 of them pathogenic, including the common G2019S	~65
PARK2	6q25.2-q27	Parkin	Juvenile and EOPD AR and sporadic	UPS, E3-ligase	>100 mutations (point mutations, exonic rearrangements)	<40
PARK6	1p35-p36	PINK 1	ARPD	Mitochondria, kinase	>40 point mutations, rare large deletions	32 ± 7
PARK7	1p36	DJ-1	EOPD AR	Oxidative stress	>10 point mutations and large deletions	27–40
PARK9	1p36	ATP13A2	Juvenile AR Kufor–Rakeb syndrome and EOPD	Lysosome? Autophagy?	>5 point mutations	11–16
PD-associated loci and genes with unknown evidence						
PARK3	2p13	Unknown	LOPD AD		Not identified	35–89
PARK5	4p14	UCHL 1	LOPD AD	UPS, Ubiquitin hydrolase	One mutation in a single PD sibling pair	~50
PARK10	1p32	Unknown	Unclear		Not identified	~65
PARK11?	2q36-q37	GIGYF2	LOPD AD	IGF-1 signaling	Seven missense variants	Late
PARK13	2p13	Omi/ HTRA2	Unclear	Mitochondria, protease	Two missense variants	Late
PARK14?	22q13.1	PLA2G6	Juvenile AR levodopa-responsive dystonia-parkinsonism	Phospholipase enzyme	Two missense mutations	20–25
PARK15?	22q11-q13	FBXO7	EO AR parkinsonian-pyramidal syndrome	UPS, E3-ligase	Three point mutations	10–19
PARK12	Xq21-q25	Unknown	Unclear		Not identified	Late

EO, early-onset; **LO**, late-onset; **AD**, autosomal dominant; **AR**, autosomal recessive; **PD**, Parkinson's disease; **SNCA**, α-Syn; **UPS**, ubiquitin-proteasome system. Based on Lesage and Brice, 2009 and Hatano *et al.*, 2009 for review.

1.2.1.1. α-Synuclein

α-Syn is a natively unfolded presynaptic protein believed to play a role in synaptic vesicle recycling, storage and compartmentalization of neurotransmitters that associates with vesicular and membranous structures (Abeliovich *et al.*, 2000; Yavich *et al.*, 2004; Yavich *et al.*, 2006). Structurally, α-syn consists of an N-terminal amphipathic region, an acidic C-terminal domain and a hydrophobic middle region (containing the non-amyloid-β component domain, NAC). Three missense mutations (Figure 1.2) in α-syn gene (A53T, A30P and E46K) (Polymeropoulos *et al.*, 1997; Kruger *et al.*, 1998; Zarranz *et al.*, 2004) were identified as a cause for autosomal dominant PD.Furthermore, genomic duplications/triplications in the same gene were also associated with autosomal dominant PD (Singleton *et al.*, 2003), leading to a pathogenic overexpression of the wild-type protein, however, single nucleotide polymorphisms in the SNCA promoter have been described to be related with sporadic PD (Edwards *et al.*, 2010).

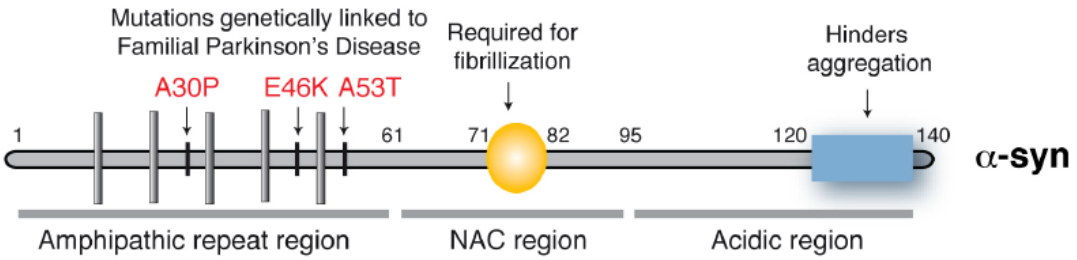


Figure 1.2 - Schematic representation of α-syn structure and domains. Missense mutations genetically linked to Familial PD are represented. Based on Greggio *et al.*, 2011.

α-Syn has a high propensity to aggregate due to its hydrophobic NAC domain. α-Syn fibrils constitute the major structural component of LBs and suggest a role for aggregated α-syn in PD pathogenesis (Spillantini *et al.*, 1998). Several recent studies provide evidences for the NAC domain and the truncated forms of α-syn in mediating neurodegeneration *in vivo*. Overexpression of truncated α-syn lacking residues 71–82 (within NAC domain) failed to aggregate and form oligomeric species in flies, leading to an absence of dopaminergic pathology (Periquet *et al.*, 2007). On the other hand, expression of truncated α-syn, containing the NAC domain induced increased aggregation into large inclusion bodies, accumulation of high molecular weight α-syn species and enhanced dopaminergic neurotoxicity in flies (Periquet *et al.*, 2007). Another study that supports these observations, refers progressive loss of nigral dopaminergic neurons with pathological inclusions and associated behaviours in mice expressing C-terminal truncated human α-syn (containing residues 1-120), on mouse α-syn null background, suggesting a critical role for C-terminal truncated α-syn in aggregation and dopaminergic toxicity *in vivo* (Tofaris *et al.*, 2006). This suggests that alpha-syn C-terminal

region may be an important regulator of its aggregation *in vivo* and pathogenic α-syn mutations may enhance C-terminal truncation-induced aggregation (Li *et al.*, 2005).

Currently, it is not clear whether accumulation of misfolded proteins that lead to LB-like α-syn aggregates formation is toxic or protective in PD. Pharmacological strategies known to promote inclusion formation seem to protect against α-syn toxicity (Bodner *et al.*, 2006). A recent study using a protein aggregate filtration assay demonstrated that synaptic pathology and neurodegeneration occurs due to abundant α-syn associated with presynaptic terminals, in contrast to α-syn aggregates from LBs in *postmortem* brains from patients with Lewy body dementia (LBD), supporting the idea that LBs play a less important role in toxicity (Kramer and Schulz-Schaeffer, 2007). However it has shown that α-syn can misfold and subsequently aggregate and the resultant aggregates are neurotoxic (Fink, 2006).

The mechanisms by which abnormal processing and accumulation of α-syn impair basic cellular functions leading to dopaminergic neurodegeneration have been intensely studied. One of the earliest defects following α-syn accumulation *in vivo* is the disruption of the trafficking between endoplasmic reticulum (ER) and Golgi apparatus, causing ER stress (Cooper *et al.*, 2006). Furthermore, mitochondrial pathology was previously observed in transgenic mice expressing human A53T α-syn (Martin *et al.*, 2006; Stichel *et al.*, 2007), providing a crucial role for α-syn in modulating mitochondrial function in neurodegeneration. This can result from the fact that α-syn may also be a modulator of oxidative damage, since mice lacking α-syn were shown to be resistant to mitochondrial toxins (Williams *et al.*, 2006). Moreover, in human α-syn transgenic mice, nigral dopaminergic neurons were reported to be vulnerable to degeneration and mitochondrial dysfunction following parkinsonian neurotoxin MPTP (Klivenyi *et al.*, 2006; Nieto *et al.*, 2006). In addition, β-synuclein (β-syn) seems to protect α-syn-induced toxicity by reducing its expression (Song *et al.*, 2004), through blockade of pore-like α-syn oligomers development (Fan *et al.*, 2006). Furthermore, overexpression of mutant α-syn (A53T and A30P) increased cytoplasmic catecholamine concentrations, leading to the disruption of pH and normal vesicular function and facilitated the toxicity of oxidized catechol metabolites, resulting in selective degeneration (Mosharov *et al.*, 2006; Tsigelny *et al.*, 2007). α-Syn and its biochemical abnormalities have been shown to activate stress-signaling protein kinases (Hasegawa *et al.*, 2006), affect age-related decrease in neurogenesis (Klegeris *et al.*, 2008), impair microtubule-dependent trafficking (Winner *et al.*, 2007), reduce intercellular communications at gap junctions (Lee *et al.*, 2006), inhibit histone acetylation in the nucleus to promote toxicity (Sung *et al.*, 2007), regulate dopamine homeostasis through regulation of dopamine transporter activity (Kontopoulos *et al.*, 2006), regulate tyrosine hydroxylase (TH) activity (Giovannoni *et al.*, 1999), or vesicle formation (Wersingerand and Sidhu, 2003). α-Syn has also been implicated in ER-Golgi trafficking (Peng *et al.*, 2005), interaction with mitochondrial proteins

(Lotharius *et al.*, 2002), and control of nuclear transcription (Cooper *et al.*, 2006, Martin *et al.*, 2006). These pathophysiological aspects are detrimental to normal functioning of dopaminergic neurons and provide implications for disease pathogenesis in α-syn-induced PD (Kontopoulos *et al.*, 2006).

1.2.1.1.1. Spreading of α-syn aggregates

Induction and spread of protein aggregates in PD brains has been a topic of intense research over the last years. The transmission of α-syn to healthy unaffected neurons from pathologically affected neurons may be a crucial step in the anatomical spread of the disease through the brain. Some evidences suggested a pathological propagation mechanism, identical to that observed in prion diseases (Angot *et al*., 2010).

In the past decades, neural grafts in the striatal region have been reported to carry out a long-term relief of some PD motor symptoms. Recently, α-syn-positive LBs and LNs were observed in grafted neurons in *post-morten* brains of PD patients who received transplants of embryonic mesencephalic tissue more than 10 years prior to death (Kordower *et al*., 2008; Li *et al*., 2008; Li *et al*., 2010). These observations were in accordance with the hypothesis postulated by Braak and colleagues which states that a neurotropic pathogen may cause the propagation of LB and LN pathology from a peripheral origin (olfactory bulb, intestine) to the brainstem and subsequently up to neocortical regions during PD progression (Braak *et al*., 2003). α-Syn could then be considered as a pathogen that disseminates from neuron to neuron, contributing to the neuropathological progression in PD and to the LB profile in grafted embryonic nerve cells (Hansen *et al*., 2011). It is not completely understood how these embryonic transplanted neurons acquired such pathologies. A possible explanation concerning the emergence of pathology in the grafted neurons is the transference of α-syn from the host nerve cells to the grafted cells, with the subsequent seeding-like process of α-syn aggregation in recipient cells, similar to the mechanisms underlying the prion diseases (Angot *et al*., 2010). α-Syn was predominantly considered a cytoplasmic protein. However, α-syn has also been observed in human cerebrospinal fluid and plasma of both PD and normal individuals (Borghi *et al*., 2000; El-Agnaf *et al*., 2003). It was recently demonstrated in mice that neurons expressing α-syn were able to transfer this protein to neurons grafted into the striatum, in analogy to the mechanism observed in the grafted neurons in PD patients (Hansen *et al*., 2011). *In vitro* experiences of co-culturing involving α-syn over-expressing cells with non-expressing cells and neuronal precursor cells have also demonstrated the transference of α-syn, according to paradigm of cell-to-cell transmission (Desplats *et al*., 2009). In both cultures of human and rodent cells, it was observed α-syn transmission and the subsequent seed aggregation of endogenous α-syn in the

recipient cells (Danzer *et al*., 2009; Luk *et al*., 2009; Nonaka *et al*., 2010; Waxman and Giasson, 2010; Hansen *et al*., 2011; Volpicelli-Daley *et al*., 2011) Several years ago, it was shown that a small but significant amount of this protein and its aggregates were secreted from neurons via exocytosis (Lee *et al*., 2005). Furthermore, cultured neurons were capable to internalize external α-syn aggregates via endocytosis (Lee *et al*., 2008a; Lee *et al*., 2008b). In addition, a recent study described the association between α-syn secretion and vesicle membranes, whose composition and biophysical properties were coincident with those in exosomes (Figure 1.3) (Emmanouilidou *et al*., 2010).

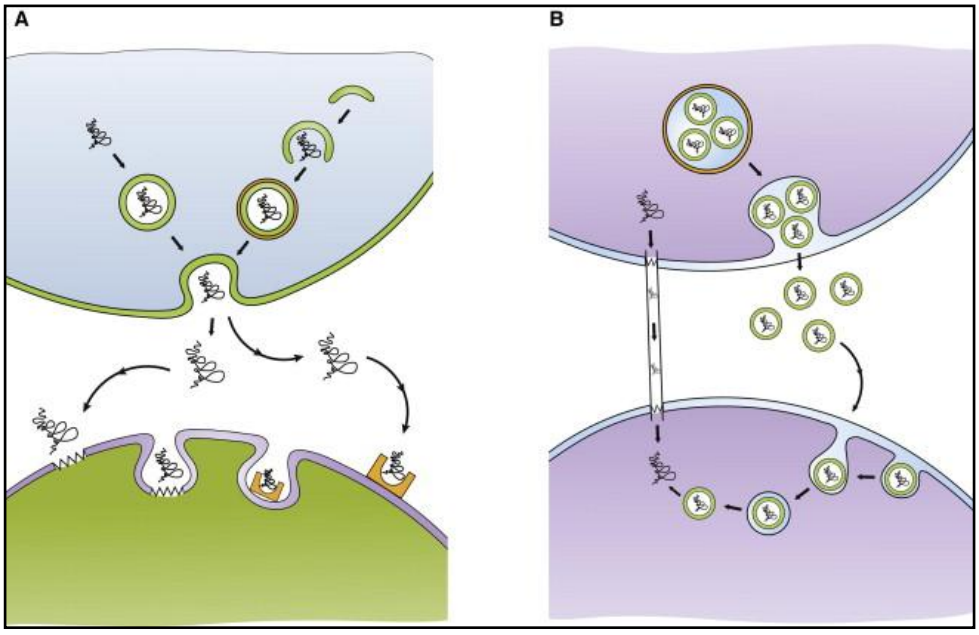


Figure 1.3 - Proposed mechanisms for cell-to-cell transmission of misfolded proteins. (A) Exocytosis and endocytosis. Misfolded proteins can be stored into exocytotic vesicles (top left arrows), which fuse with the cell membrane and release proteins into the extracellular space. Once in the extracellular medium, misfolded proteins can be taken up by cells via lipid raft-mediated endocytosis (bottom left; zigzags represent lipid rafts), or more likely by receptor-mediated endocytosis (bottom right; half rectangle with oval indentation represents receptor). (B) Another mechanism for cell-to-cell transfer of misfolded proteins is within exosomes, small membrane-bound vesicles (green circles) that can form within multivesicular bodies (orange circle), which are released and endocytosed by a surrounding cell (right; arrows). Based on Garden and La Spada, 2012.

In other recent studies, acceleration of α-syn aggregate formation in the brain of young, presymptomatic transgenic mice, along with earlier onset of neurological symptoms, have been described after intracerebral insertion of brain tissue from old transgenic mice with synucleinopathy (Mougenot *et al*., 2012; Luk *et al*., 2012). The same effects were observed after inoculation of recombinant α-syn fibrils directly into the brain of young, transgenic mice (Luk *et al*., 2012). A very recent study has demonstrated for the first time *in vivo* the sequence of events

underlying the prion-like hypothesis, i.e. the transmission of α-syn from a donor to a recipient neuron, followed by the seeding and aggregation of endogenous α-syn in the recipient neuron around a core of transferred α-syn. In the same study it was shown that transferred α-syn co-localized with a marker for early endossomes in grafted nerve cells (Angot *et al*., 2012). These studies reinforce the idea of a direct α-syn transmission from pathologically affected to healthy neurons leading to the disease progression through the nervous system.

1.2.1.2. LRRK2

Mutations in LRRK2 or dardarin cause autosomal dominant PD (Deng *et al.*, 2005; Petit *et al.*, 2005). LRRK2 gene encodes a 2527 amino acid multidomain, 280 kDa protein (Figure 1.4), which belongs to ROCO protein family that includes two characteristic domains: a Ras of complex protein (Roc) Rho/Ras-like-GTPase domain and a carboxy-terminal of Roc (COR) domain. Therefore, the ROCO family can be defined as the group of proteins containing the Roc and COR domains. LRRK2 includes also a protein kinase domain of the mitogen-activated protein kinase kinase kinase (MAPKKK) family, as well as a WD40-repeat and leucine-rich repeat domains (Marín *et al*., 2008). The occurrence of mutations in several distinct domains, as well as deletions or truncations in LRRK2 gene, suggest a gain-of-function mechanism, associated with dominant inheritance. The physiological role of LRRK2 is unknown but the existence of multiple functional domains indicates an involvement in a variety of cellular functions. This protein is expressed in the majority of forebrain structures, including nigrostriatal dopaminergic neurons and is predominantly a cytoplasmic protein, mainly localized in the Golgi apparatus, synaptic vesicles, plasma membrane, lysosomes, but also associates with the outer mitochondrial membrane (Zimprich *et al.*, 2004; Muqit *et al.*, 2006; Tang *et al.*, 2006; Wang *et al.*, 2006; Murakami *et al.*, 2007).

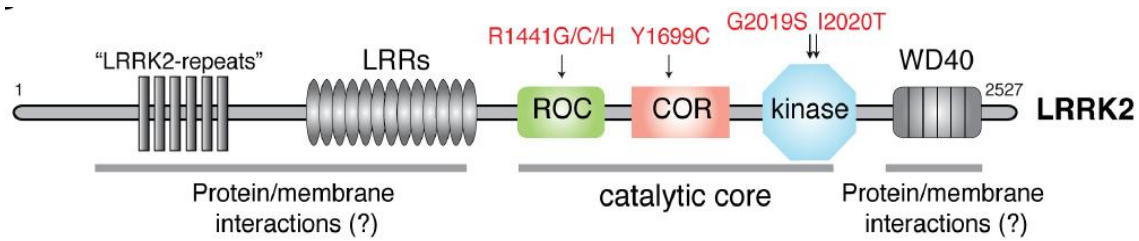


Figure 1.4 - Schematic representation of LRRK2 structure and domains. Point mutations are represented in different domains. Based on Greggio *et al*., 2011.

Point mutations have been identified in almost all domains of LRRK2 gene, such as R1441C, R1441G in the Roc domain, Y1699C in the COR domain, and G2019S and I2020T in

the kinase domain (Kondo *et al*., 2011). Overexpressed mutant LRRK2 showed to be toxic in cultured cells (Cookson *et al.*, 2007; Sakamoto *et al.*, 2009) and *Drosophila*, (Greggio *et al.*, 2006; Cookson, 2010), however loss of neurons was not observed in transgenic mice overexpressing R1441G and R1441C mutants (Smith *et al.*, 2006; Imai *et al.*, 2008). LRRK2 mutations have been related to clinically typical PD features, ranging from nigral degeneration without LBs, to nigral degeneration with widespread LBs or neurofibrillary tangles (Liu *et al.*, 2008).

Due to its interaction with Rab5a, LRRK2 interferes with vesicle endocytosis (Wang *et al.*, 2008), plays a role in activation of apoptosis through interaction with death adaptor Fas-associated protein with death domain (FADD) (Parisiadou *et al.*, 2009), participates in the control of protein translation through phosphorylation of 4E-BP1 (Shin *et al.*, 2008) and interacts with the microRNA pathway to regulate protein synthesis (Sakaguchi-Nakashima *et al.*, 2007). Several groups also reported that LRRK2 interacts with alpha and beta tubulins, (Imai *et al.*, 2008; Ho *et al.*, 2009; Gehrke *et al.*, 2010), suggesting that LRRK2 may play a role in cytoskeleton dynamics. Deletion mutants of LRK-1, a LRRK2 homolog protein in *Caenorhabditis elegans*, led to the depletion of synaptic vesicle proteins in dendritic endings of neurons, defining a possible role in polarized sorting of synaptic vesicle proteins (Paisan-Ruiz *et al.*, 2004). Some studies demonstrated the capacity of LRRK2 to associate with lipid rafts, localize to LBs and regulate neurite length and branching (Biskup *et al.*, 2006; Galter *et al.*, 2006; Higashi *et al.*, 2007). These findings suggest that LRRK2 modulates synaptic vesicle recycling, neurite outgrowth and operates in an inherent manner to Golgi, lysosomes and mitochondria, which dysfunction may compromise dopaminergic neuron survival (Taymans *et al.*, 2006). It is also evident from several studies that altered LRRK2 kinase activity due to disease-causing mutations affected cell viability through apoptosis, imputing a direct role of pathological activation of LRRK2 kinase causing neurodegeneration (MacLeod *et al.*, 2006; Simon-Sanchez *et al.*, 2006; Zhu *et al.*, 2006; Hatano *et al.*, 2007; Sakaguchi-Nakashima *et al.*, 2007).

1.2.1.3. PARKIN

The parkin protein is constituted by a 465 amino acid sequence, containing an N-terminal ubiquitin like domain, a central linker region and a C-terminal RING (Really Interesting New Gene) domain consisting of two RING finger motifs separated by an in between RING domain. Parkin acts as an E3 ubiquitin protein ligase by targeting abnormal proteins to the ubiquitn proteasome system for degradation. Mutations in this protein are described to cause the loss of its E3 ligase activity and lead to autosomal recessive early-onset PD (Zhang *et al.*, 2000; Iwata *et al.*, 2001; Thomas and Beal, 2007). Several parkin substrates have been identified and the

accumulation of one or several of these substrates was shown to be implicated in neurodegeneration (Shimura *et al.*, 2000). Indeed, Kitada and colleagues (1998) reported that when dopaminergic neurons were subjected to a variety of toxic insults, parkin increased neuronal survival (Kitada *et al.*, 1998). Parkin appears to have a neuroprotective role through activation of IkappaB kinase/nuclear factor-kappaB signaling, since parkin mutants failed to stimulate this pathway (Dawson, 2006). Furthermore, the ubiquitin-like (UBL) domain of parkin connects with ubiquitin interacting motifs (UIM) of Eps15 [an adaptor protein involved in epidermal growth factor receptor (EGFR) endocytosis and trafficking] and ubiquitinates the substrates in a proteasome-independent manner. Parkin impairs the binding between Eps15 UIMs and ubiquitinated EGFR delaying EGFR internalization and degradation, promoting phosphatidylinositol 3-kinase/Akt cell survival signaling (Feany and Pallanck, 2003). Parkin also seems to have a role in modulation of key mitochondrial functions, including a role in mitochondrial morphogenesis during spermiogenesis, the final stage of spermatogenesis (Henn *et al.*, 2007), and the enhancement of mitochondrial biogenesis in proliferating cells through transcription and replication of mitochondrial DNA (Fallon *et al.*, 2006). In cases of inactivation of the putative mitochondrial serine/threonine kinase (PINK1), that causes autosomal recessive PD, parkin was shown to rescue mitochondrial dysfunction, muscle degeneration and dopaminergic loss in flies (Clark *et al.*, 2006; Kuroda *et al.*, 2006; Yang *et al.*, 2006; Riparbelli and Callaini, 2007). In addition, a study from Stichel and collaborators (2007) demonstrated that α-syn-induced mitochondrial dysfunction was further enhanced due to lack of parkin activity *in vivo*, implicating a crucial role of this protein in modulating mitochondrial function in α-syn-induced PD (Stichel *et al.*, 2007). Post-translational modifications of parkin either due to oxidative or nitrative stress also compromise its protective function by impairing the E3 ligase activity (Casarejos *et al.*, 2006; Park *et al.*, 2006). Some studies also provided important insights on the role of mutant parkin *in vivo*. Expression of mutant human parkin in flies (but not the wild-type (WT) protein), led to an age-dependent dopaminergic neurodegeneration accompanied by motor impairment, implying a toxic gain of function mechanism (La Voie *et al.*, 2005). This is in contrast with the lack of nigral dopaminergic degeneration in mouse models generated by targeted deletion of parkin, causing a loss of function phenomenon (Chung *et al.*, 2004). Surprisingly, catecholaminergic neurons from parkin knockout mice failed to show increased susceptibility to neurodegeneration against neurotoxins (Moore *et al.*, 2005; Sang *et al.*, 2007) and human α-syn-induced disease (Chung *et al.*, 2004; Stichel *et al.*, 2007). Although parkin may be considered as a neuroprotective agent, these considerations suggest that its neuroprotective efficiency is quite selective. Finding the specific neuroprotective pathways that are affected due to parkin deficiency will help to identify its role in PD pathogenesis (Kontopoulos *et al.*, 2006).

1.2.1.4. DJ-1

Loss -of-function mutations in the DJ-1 locus are related with rare forms of autosomal recessive early-onset parkinsonism (Moore *et al.*, 2005a). DJ-1 is a highly conserved protein of 189 amino acids that belongs to the DJ-1/Thi/PfpI protein super family. It has ubiquitous expression in a variety of mammalian tissues, including brain and is localized in cytosol, mitochondria and nucleus (Perez *et al.*, 2005; Thomas *et al.*, 2007). Association of DJ-1 pathogenic mutations to familial PD has helped to identify some of its functions that shed light in disease pathogenesis. These include antioxidant, transcriptional co-activation and chaperone activity.

Many lines of evidence suggest that DJ-1 acts as an antioxidant protein (von Coelln *et al.*, 2006). Due to its inherent ability to undergo self-oxidation and eliminate hydrogen peroxide (H_2O_2), it was suggested that it may function as a scavenger of other reactive oxygen species (ROS) (Solano *et al.*, 2006). Overexpression of WT DJ-1 either in cell culture or dopaminergic neurons *in vivo* protected against a wide variety of toxic injury induced by oxidative stress (Bonifati *et al.*, 2003; Bandopadhyay *et al.*, 2004; Thomas and Beal, 2007). This antioxidant capacity appears to be related to the stabilization of the antioxidant transcriptional regulator Nrf2 (nuclear factor erythroid 2-related factor) by DJ-1, thus preventing association with its inhibitor, Keap1 (Kelch-like ECH-associated protein 1) and Nrf2 ubiquitination (Zhang *et al.*, 2005). This is in accordance with the ability of DJ-1 to increase cellular glutathione levels by activating glutamate-cysteine ligase (GCL) (Canet-Aviles *et al.*, 2004). α-Syn aggregation and subsequent cell death were described to be inhibited by DJ-1 redox-dependent chaperone activity (Taira *et al.*, 2004). Moreover, DJ-1 associates with parkin during oxidative stress suggesting a common neuroprotective role (Paterna *et al.*, 2007).

DJ-1 familial PD-linked mutations have been considered to cause nigral neuron degeneration through a loss-of-function mechanism. Furthermore, methionine oxidized DJ-1 was identified in sporadic PD brains in mass spectrometric studies, suggesting a role for methionine oxidation in disease pathogenesis (Inden *et al.*, 2006). Mouse models lacking DJ-1 developed age-dependent motor deficits, hypokinesia and dopaminergic dysfunction without neuronal loss (Zhou and Freed, 2005; Clements *et al.*, 2006). In these mice, the nigrostriatal dopaminergic neurons showed an increased susceptibility to the parkinsonian neurotoxin MPTP via an unknown mechanism (Shendelman *et al.*, 2004). Increased vulnerability of DJ-1 knockout mice could be related with increased p53 (protein 53) and Bax (Bcl-2-associated X protein) expression (Zhou *et al.*, 2006), deficits in phase II detoxification enzyme NQO1 (NADPH quinone oxidoreductase 1) (Zhang *et al.*, 2005), irreversible changes in membrane potential due to impaired Na^+/K^+ ATPase (Moore et al., 2005b), defective phosphatidylinositol

3-kinase/Akt signaling (Choi *et al.*, 2006) and incapacity of Daxx (Death domain-associated protein 6) protein to inhibit ASK1- (apoptosis signal regulating kinase 1) induced cell death (Goldberg *et al.*, 2005). Concerning dopaminergic neuronal function, it is important to consider the ability of DJ-1 to transcriptionally upregulate TH expression by suppressing the sumoylation of pyrimidine tract-binding protein-associated splicing factor (Chen *et al.*, 2005).

1.2.1.5. PINK1

Mutations in the PINK1 gene were identified to cause early-onset familial PD (Kim *et al.*, 2005). PINK1 gene encodes a 581 amino acid protein that contains an N-terminal mitochondrial targeting sequence and a highly conserved protein kinase domain similar to serine/threonine kinases of the Ca^{2+} calmodulin family. It has an expression pattern that suggests mitochondrial localization (Bretaud *et al.*, 2007). Little is known about the precise function of PINK1 in the pathogenesis of PD. However, its mitochondrial localization, the presence of a kinase domain where the majority of mutations occur, indicate a role in mitochondrial dysfunction, protein stability and kinase pathways (Yang *et al.*, 2005; Pisani *et al.*, 2006). Truncation of PINK1 C-terminal and disease-related mutations seem to downregulate its serine/threonine kinase activity and confer different autophosphorylation patterns, suggesting an important involvement of its kinase activity in mitochondrial function (Junn *et al.*, 2005; Zhong *et al.*, 2006).

In vitro studies suggest that overexpression of WT PINK1 can prevent staurosporine-induced cell death, mitochondrial cytochrome *c* release and subsequent caspase 3 activation leading to apoptosis (Gandhi *et al.*, 2006). This is consistent with increased vulnerability of neuroblastoma SH-SY5Y cells to the mitochondrial complex I inhibitor rotenone and MPP^+ (1-methyl-4-phenyl pyridinium ion) following suppression of PINK1 function by small interference RNA (Leutenegger *et al.*, 2006), or expression of PINK1 mutants (Beilina *et al.*, 2005). Proteasomal stress impaired PINK1 function by mechanisms that lead the protein to undergo altered cleavage (Silvestri *et al.*, 2005), an abnormal event that may enable this protein to be accumulated in LBs (Yang *et al.*, 2005, Sim *et al.*, 2006). *In vivo* studies in flies showed that PINK1 loss of function either due to expression of disease-causing mutations or due to its inactivation by siRNA results in muscle and dopaminergic neuron degeneration as a consequence of mitochondrial dysfunction. Interestingly, this degenerative phenotype was rescued by overexpression of parkin, revealing the importance of both parkin and PINK1 in regulating mitochondrial physiology and survival in flies (Clark *et al.*, 2006; Kuroda *et al.*, 2006; Yang *et al.*, 2006; Riparbelli and Callaini, 2007). In addition, experiments in flies showed that overexpression of human superoxide dismutase 1 (SOD1 or Cu/Zn-SOD) prevented the loss of dopaminergic neurons due to PINK1 inactivation, suggesting that the oxidative damage pathways are modulated by mitochondrial dysfunction (Scheele *et al.*, 2007). These findings

were supported by the fact that oxidative damage induced by PINK1 dysfunction recruited the antioxidant DJ-1, maintaining the steady-state levels of PINK1 through a physical interaction between these proteins (Beilina *et al.*, 2005).

PINK1 and parkin have a common signaling pathway and further reports suggested their direct interaction (Shiba *et al*., 2009; Sha *et al*., 2010), giving rise to the hypothesis that parkin is not only recruited for the cytosolic ubiquitination process, but is also involved in mitochondria homeostasis. The discovery of parkin translocation from cytosol to depolarized mitochondria and subsequent promotion of their degradation by the autophagy-lysosome pathway (also known as mitophagy) contributed to support this notion (Narendra *et al*., 2008). This is an intriguing possibility, since parkin has been described as a cytosolic protein (Darios *et al*., 2003; Narendra *et al*., 2008). On the other hand, it was demonstrated that PINK1 is a transmembrane protein located in the outer mitochondrial membrane, with its kinase domain facing the cytosol (Zhou *et al*., 2008). These details related with PINK1 localization and topology can be relevant to the reported genetic interaction between parkin and PINK1 since the former and the functional domain of the latter are in the same subcellular compartment. Subsequently, it has been proved that the recruitment of parkin to depolarized mitochondria is a PINK1-dependent process in mammals (Geisher *et al*., 2010; Vives-Bauza *et al*., 2010) and *Drosophila* cells (Ziviani *et al*., 2010).

Increased oxidative stress or mitochondrial activity impairment are indicated as consequences of PINK1 absence or a deficiency in protein activity. Indeed, a report in which PINK1 was silenced in the human neuroblastoma SH-SY5Y cell line resulted in oxidative stress and mitochondrial dysfunction, which increases over the time (Gegg *et al*., 2009). PINK1 deficiency in differentiated neurons also resulted in increased oxidative stress along with a substantial enhancement in mitochondrial mass, after the silencing process (Wood-Kaczmar *et al*., 2008). Furthermore, in PINK1 knockout mouse models, it was reported a progressive increase in oxidative stress, accumulation of abnormal mitochondrial morphology and inhibition of mitochondrial respiration (Gautier *et al*., 2008; Gispert *et al*., 2009). A recent study proposed a physiological scenario in which PINK1 is needed to recruit parkin to the mitochondria afterwards dysfunctional mitochondria are delivered to the perinuclear area, once there they are degraded by autophagy (Vives-Bauza *et al*., 2010). Impairment in the turnover of dysfunctional mitochondria, due to mutations in either parkin or PINK1could results in neurodegeneration in familial forms of PD (Vives-Bauza *et al*., 2010).

At this point of knowledge it is premature to infer about the physiological function of PINK1 through its direct interaction with parkin and DJ-1. However, this interaction suggests

the involvement of three different proteins causing familial PD in sharing common pathways for PD pathogenesis.

1.2.2. Mitochondrial dysfunction and oxidative damage in PD

Multiple evidences suggest a pathogenic role for oxidative damage and mitochondrial dysfunction in PD. Deficits in the subunits and activity of mitochondrial complex I in blood platelets and SNpc of PD patients have been consistently described (Li and Beal, 2005; Greggio *et al.*, 2006). Reduction of complex I activity was also observed in cytoplasmic hybrid (cybrid) cell lines containing mitochondrial DNA (mtDNA) from parkinsonian patients (Smith *et al.*, 2006). Epidemiological studies indicate that exposure to industrial wastes, pesticides and environmental toxins are also involved in sporadic PD pathogenesis (West *et al.*, 2007). A paradigmatic example is the inadvertent discovery that MPTP induced parkinsonism in designer-drug abusers. Indeed, MPP^+ is the toxic metabolite of MPTP, which is selectively taken up by dopaminergic neurons, causing mitochondrial dysfunction (Smith *et al.*, 2005a). Like MPTP, complex I inhibitors such as rotenone and paraquat induced dopaminergic degeneration in rodents, suggesting a pivotal role of mitochondrial dysfunction in PD pathogenesis (Iaccarino *et al.*, 2006; Keeney *et al.*, 2006). Multiple lines of evidence support the notion of mitochondrial dysfunction in triggering PD (Beal, 2005). Some findings demonstrate that neurons of the SNpc have high amounts of mtDNA deletions in *postmortem* brains from PD patients when compared with other neuronal populations in brain- and age-matched controls (Swerdlow *et al.*, 1996). A related study revealed that nigral dopaminergic neurons from PD patients contained higher levels of clonally expanded somatic deletions in mtDNA, leading to mitochondrial dysfunction (Gorell *et al.*, 1998). These results are supported by a study where targeted deletion of mitochondrial transcription factor A (TFAM) in midbrain dopaminergic neurons led to progressive PD in mice, due to deficiencies in mitochondrial respiratory chain and reduced expression of mtDNA (Langston *et al.*, 1983). Moreover, an unexpected low mitochondrial mass observed in SNpc of mice might be a contributing factor to vulnerability of these selective neurons and could indicate mitochondrial dysfunction (Betarbet *et al.*, 2000). Together, these findings suggest that factors which modulate normal mitochondrial functioning may significantly compromise neuronal viability, suggesting a pivotal role for mitochondria in PD pathogenesis.

Nigrostriatal dopaminergic neurons generate increased amounts of ROS, probably due to redox cycling of catechols. The presence of dopamine is considered one of the sources of oxidative stress that is unique and characteristic of dopaminergic neurons. Dopamine metabolism via monoamine oxidase (MAO) or amine autoxidation produces radical species:

H_2O_2, quinones and semiquinones (Graham, 1978), which promote the oxidation of lipids and other compounds, impairing mitochondrial function and increasing oxidative stress (Gluck *et al*., 2002; Sulzer *et al*., 2007; Naoi *et al*., 2009). Furthermore, dopamine autoxidation occurs at neutral pH. Therefore, the uptake reduction of dopamine into synaptic vesicles, in which dopamine cannot autooxidize due to vesicle acid pH, may represent a vulnerability factor for these neurons. In agreement, neurons with reduced dopamine transporter activity in the cell membrane are less affected by oxidative stress caused by dopamine or neurotoxins (González-Hernandez *et al*., 2004) and are also less affected in PD (Damier *et al*., 1999).

Interconnection between oxidative stress and mitochondrial dysfunction is further suggested by an impairment of mitochondrial complex I due to chronic decrease of the antioxidant glutathione (Thiruchelvam *et al.*, 2000). Furthermore, PPARgamma coactivator 1 alpha (PGC-1α), which is involved in mitochondrial biogenesis and respiration, acts as a modulator of ROS production during oxidative stress (Lin and Beal, 2006). It was demonstrated that many ROS detoxifying enzymes like glutathione peroxidase-1, catalase and superoxide dismutase 2 (SOD2 or Mn-SOD) are regulated by PGC-1α upon oxidative stress. Concordantly, nigrostriatal dopaminergic neurons were more susceptible to parkinsonian neurotoxin MPTP in mice lacking PGC-1α. Moreover, neural cells were shown to be protected from oxidative stress-induced cell death upon PGC-1α overexpression, evidencing its role as a potent regulator of ROS metabolism. The capacity of PGC-1α to increase the activity of mitochondrial electron transport chain and simultaneously stimulate a wide anti-ROS program, turns it an important target to restrict the damage that has been associated with defective mitochondrial function and oxidative impairment observed in PD and other neurodegenerative diseases (Bender *et al.*, 2006).

Studies in *postmortem* brains suggest a prominent role for Nrf2/ARE (antioxidant response element) signaling in PD pathogenesis (Choi *et al.*, 2006). The leucine-zipper transcription factor Nrf2 regulates the coordinated induction of the ARE activating the transcription of cytoprotective genes, including both antioxidant and anti-inflammatory proteins (Kraytsberg *et al.*, 2006). Briefly, oxidation of a specific cysteine in Keap1 enables Nrf2 to migrate into the nucleus, where it activates the transcription of some genes that encode phase II detoxification enzymes, like NQO1, heme oxygenase 1 (HO-1), GCL, glutathione S-transferase (GST), and downregulates inflammatory enzymes such as inducible nitric oxide synthase (iNOS), cycloxygenase-2 (COX-2), among others (Chinta and Andersen, 2006; Ekstrand *et al.*, 2007; Liang *et al.*, 2007). In Nrf2 knockout mice, neurons were reported to be more susceptible to death due to mitochondrial dysfunction and oxidative stress (McGill and Beal, 2006; St-Pierre *et al.*, 2006; Ramsey *et al.*, 2007). Since both oxidative damage and inflammation have been implicated in PD pathogenesis, Nrf2/ARE pathway may constitute an important target for

therapeutic strategies (Lee and Johnson, 2004; Kobayashi *et al.*, 2006). Phosphatidylinositol 3-kinase (PI3K)/Akt (or protein kinase B) pathway is another promising pathway that has emerged in dopamine neuronal survival. Several findings reveal that overexpression of the oncoprotein Akt protected against 6-hydroxydopamine (6-OHDA)-induced dopaminergic toxicity. Akt seems to acts as a neurotrophic factor in dopaminergic neurons of adult and aged mice, leading to increased neuron size and sprouting (Itoh *et al.*, 1999). In addition, familial PD-linked proteins such as parkin (Feany and Pallanck, 2003), DJ-1 (Choi *et al.*, 2006) and PINK1 (Kim *et al.*, 2005) are involved in mediating cell survival through the Akt pathway, sustaining an important role for Akt regulation in PD. Thus, it is becoming increasingly clear from multiple findings that both mitochondrial dysfunction and oxidative damage take a pivotal role in disease pathogenesis, contributing to sporadic and familial forms of PD.

To sum up, the complexity of PD arises from a multitude of etiological factors involved in disease pathogenesis. Studies related with familial PD-linked genes tightly improved our knowledge of disease development beyond the more common sporadic form. At this point, there are several distinct pathways that are important in modulating pathogenic events causing the death of dopaminergic nerve cells in PD (Figure 1.5).

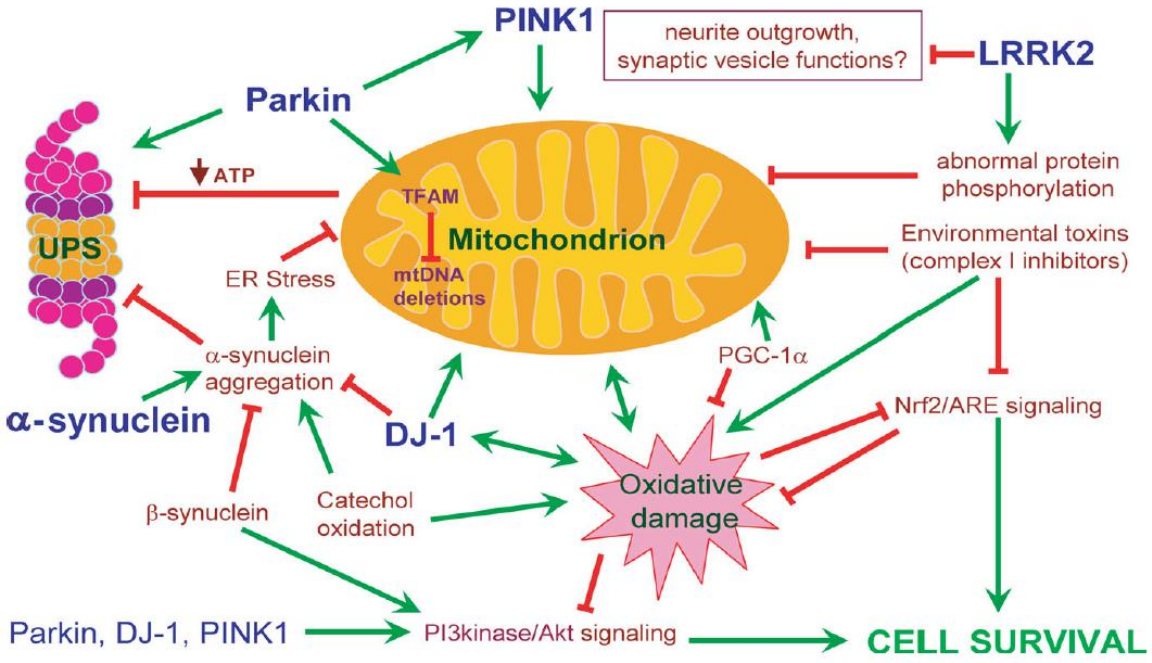


Figure 1.5 - Schematic summary of the established intersecting pathways underlying PD pathogenesis in dopaminergic neurons. These pathways seem to converge on aspects that affect the function and survival of dopaminergic neurons due to oxidative damage, mitochondrial dysfunction and abnormal protein accumulation and phosphorylation. Promoting or activating effects are indicated by green arrows, while inhibitory effects are indicated by red lines with blunt ends. Based on Thomas and Beal, 2007 for review.

1.2.2.1. Iron homeostasis in neurodegeneration and relation with α-syn

Many studies have confirmed an increase in iron (Fe) levels in the SN of most PD cases (Dexter *et al*., 1987; Riederer *et al*., 1989; Hirsch *et al*., 1991); however, the stage during disease progression at which nigral Fe alterations occurs is still debatable. It is known that total nigral Fe levels increase in PD, possibly contributing to nigrostriatal neurodegeneration as a result of its ability to generate ROS and cause lipid peroxidation (Youdim *et al*., 1991; Jomova *et al*., 2010). These raised Fe levels, besides contributing to the increase of oxidative stress, also enhance *α*-syn aggregation (Hashimoto *et al*., 1999). Fe is one of the most important elements for almost all types of cells, including neurons. It is an essential cofactor of various proteins related with normal function of neuronal tissues, such as the non-heme Fe enzyme and TH essential for the synthesis of the neurotransmitters dopamine, norepinephrine, and serotonin (Beard *et al*., 2003). In a healthy brain, Fe appears widely distributed by region and cell-type and its accumulation occurs progressively during aging and the neurodegenerative processes (Dexter *et al*., 1993). In mammals, Fe homeostasis (Figure 1.6) is mediated by the iron-regulatory proteins (IRP1 and IRP2), which post-transcriptionally modulate the expression of specific mRNAs in response to intracellular Fe content (Hentze *et al*., 2004; Rouault, 2006). IRP1 and IRP2 proteins bind to structural elements called iron-responsive elements (IREs). IREs are found in the untranslated region (UTR) of the mRNAs of the transferrin receptor and ferritin, the major proteins that regulate cellular iron homeostasis, involving the plasma-to-cell iron transport, and the iron-storage, respectively (Aguirre *et al*., 2005). It has been demonstrated that α-syn harbors an IRE element in its 5'- UTR (Friedlich *et al*., 2007). Alternatively spliced α-syn mRNAs, which lack the IRE-like sequence encode 112 and 126 amino acid proteins that do not appear to nucleate and fibrillize with the full-length α-syn (Jellinger, 2003; Lee and Trojanowski, 2006). Thus, high intracellular Fe content may also regulate α-syn aggregation through the IRE/IRP system, leading to degeneration of dopaminergic neurons (Li *et al*., 2011). Other studies have revealed a potentially significant association between α-syn and iron (Binolfi *et al*., 2006; Bharathi, 2007). Indeed, α-syn can bind iron and this may be important for the normal activity of the protein, moreover α-syn overexpression significantly increased the percentage of Fe in cells (Davies *et al*., 2011). The same authors reported that α-syn is a cellular ferrireductase, responsible for reducing iron (III) to bio available iron (II) (Davies *et al*., 2011). As Fe deposits are commonly found in LBs (Castellani *et al*., 2000; Gaeta and Hider, 2005), Fe may play a pivotal role in α-syn pathogenicity in PD.

As stated above, Fe can lead to the formation of ROS. Ferric iron (Fe^{3+}) can be reduced to ferrous iron (Fe^{2+}) by the superoxide radical ($O_2^{\bullet -}$) ($Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2$). Fe^{2+} can also react with H_2O_2 generating the highly reactive hydroxyl free radical ($^{\bullet}OH$) ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$), through the Fenton reaction (Jomova and Valko, 2011). Combining these reactions

will result in the Haber-Weiss reaction ($O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^-$), which along with dopamine oxidation can trigger neurotoxicity (Núñez *et al*., 2012). Therefore, the control of Fe homeostasis is a pivotal process to keep a healthy brain.

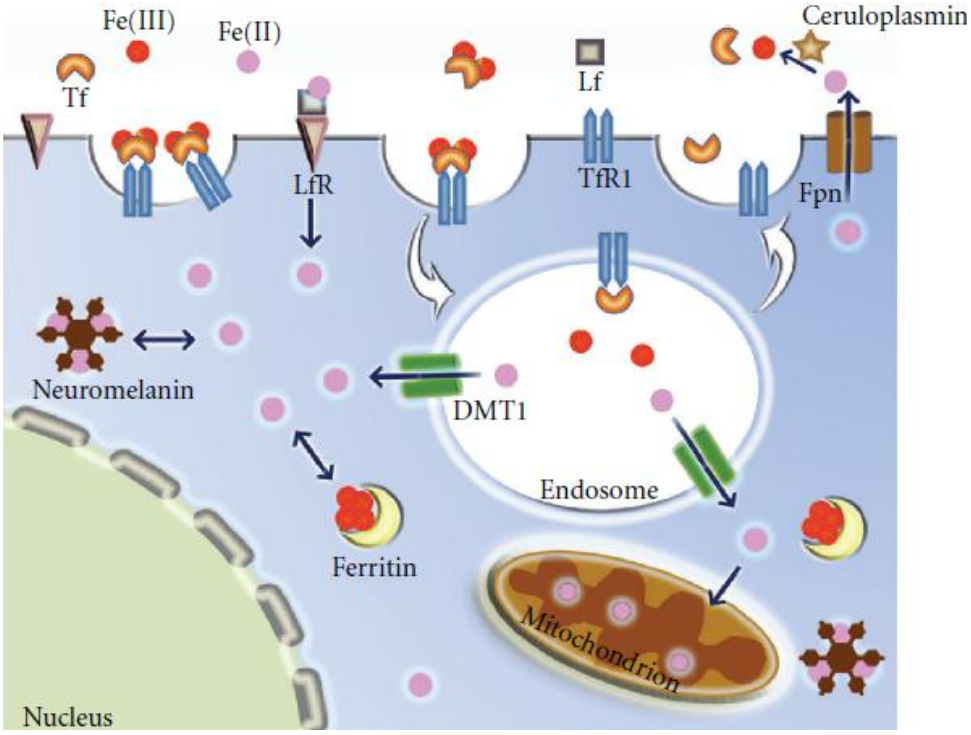


Figure 1.6 - Homeostasis of Fe in the brain. Fe binds to transferrin (Tf), enters in the cell by endocytosis, through the transferrin receptors (TfR), and translocates across the endosomal membrane through the divalent metal transporter 1 (DMT1). Other pathway related to Lactoferrin receptors (LfR) that provides Fe transport from Fe containing lactoferrin across the cell membranes. Inside the cell Fe binds to H-ferritin and accumulates around the neuromelanin. Ferroportin (Fpn) transports Fe^{2+} outside the neuron that is oxidized to Fe^{3+} by ceruloplasmin enabling its binding to Tf. Based on Batista-Nascimento *et al*., 2012.

1.2.3. Environmental factors

Environmental factors were long thought to be the main cause that predispose to the development of PD. Rural environment appears to increase the risk of developing PD, and some epidemiological studies have demonstrated a correlation between exposure to pesticides and wood preservatives and a pre-disposition to develop the disease (Teismann *et al.*, 2003).

People intoxicated with MPTP developed a syndrome identical to PD (Langston *et al*., 1983), this is an example of how an exogenous toxin can mimic the clinical and pathological features of PD. In humans and monkeys, MPTP exposure produced an irreversible and severe parkinsonian syndrome characterized by some features of PD, including slowness of movement,

postural instability, tremor, rigidity and freezing, accompanied by relatively selective dopaminergic neurodegeneration (Dauer and Przedborski, 2003 for review). In MPTP-treated non-human primates was observed the destruction of striatal terminals that preceded that of SNpc cell bodies (Herkenham *et al*., 1991). Moreover, and as in PD, monkeys treated with low-doses of MPTP exhibited preferential degeneration of putamenal dopaminergic nerve terminals compared with those from caudate (Moratalla *et al*., 1992). This drug damaged the dopaminergic pathways in a pattern similar to that seen in PD, including greater cell loss in the SNpc and a preferential loss of neurons in the ventral and lateral parts of the SNpc (Sirinathsinghji *et al*., 1992; Varastet *et al*., 1994) this regional neurological loss pattern was also found in MPTP-treated mice (Seniuk *et al*., 1990; Muthane *et al*., 1994). After the discovery of MPTP-induced PD, subsequent research exploring the molecular basis of MPTP-induced neurodegeneration established relationships between oxidative stress, mitochondrial function and neurodegeneration. MPTP blocks the mitochondrial electron transport chain by inhibiting complex I (Nicklas *et al*., 1987), and altered energy metabolism and generation of ROS were observed after toxin administration (Jackson-Lewis *et al*., 1995). Moreover, prolonged administration of low to moderate doses of MPTP to mice led to apoptosis of SNpc dopaminergic neurons (Tatton and Kish, 1997). MPTP and α-syn were also linked, indeed administration of this toxin led to the accumulation and nitration of α-syn in SNpc dopaminergic neurons (Vila *et al*., 2000; Przedborski *et al*., 2001), and ablation of α-syn in mutant mice prevents MPTP-induced dopaminergic neurodegeneration (Dauer *et al*., 2002).

Rotenone, that inhibits mitochondrial complex I, is a mitochondrial poison present in the environment, and it is used as an insecticide (Sherer *et al*., 2003). Betarbet and colleagues (2000) showed that rotenone-treated rats developed slowness of movement and abnormal postures, and these symptoms were accompanied by selective degeneration of nigrostriatal dopaminergic neurons containing α-syn-positive LB-like inclusions. Nevertheless, this model was the first to link an environmental toxin to the PD pathological hallmark of α-syn aggregation, an association also observed in cell culture based experiments (Uversky *et al*., 2001; Sherer *et al*., 2002; Lee *et al*., 2002). Paraquat is an herbicide present in the environment and shows structural similarity to MPP^+ (Dauer and Przedborski, 2003). Liou and coworkers (1997) reported that exposure to paraquat may confer an increased risk for PD. The toxicity of paraquat seems to be mediated by the formation of superoxide radicals (Day *et al*., 1999). Some reports indicated that administration of paraquat to mice led to SNpc dopaminergic neuron degeneration accompanied by α-syn containing inclusions, as well as α-syn immunostaining in frontal cortex (Manning-Bog *et al*., 2002; McCormack *et al*., 2002).

Factors decreasing the risk of developing PD can also provide useful information to the understanding of the disease etiology. Many epidemiological studies have shown an inverse

association between cigarette smoking and PD (Grandinetti *et al*., 1994; Hernan *et al*., 2001; Paganini-Hill, 2001), reporting that patients who smoke are 50% less susceptible to develop PD when compared to non-smokers (Miller and Das, 2007).

Some studies also found an association between coffee consumption and the risk for developing PD. It was reported in two cohort studies in men, a robust and significant inverse association between caffeine consumption and PD (Ross *et al*., 2000; Ascherio *et al*., 2001), while in a cohort of studies in women, a poor association was found (Ascherio *et al*., 2001).

1.3. SELECTIVE KINASES AND α-SYN PHOSPHORYLATION

Increasing evidence suggest that phosphorylation may play a critical role in modulating α-syn aggregation, LB formation, and toxicity (Fujiwara *et al*., 2002; Anderson *et al*., 2006). It has been demonstrated that α-syn deposited in LBs is highly phosphorylated at Serine (Ser)129 (Kahle *et al*., 2000; Okochi *et al*., 2000; Fujiwara *et al*., 2002; Anderson *et al*., 2006) and Ser87 (Paleologou *et al*., 2010). However, it is still not clear whether phosphorylation protects or enhances against α-syn toxicity *in vivo*. It was recently demonstrated that insufficiency of a pre-synaptic scaffold protein Septin 4 (Sept4), involved in dopaminergic neurotransmission, can potentiate α-syn Ser129 phosphorylation, aggregation and toxicity *in vivo*, while a direct association of Sept4 with α-syn prevented Ser129 phosphorylation and α-syn aggregation *in vitro* (Ihara *et al*., 2007). However, it seems that the pathological modification of phosphorylated Ser129 of α-syn can be selective for neurons, and not for platelets from PD or multiple system atrophy patients (Shults *et al*., 2006).

The role of phosphorylation in promoting or inhibiting fibril formation is still controversial. Phosphorylated α-syn at Ser129 has been reported to promote fibril formation more readily than unmodified protein, *in vitro* (Meulener *et al*., 2006), but inhibition of oligomerization and fibril formation has been also described for Ser87 or Ser129 phosphorylated forms of α-syn. An additional study using an *in vivo* model suggests a lack of correlation between the levels of α-syn fibrillation and phosphorylation at Ser129 (Dick, 2006). More research is needed in order to obtain a coherent view of how phosphorylation alters the physiochemical properties of α-syn. Kinases and phosphatases responsible for regulating α-syn phosphorylation at Ser129 and Ser87 *in vivo* are still not known; however, a series of *in vitro* and cell culture-based studies have identified several kinases, which phosphorylate α-syn at Ser129 and/or Ser87, including casein kinases (CK)-1(at Ser87 and Ser129) and CK-2 (at Ser129) (Okochi *et al*., 2000) and the GRKs (1, 2, 5, and 6, at Ser129) (Pronin *et al*., 2000), LRRK2 (at Ser129) (Qing *et al*., 2009a) and polo-like kinases (PLKs, at Ser129) (Inglis *et al*., 2009; Mbefo *et al*., 2010).

1.3.1. Casein kinases

CK-1 and CK-2 have an ubiquitous expression; they are constitutively active kinases localized in the nucleus and the cytosol (Rinne *et al.*, 1998; Gross and Lozano, 2000; Nyholm *et al.*, 2005). Plasma membrane and cytoskeleton association of CK-1 and CK-2 has been also reported (Tuazon and Traugh, 1991; Ahmed, 1994; Limousin *et al.*, 1998; Paleologou *et al.*, 2010). CKs are involved in diverse regulatory processes, suggesting a broad specificity and a variety of cellular protein substrates. Some findings indicate that CK-1, and specifically CK-2, are key players in the regulation of cell proliferation and transcriptional activity (Tawfic and Ahmed, 1994; Vancura *et al.*, 1994; Walter *et al.*, 1996). However, CK-1 and CK-2 have also been localized to synaptosomes (Issinger, 1993; Hollander *et al.*, 1999) and shown to be capable to phosphorylate a number of synaptic vesicle proteins, like vesicle-associated membrane protein (VAMP), p65 and synaptic vesicle protein 2 (SV-2). Since α-syn was described to bind to brain vesicles (Guerra and Issinger, 1999) as well as small synthetic unilamellar liposomes (Bennett *et al.*, 1993) and PD-associated mutations appear to affect α-syn membrane binding (Gross *et al.*, 1995) these findings may be of particular interest.

CK-2 is an ubiquitous and pleiotropic serine/threonine protein kinase, which was highly conserved during evolution, suggesting vital biological roles (Jensen *et al.*, 1998). CK-2 activity and expression are significantly high in the brain (Davidson *et al.*, 1998; Litchfield, 2003), where it is broadly distributed and primarily localized in neurons (Nakajo *et al.*, 1987; Krebs *et al.*, 1988). Furthermore, CK-2 has innumerous substrates and consequently controls many neuronal functions, including synaptic transmission, plasticity, development, neuritogenesis, information storage and survival (Girault *et al.*, 1990). In addition, CK-2 phosphorylation has been related to the formation of neuronal cytoplasmic aggregates. For example, in AD studies, increased CK-2 immunolabeling has been demonstrated in neurofibrillary tangle-bearing neurons (Martin *et al.*, 1990; Blanquet, 2000). In other neurodegenerative disorders, including the Guamanian Parkinson dementia complex, progressive supranuclear palsy and Pick disease, the association of CK-2 with tangles and other inclusions has also been reported (Iimoto *et al.*, 1990). Additionally, CK-2 is highly expressed in the hippocampus and rapidly activated during long-term potentiation (Masliah *et al.*, 1992). Some neuronal proteins such as neuromodulin, dopamine- and cyclic AMP-regulated phosphoprotein of 32 kDa (DARPP-32) (Baum *et al.*, 1992) and microtubule-associated protein 1B (MAP-1B) (Charriaut-Marlangue *et al.*, 1991) were shown to be phosphorylated by CK-2. Okochi and colleagues (2000) reported in an *in vitro* experiment that α-syn Ser129 phosphorylation was mediated by CK-1 and CK-2. Moreover, the same study revealed that phosphorylation of Ser129 was reduced *in vivo* upon inhibition of CK-1 or CK-2. Furthermore, CK-1 phosphorylation was reported to be activated in striatonigral neurons where dopaminergic neuron degeneration takes place specifically during

PD (Girault *et al.*, 1989). Therefore, Ser129 phosphorylation may also be increased, particularly in these neurons, which may interfere in trafficking of dopamine-containing synaptic vesicles. Together, these findings suggest that CK-1 and CK-2 are involved in the regulation of neuronal function, and that phosphorylation of α-syn may affect its binding to membranes.

α-Syn can be phosphorylated majorly at Ser129 and Ser87. Both Ser129 and the acidic sequence motif required for substrate recognition by CK-1 or CK-2 are also well conserved between several distinct mammalian species (Diaz-Nido *et al.*, 1988). Indeed, a cognate recognition sequence for CK-1 encompasses Ser129 (Desdouits *et al.*, 1995; Okochi *et al.*, 2000). Thus, Ser129 may be alternatively phosphorylated by CK-1 or CK-2. Interestingly, Ser87 phosphorylation seems to be specific for human α-syn and is the only one outside the C-terminal region reported to undergo phosphorylation by CK-1 (Pearson and Kemp, 1991; Okochi *et al.*, 2000).

1.3.2. Polo-like kinases

PLKs encompass a family of conserved serine/threonine protein kinases that play pivotal roles in cell cycle regulation, cellular response to stress and carcinogenesis. In mammalians, the PLK family consists of three closely related kinases PLK1, PLK2/Snk (serum-inducible kinase), PLK3/Fnk (fibroblast growth factor-inducible kinase) also designated Prk (proliferation-related kinase), and a distant member PLK4/Sak (Snk akin kinase) (Marshak and Carroll, 1991; Clay *et al.*, 1993; Golsteyn *et al.*, 1994; Hamanaka *et al.*, 1994; Okochi *et al.*, 2000). These PLKs share a conserved sequence motif characterized by two regions: a highly conserved N-terminal serine/threonine catalytic domain and a non-catalytic C-terminal domain designated Polo box domain (PBD) (Fode *et al.*, 1994; Li *et al.*, 1996). This PBD plays key roles in the regulation of substrate interactions, subcellular localization, targeting, and PLKs autoinhibition (Barr *et al.*, 2004).

In addition to their key role in the regulation of cell cycle (Jackson *et al.*, 2007 for review), PLKs are also expressed in different levels in post-mitotic cells, including neurons. PLK2 and PLK3 expression are induced by synaptic activation and appear to be involved in synaptic plasticity, remodeling, and homeostasis (Kauselmann *et al.*, 1999; Cheng *et al.*, 2003; Xie *et al.*, 2005) and have been also involved in the regulation of dendritic spine morphology. Furthermore, some findings using whole-genome microarray hybridization analysis revealed that PLK2 is indispensable for nerve growth factor-driven neuronal differentiation, since its silencing leads to neuronal differentiation inhibition (Seeburg *et al.*, 2005). Previous studies showed that PLK2 has several properties that are particularly intriguing for an α-syn kinase as it was induced by excitotoxic glutamate agonists (Seeburg *et al.*, 2008). In addition, some authors

have proposed that PLK2 has a pivotal role in maintaining dendritic spine stability (Draghetti *et al.*, 2009) and modulating excitatory glutamatergic synaptic connections (Kauselmann *et al.*, 1999; Pak and Sheng, 2003). Thus, PLK2 involvement in phosphorylation of α-syn provides a potential link between excitotoxic responses and LB pathology.

Some recent findings demonstrate that PLK2 can phosphorylate α-syn Ser129 (Inglis *et al.*, 2009; Mbefo *et al*., 2010). A study confirming these findings, demonstrated using *in vitro* kinase assays, co-transfection, and siRNA-mediated knockdown of PLKs, that α-syn is phosphorylated by specific members of the PLK family. PLK phosphorylates α-syn specifically at Ser129 and this mechanism seems to be mediated by specific interactions between the PLKs and the α-syn N-terminal region (residues 1–95) (Mbefo *et al*., 2010). These results were validated by co-localization assays, in which α-syn and PLKs co-localized in different subcellular compartments, as well as by co-transfection studies and siRNA-mediated knockdown of PLKs in primary neurons and other mammalian cells. PLK2 and PLK3 partially co-localized with α-syn phosphorylated at Ser129 in primary hippocampal neurons and also in cortical brain areas of α-syn transgenic mice. These results point out to PLK2 and PLK3 as the primary PLKs responsible for phosphorylation of α-syn at Ser129 (Inglis *et al.*, 2009). Therefore, the physiological importance of α-syn phosphorylation by members of the PLK family can be supported by the following observations: i) PLK2 and PLK3 partially co-localized with α-syn phosphorylated at Ser129 in primary hippocampal neurons, mammalian cells and in cortical brain areas of mice overexpressing α-syn; ii) siRNA-mediated silencing of PLK2 or PLK3 resulted in marked reduction of nuclear and cytoplasmic Ser129 α-syn phosphorylation levels in mammalian cells and primary neurons; iii) PLK2 was expressed in normal brain and increased in AD and LBD brains, accordingly with previous reports demonstrating raised levels of PLK in the cytosol of vulnerable hippocampal and cortical neurons and homogenates of AD brains (Mbefo *et al.*, 2008); iv) PLK2 was identified as a major contributor for α-syn phosphorylation *in vivo* and v) in PLK2 knock-out mice the levels of Ser129 α-syn phosphorylation were reduced by ~70% (Pak and Sheng, 2003).

1.3.3. G protein-coupled receptor kinases

G protein-coupled receptor (GPCR) signaling is regulated by GRKs. These kinases specifically recognize and phosphorylate agonist occupied GPCRs. Phosphorylation of the receptor and subsequent binding of another protein, arrestin, uncouples G protein from the activated receptor. These events can also promote receptor endocytosis. Internalized receptors are then dephosphorylated and recycled back to the cell membrane or targeted to lysosomes for degradation. Seven mammalian GRKs have been identified and can be divided into three

subfamilies based on their overall structural homology and organization: GRK1 (rhodopsin kinase) and GRK7; GRK2 (bARK1) and GRK3 (bARK2); GRK4, GRK5, and GRK6. GRKs share common features including a centrally localized catalytic domain of approximately 270 amino acids, an N-terminal domain of around 190 amino acids that has been involved in receptor interaction and GRK regulation, and a C-terminal domain with a variable length of 105–233 amino acids that is implicated in phospholipidic association (Pronin *et al.*, 2000).

Cytoskeleton plays a prominent role in assembly of signaling networks and is also involved in endocytosis and vesicular trafficking. Phosphorylation of tubulin mediated by GRK during a signaling event activates rearrangement of microtubules, thus contributing to receptor internalization, recycling or degradation (Carman *et al*., 1998; Haga *et al*., 1998; Pitcher *et al*., 1998). It is also possible that GRKs may be involved in the regulation of signaling and cytoskeleton dynamics through phosphorylation of additional protein substrates. Thus, previous reports have demonstrated that co-expression of α-syn with GRK2 or GRK5 in COS-1 cells were able to phosphorylate α-syn at Ser129 (Pronin *et al.*, 2000).

Some findings have shown that GRK5 is accumulated in LBs in the brains of patients with non-familial forms of PD and can co-localize with α-syn in the pathological structures of the SN (Harris *et al.*, 2000). In the same study, using a cell line stably expressing α-syn, the authors demonstrated that phosphorylation of α-syn at Ser129 depended on the expression levels of GRK5 and its kinase activity. Phosphorylated α-syn co-localized with GRK5 and α-syn, next to the plasma membrane. It was also concluded that GRK5 promoted the formation of α-syn soluble oligomers and aggregates through its phosphorylation.

In a *Drosophila* model of PD, Ser129 phosphorylation of α-syn appeared to be critical for dopaminergic neurons toxicity. Furthermore, overexpression of *Drosophila* GRK2 increased α-syn phosphorylation at Ser129, enhancing α-syn neurotoxicity (Pronin *et al.*, 2000). Other results suggest no genetic association between GRK2 and sporadic PD, since no detection of GRK2 was observed in LBs from sporadic PD brains. Alternatively, it was found that GRK5 co-localized with α-syn in double-transfected cells and in LBs of sporadic PD and that the susceptibility to sporadic PD was associated with haplotype of SNPs in the *GRK5* gene. Some other findings pointed out that co-expression of human α-syn and WT GRK5 in dopaminergic neurons from *C. elegans*, resulted in phosphorylation of α-syn and produced a neurotoxic effect in these cells. These conclusions strongly support the hypothesis that phosphorylated α-syn by GRK5 may have a noxious effect on dopaminergic neurons (Harris *et al.*, 2000). In contrast, other lines of evidence reported that endogenous GRK3 or GRK6 can phosphorylate α-syn at Ser129 in HEK293 cells, while endogenous GRK2 or GRK5 did not seem to have an essential role in the phosphorylation of α-syn in the same cell line (Sakamoto *et al.*, 2009).

1.3.4. Leucine-rich repeat kinases

While the pathogenesis of mutant α-syn is, at least in part, understood, the mechanism by which mutant LRRK2 can induce PD is not well clarified. LRRK2 is a large, multidomain GTPase/kinase protein that has the capacity to autophosphorylate *in vitro* (Chandra *et al.*, 2004; West *et al.*, 2005; Gillardon, 2009a; Gillardon, 2009b). The notion that altered signaling can be implicated in the disease came from the fact that the pathological mutations are within the two enzymatic domains of LRRK2 (Gloeckner *et al.*, 2006). One of these mutations, G2019S, clearly increases kinase activity and is involved in PD pathology. At least, in neuronal cell models, kinase activity seems to be required for the toxicity of mutant proteins, (Greggio *et al.*, 2006; Greggio *et al.*, 2008) further supporting the notion that alteration of LRRK2 signaling may have pathological implications.

Several studies have focused on the functional link between α-syn and LRRK2. As described before in this section, α-syn present in LBs is highly phosphorylated at Ser129 and phosphorylated proteins appear to be more prone to aggregation *in vitro* (Fujiwara *et al*., 2002), suggesting a role in the neurodegenerative process. LRRK2 was hypothesized to be the kinase that mediates phosphorylation of α-syn. However, only one study indicated that recombinant α-syn was directly phosphorylated by overexpressed LRRK2 in HEK293 cell lysates (Qing *et al*., 2009a), while no other evidences exist for LRRK2-induced α-syn phosphorylation in cell or animal systems. It would be of interest to investigate whether pathological brain tissues from LRRK2 PD cases would lead to increased levels of phosphorylated α-syn. Some results reported that LRRK2 induced α-syn expression via the extracellular signal-regulated kinase pathway, although with a modest effect (Carballo-Carbajal *et al*., 2010). In one report, the authors (Qing *et al*., 2009b) successfully co-immunoprecipitated LRRK2 and α-syn from HEK293 cells exposed to oxidative stress and also from pathological tissue of diffuse LBs cases. These results are quite interesting since they leave the possibility that these two proteins can localize to the same cellular compartment upon stress, participating in a common biological process. Moreover, LRRK2 kinase activity may, directly or indirectly, influence α-syn phosphorylation state. Some other findings suggested that LRRK2 inclusions did not recruit α-syn when both proteins were co-overexpressed in cell systems (Waxman *et al.*, 2009), leading to the idea that LRRK2 and α-syn deposition may constitute two independent processes.

1.4. AVAILABLE THERAPIES FOR PD TREATMENT

Several therapies have been proposed to treat or alleviate PD symptomatology. There are several treatment strategies available for PD patients, including: oral medications (levodopa, MAOB inhibitors, dopamine agonists, anticholinergics, antiglutamatergics), surgical therapies

such as deep brain stimulation, continuous delivery therapies (transdermal patch, intraduodenal levodopa), among others (Obeso *et al*., 2010 for review). The use of carotid body tissue to treat PD has also been suggested because it is composed of glomus cells, which produce and secrete high levels of dopamine and, in addition, are resistant to hypoxia (Lopez-Barneo *et al*., 2001). Currently, clinical trials are being conducted with putative neuroprotective drugs, where glial cell line-derived neurotrophic factor (GDNF) can be included (Schapira *et al*., 2009), however none of these drugs has shown neuroprotective effects in these clinical trials (LeWitt and Taylor, 2008).

1.4.1. GDNF

GDNF and related molecules, such as Neurturin (NRTN), Artemin (ARTN) and Persephin (PSPN) belong to a structurally related family of neurotrophic factors (GDNF family ligands, GFLs) (Airaksinen and Saarma, 2002). GDNF is known to signal (Figure 1.7) through a multi-component receptor system consisting of the glycosylphosphatidyl–inositol (GPI)-linked receptor GFRα1 (GDNF family receptor α1), and the co-receptor Ret (Takahashi, 2001; Paratcha *et al*., 2003; Sariola and Saarma, 2003). In embryonic and adult tissues from rodent origin, Ret is highly expressed in peripheral enteric, sympathetic and sensory neurons as well as central motor, dopamine and noradrenalin neurons, hence suggesting an important role of Ret in differentiation and survival of these cells (Takahashi, 2001).

Since the discovery of GDNF by Lin and collaborators (1993) numerous cell culture experiments and rodent studies have demonstrated that this neurotrophic factor is active on different cell types in multiple tissues. GDNF is a neurotrophic factor for survival and axonal growth of mesencephalic dopaminergic neurons and has been shown to ameliorate motor symptoms and reduce brain damage in several animal models of PD (Gash *et al*., 1996; Björklund *et al*., 1997; Björklund *et al*., 2000; Kordower *et al*., 2000; Zurn *et al*., 2001). Grondin and co-workers (2002) presented evidence for structural and functional benefits of infusions of GDNF in MPTP-treated parkinsonian monkeys. This was the first demonstration that GDNF infused directly into the brain parenchyma of non-human primates is effective in restoring dopaminergic function. In the same study animal behavior recovery was accompanied by only a small increase in striatal dopamine levels, suggesting that GDNF may induce additional functional plasticity in the basal ganglia beyond mere increases in dopamine levels. Moreover, the authors suggested that GDNF may affect TH expression levels in neurons that were injured, by the MPTP treatment (Grondin *et al*., 2002). More recently, GDNF has demonstrated potent neuroprotective and restorative effects on dopaminergic nigral neurons, and thus may constitute a potential therapeutic strategy for PD (Deierborg *et al*., 2008; Lindvall

and Wahlberg, 2008; Marks *et al*., 2008). However, other reports have demonstrated the GDNF ineffectiveness on the amelioration of PD symptoms. Indeed, monthly injection of GDNF into the ventricles has been shown to be ineffective to ameliorate the symptoms of PD patients. Furthermore, several side effects were reported (Nutt *et al*., 2003), and there was no evidence of striatal dopaminergic reinnervation in a *post-mortem* studies (Kordower *et al*., 1999). Moreover, it has been demonstrated that excessive striatal GDNF expression resulted in downregulation of TH and abnormal axonal sprouting in downstream brain areas (Georgievska *et al*., 2002; Rosenblad *et al*., 2003).

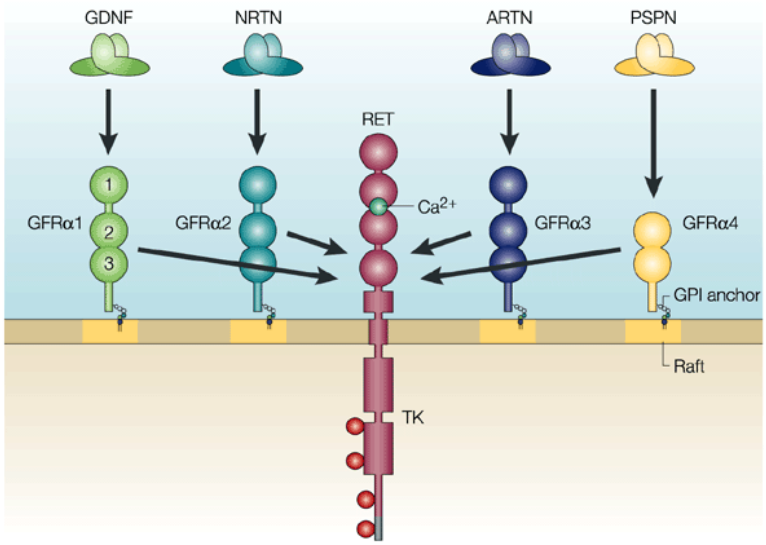


Figure 1.7 - Homodimeric GFLs activate Ret tyrosine kinase (TK) receptor by first binding their cognate GFRα receptors. Arrows indicate the preferred ligand–receptor interactions that are known to occur physiologically *in vivo*. Binding of Ca^{2+} ions to one of the four extracellular cadherin-like domains of Ret is required for its activation by GFLs. Four tyrosine residues in the Ret intracellular domain (Tyr905, Tyr1015, Tyr1062 and Tyr1096; red balls) serve as docking sites for different adaptors. One of them (Tyr1096) is in the carboxy-terminal end of the long isoform of Ret (grey). Membrane rafts are shown in yellow. ARTN, artemin; NRTN, neurturin; PSPN, persephin. Based on Airaksinen and Saarma, 2002.

1.5. OBJECTIVE

As described throughout the literature review, α-syn aggregation and phosphorylation state play an important role in PD pathogenesis. Some reports demonstrated that α-syn deposited in LBs is highly phosphorylated at Ser129 residue, suggesting a role for phosphorylation in α-syn aggregation, LBs formation, and toxicity, although the later is still controversial (Fujiwara *et al*., 2002; Anderson *et al*., 2006; Chen *et al*., 2009). A group of kinases have been suggested to be responsible for α-syn phosphorylation and subsequent aggregation, including PLKs (PLK1 and PLK2) (Inglis *et al*., 2009; Mbefo *et al*., 2010), CKs (CK-1 and CK-2) (Okochi *et al*., 2000) and LRRK2 (Qing *et al*., 2009a). On the other hand, several studies have confirmed an increase of Fe levels in the *substantia nigra* of PD patients, which may be involved in nigrostriatal neurodegeneration. Relatively recent investigations have found that $FeCl_2$ exposure promoted the accumulation of Ser129 phosphorylated α-syn on 3D5 neuronal cell line overexpressing human WT α-syn (Takahashi *et al*., 2007). To reinforce this key aspect, our group has recently shown that α-syn phosphorylation at Ser129 is induced by $FeSO_4$-induced oxidative stress in SH-SY5Y cells (Perfeito *et al*., 2010). Moreover, currently there is no effective treatment to PD. However, since the discovery of GDNF in 1993 (Lin *et al*., 1993), it has been proposed as a particularly potent neurotrophic factor for survival and axonal growth of midbrain dopaminergic neurons. Some evidences suggested that GDNF may offer potential protective strategies against the disease, although its efficacy was not always evident. According to these evidences, we hypothesized that in the presence of an oxidative stress agent (Fe^{2+}) the phosphorylation state of α-syn could be affected, along with modified cell toxicity. On one hand, GDNF triggers a series of phosphorylation cascades and thus could promote α-syn phosphorylation, modifying its cytoprotective effect. On the other hand GDNF could prevent Fe^{2+}-induced changes in α-syn phosphorylation. Another hypothetical assumption was that in the presence of α-syn phosphorylating kinase inhibitors, α-syn phosphorylation state as well as cell toxicity could be decreased.

Based on these hypotheses, our main objective was to evaluate the role of selective kinases and GDNF in regulating the phosphorylation of α-syn in a specific serine residue (Ser129) in the absence and presence of Fe^{2+} using human neuroblastoma (SH-SY5Y) cells conditionally overexpressing wild-type α-syn (hereinafter referred to as α-syn), as a cell model of PD.

CHAPTER 2 – MATERIALS AND METHODS

2.1. MATERIALS

Optimem, Roswell Park Memorial Institute's 1640 medium (RPMI 1640), fetal bovine serum (FBS) and Geneticin were purchased from GIBCO (Paisley, Scotland, UK). Protease inhibitor cocktail (chymostatin, pepstatin A, leupeptin and antipain), penicillin/streptomycin (P/S), albumin from bovine serum (BSA), nicotinamide adenine dinucleotide hydrate (NADH), pyruvate, Phenylmethanesulfonyl fluoride (PMSF), Bicine, 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS), TWEEN, BIS-TRIS, Dithiothreitol (DTT), Tetramethylethylenediamine (TEMED), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), doxycycline (Dox), hygromycin B, and alamar blue (resazurin) were from Sigma-Aldrich (St. Louis, MO, USA). Fugene 6 was acquired from Roche Diagnostics GmbH (Mannheim, Germany). Secondary antibodies for western blotting anti-rabbit IgG, anti-mouse IgG+IgM and Enhanced ChemiFluorescence reagent (ECF) were purchased from GE Healthcare (Little Chalfort, UK). Fluorogenic caspase-3 substrate VII Ac-Asp-Glu-Val-Asp-AFC (Ac-DEVD-AFC), CK-1 inhibitor 4-(4-(2,3-Dihydrobenzo[1,4]dioxin-6-yl)-5-pyridin-2-yl-1H-imidazol-2-yl)benzamide (D4476) and LRRK2 inhibitor 2-((2-Methoxy-4-(4-(4-methylpiperazin-1-yl)piperidine-1-carbonyl) phenyl) amino)-5,11-dimethyl-5H-benzo [e] pyrimido [5,4-b] [1,4] diazepin-6 (11H) - one (LRRK2-IN-1) was from Calbiochem (Darmstadt, Germany). PLK1 inhibitor sodium (E)-2 - { 2-methoxy -5- [(2',4',6'-trimethoxystyrylsulfonyl) methyl] phenylamino } acetate (ON-01910) and PLK2 inhibitor (R)-4- (8-cyclopentyl- 7- ethyl- 5- methyl - 6 - oxo - 5, 6, 7, 8 - tetrahydropteridin – 2 – ylamino) -3-methoxy-N-(1-methylpiperidin-4-yl)benzamide (BI2536) and CK-2 inhibitor 5-(3-chlorophenylamino)benzo[c][2,6]naphthyridine-8-carboxylic acid (CX-4945) were purchased from Selleck Chemicals (Houston, Texas, USA). Glial cell-derived neurotrophic factor (GDNF) was from Peprotech (Hamburg, Germany). Polyvinylidene Difluoride (PVDF) membrane was acquired from Millipore (Billerica, MA, USA). Methanol was from Merck (Darmstadt, Germany). Bio-Rad Protein Assay was from Bio-Rad (Hemel Hempstead, UK). All other reagents were of analytical grade.

2.2. METHODS

2.2.1. Cell culture and maintenance of stable neuroblastoma SH-SY5Y cell line overexpressing WT alpha-syn

Vekrellis and colleagues previously described (Vekrellis *et al.*, 2009) the generation of a stable cell line conditionally overexpressing Wt α-syn. In resume, naïve SH-SY5Y cells were

transfected with the Tet-Off vector (Clontech, Mountain View, CA, USA) using the Lipofectamine 2000 reagent, and the selection process was carried out with 500 µg/ml geneticin (also known as G418). Inducibility of G418 resistant clones was determined by transient transfection of a pTRE-LUC vector, with or without the presence of Dox (2 µg/ml). One clone (2-22) was later used for generation of stable pTRE-α-syn expression. Wt α-syn was subcloned into the *Hind*III and *Xba*I 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.

The instructions for maintenance and culture of stable SH-SY5Y cell line conditionally overexpressing Wt α-syn (SH-SY5Y Wt α-syn) were 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. α-Syn overexpression was switched off with Dox (2 µg/ml). Stock cultures were always kept in the presence of Dox. For all the experiments cells were maintained for five to six days in the absence of Dox, a necessary condition for the maximal expression of WT α-syn in this particular cell line. The cells showed a highly tenuous basal expression in the presence of Dox and a strong expression of recombinant α-syn in its absence at day five/six.

2.2.2. Incubation and stimulation of SH-SY5Y cell line

In this section, incubation with compounds and stimulation procedures will be discriminated for all the subsequent assays. Kinase inhibitor solutions were prepared in 100% dimethyl sulfoxide (DMSO), and in all cases the % of DMSO in the culture medium was equal or inferior to 0.5%. CK-1 inhibitor was directly diluted into the aqueous cell culture medium, however, in order to promote its solubility and a better cell delivery, this compound was prepared accordingly to Rena and colleagues (2004).

Cells were cultured as described above for all the experiments. As previously mentioned, the maximal expression of WT α-syn occurs at day five-six. As such, and for the cases with 24h of compound incubation, cells were plated at day four, incubated at day five and cell extracts were performed at day six. The respective assay was performed at day six or in the following days. For the cases with incubation for 2:30h or less, cells were plated at day four, the incubation and cell extracts were done at day five. The respective assay was performed at day five or in the following days.

In Alamar Blue experience, $8.0x10^4$ cells/well were plated onto 48-multiwell plate. Incubations were done for 24h for all kinase inhibitors (ON-01910, BI 2536; D4476; CX-4945

and LRRK2-IN-1). A range of concentrations were tested (Table 2.1) for all inhibitors, in order to define which concentration could be used in all subsequent assays without cytotoxic effects.

For the lactate dehydrogenase activity assay, $1.0x10^6$ cells/well were plated onto 6-multiwell plate. Cell incubations were firstly done with $FeSO_4$ (Iron (II) sulphate) 500μM (prepared in sterile water) for 24h and 2h in order to address its potential cytotoxic effect. In further experiences cells were incubated with selected inhibitors along with $FeSO_4$. Inhibitors were incubated 30 min prior incubation with $FeSO_4$ for 2h. Likewise, the procedure was repeated for the incubation with GDNF 20ng/ml (prepared in sterile water).

In caspase-3 activity assay, $8.0x10^4$ cells/well were plated onto 48-multiwell plate. Cells were incubated for 24h with selected inhibitors or GDNF (20ng/ml), separately.

For western blotting analysis, $5.0x10^6$ cells/well were plated onto 100 mm Petri dishes. For the time-course incubation with GDNF (20ng/ml), cells were incubated for different periods ranging from 5 min to 24h. In other immunoblotting experiences cells were incubated in different conditions, with selected inhibitors or GDNF 30 min prior incubation with $FeSO_4$ for 2h, and NaF 20mM (positive control for phosphorylation), 30 min before the end of incubation.

Table 2.1 - Range of kinase inhibitors concentrations tested on SH-SY5Y WT alpha-syn neuroblastoma cells.

Kinase inhibitors	Range of final concentrations in culture medium (μM)
PLK1 (ON-01910)	0.05; 0.1; 0.5; 1; 2; 5
PLK2 (BI2536)	0.01; 0.1; 0.5; 1; 2; 5
CK-1 (D4476)	0.01; 1; 10; 100
CK-2 (CX-4945)	0.01; 0.1; 1; 10; 100; 250
LRRK2 (LRRK2-IN-1)	0.05; 0.1; 1; 2; 5; 10

2.2.3. Subcellular fractionation - Total extracts

Adherent cells were washed twice in ice-cold phosphate-buffered saline (PBS, in mM: 137 NaCl, 2.7 KCl, 1.4 KH_2PO_4, 4.3 $Na_2HPO_4.2H_2O$, pH 7.4) and then scraped in lysis buffer containing (in mM): 100 NaCl; 20 Tris (pH 7.0); 2 EDTA; 2 EGTA and supplemented with 1% Triton X-100, 0.1% SDS, 1 mM PMSF, 1 mM DTT, 50 mM NaF, 1.5 mM sodium orthovanadate, 0.1 μM okadaic acid and 1 μg/ml protease cocktail inhibitor (containing chymostatin, pepstatin A, leupeptin and antipain). Cellular extracts were frozen/unfrozen 3 times in liquid nitrogen, centrifuged at 14000 rpm, 10 min, 4ºC (Eppendorf Centrifuge 5417R) to remove cellular debris. The pellet was discarded, the supernatant (total extract) was collected and protein content quantified by the Bio-Rad method, according to the manufacturer's instructions. Extracts were stored at -20ºC.

2.2.4. Bio-Rad protein assay

The Bio-Rad protein assay is a simple procedure with an appropriate degree of accuracy for determining the concentration of solubilized protein. This method is a dye-binding assay, in which a differential color change of a dye occurs in response to different concentrations of protein (Bradford, 1976).

A standard curve was prepared by adding 0, 1, 2, 3, 4, 5 µl of BSA 0.1% into each well (96-multiwell plate), 1 µl of lysis buffer and 120 µl of Bio-Rad reagent (diluted 3x). Final volume was adjusted to 200 µl/well with distilled water, to generate 0, 1, 2, 3, 4, 5 µg/µl of protein. Protein content was determined by adding 1 µl sample, 79 µl distilled water and 120 µl Bio-Rad reagent into each well. Subsequently, multiwell plate was incubated during 15 min at room temperature and protected from light. Optical density was measured at 595 nm in a SpectraMax Plus 384 spectrophotometer. Protein concentrations (µg/µl) were calculated accordingly: x=(y-b)/m, where y is the optical density, m is the slope of straight pattern and b is the intersection point with yy axis.

2.2.5. 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 exhibits a dark blue color and has little intrinsic fluorescence. When taken into cells, resazurin is reduced to resorufin and turns red. Viable cells continuously convert resazurin to resorufin, thereby generating a quantitative measure of viability and cytotoxicity, based on its optical density or fluorescence (Fields and Lancaster, 1993; Al-Nasiry *et al.*, 2007).

Resazurin (1 mg/ml) was reconstituted in PBS and subsequently diluted (1:100) in RPMI 1640 medium containing the selection antibiotics G418 (250 µg/ml) and Hygromycin B (50 µg/ml), and finally added to SH-SY5Y WT α-syn cells in the presence or in the absence of Dox and incubated for 2 h at 37ºC. Absorbance was detected at 570 nm and 600 nm in a SpectraMax Plus 384 microplate scanning spectrophotometer (Molecular Devices, USA). After the readings, cell protein content was determined by Bio-Rad method, as described above. Resazurin reduction was calculated upon subtraction of the absorbance at 570 nm by 600 nm. Results were expressed as O.D./mg protein or in % of control in accordance with the formula:

$$\frac{\left(\mathrm{Abs}_{570}-\mathrm{Abs}_{600}\right)_{\text{treated cells}}}{\overline{\times}\left(\mathrm{Abs}_{570}-\mathrm{Abs}_{600}\right)_{\text{control cells}}}\times 100$$

2.2.6. Lactate dehydrogenase (LDH) activity assay

Membrane integrity was assessed by determining the release of the cytosolic enzyme LDH from the injured cells into the extracellular medium. This method represents a common assay to determine membrane leakage and cellular damage, typical of apoptotic or necrotic cell death.

After the incubation periods, the culture medium was collected for further assessment of extracellular LDH activity, whereas the adherent cells were scraped (with 100 µl of 10 mM HEPES, pH 7.4 with 0.5% of Triton X-100) in order to measure the intracellular LDH activity. All the samples were centrifuged at 14000 rpm, 10 min, 4ºC. The respective pellets were discarded and the supernatants were stored at -80ºC until used for the assessment of enzyme activity. For LDH intra and extracellular activity, and considering a final reaction volume of 200 µl, 6.45 µl of each sample was mixed with 32.25 µl of pyruvate (9.76 mM). The reaction was started immediately after addition of 161.3 µl of NADH (0.24 mM). For the controls (blanks), NADH was replaced for Tris/NaCl pH 7.2 (81.3 mM of Tris and 203.3 mM of NaCl) which was also used to prepare pyruvate and NADH solutions.

Enzyme activity was determined by following the rate of NADH consumption at 340 nm, performed at 30ºC, using a microplate reader SpectraMax Plus 384. The percentage of LDH released into the extracellular medium was calculated accordingly to the formula: $\%\ \text{released LDH} = \frac{\text{Extracellular LDH}}{(\text{Extracellular LDH} + \text{Intracellular LDH})} \times 100$.

2.2.7. Caspase-3 activity assay

The assessment of caspase-3 activity was determined using a specific 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 1 µg/ml protease cocktail inhibitor, 2 mM DTT and 0.1 mM PMSF. Then, a reaction buffer composed by 25 mM HEPES, 10% sucrose and 0.1% CHAPS, supplemented with 15 mM DTT and 15 µM Ac-DEVD-AFC was added to cell samples. The fluorescence was taken during 1:30 h at 37ºC, with 400 nm excitation and 505 nm emission, using a Microplate Spectrofluorometer Gemini EM (Molecular Devices, USA). After the readings, cell protein content was determined by Bio-Rad method, as previously described. The values were obtained as relative fluorescence units (RFU) per minute per mg protein for each condition, and subsequently expressed as % of control.

2.2.8. Western blotting

Total extracts, obtained as described above, were denatured with denaturing buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 5% glycerol, 600 mM DTT, 0.01% bromophenol blue) at 95 ºC, for 5 min. Equivalent amounts of protein were separated on a 12-15% SDS-PAGE gel electrophoresis and electroblotted onto polyvinylidene difluoride (PVDF) membranes. Prior to blocking step, membranes were incubated in a paraformaldehyde 0.4% (PFA) solution for 30

min, in order to enhance protein fixation. The membranes were then blocked during 1 h at room temperature in Tris-buffered saline (TBS) solution containing 0.1% Tween (TBS-Tween) and 5% BSA, followed by an overnight incubation with primary antibodies (Table 3), at 4ºC, with gentle rotation. Membranes were further washed 3 times, for 15 min with TBS-Tween, and incubated with secondary antibodies (1:20000) for 1 h, at room temperature with gentle rotation. Proteins were visualized by using an enhanced chemifluorescent reagent (ECF) and the bands were detected by using a BioRad Versa Doc 3000 Imaging System.

Table 2.2 - List of the primary and secondary antibodies used for western blotting analysis.

Primary Antibody	Molecular Weight	Reference	Dilution	Secondary antibody
Anti-Akt	60 kDa	#9272 (Cell Signaling)	1:1000	Rabbit
Anti-P-Akt (Ser473)	60 kDa	#9271 (Cell Signaling)	1:1000	Mouse
Anti-Ret	170-175 kDa	#3220 (Cell Signaling)	1:1000	Rabbit
Anti-P-Ret (Tyr905)	170-175 kDa	#3221 (Cell Signaling)	1:1000	Rabbit
Anti-α-syn	18 kDa	#2642 (Cell Signaling)	1:1000	Rabbit
Anti-P-α-syn (Ser129)	18 kDa	#014 – 20281 (Wako)	1:500	Mouse
Anti-α-Tubulin	50 kDa	T6199 (Sigma)	1:1000	Mouse

2.2.9. Statistical analysis

Results were expressed as mean±SEM of the indicated number of independent experiments, run in duplicates or triplicates. Comparisons between multiple groups were performed using one-way or two-way analysis of variance (ANOVA), followed by Bonferroni post-hoc test, for comparison between experimental groups. Comparison between two groups was analysed using the Student´s t test or Bonferroni post-hoc test (GraphPad Prism Version 5.0). $P<0.05$ was considered significant.

CHAPTER 3 – RESULTS

In this part of the thesis we first assessed the cytotoxic effect of PLK1, PLK2, CK-1, CK-2 and LRRK2 kinase inhibitors and the oxidative stress agent (Fe^{2+}, defined in this work as Fe) and GDNF. Furthermore, the selected kinase inhibitors as well as Fe and GDNF were evaluated in order to clarify its influence on α-syn Ser129 phosphorylation.

3.1. EVALUATION OF CYTOTOXICITY OF KINASE INHIBITORS, Fe AND GDNF

3.1.1. Effect of kinase inhibitors on cell viability assessed by the Alamar Blue assay

The cytotoxic effects of the different kinase inhibitors used in this study were assessed using a cell viability assay, Alamar Blue. This assay uses the natural reducing power of living cells to convert resazurin (the active ingredient of Alamar Blue) to resorufin (Fields and Lancaster, 1993), giving a quantitative measure of cytoxicity. First of all, however, it was necessary to prove that the cell line was able to conditionally overexpress α-syn. Figure 3.1A shows clearly a highly tenuous basal expression in the presence of Dox and a strong expression of recombinant α-syn in its absence. In this way, it was proven the conditional overexpression of α-syn as well as the role of Dox as a switch for α-syn overexpression. Theoretically, –Dox condition (which leads to α-syn overexpression) mimics the duplication/triplication of α-syn gene (SNCA) observed in some familial cases of PD. In that sense, we examined if –Dox *per se* leads to an increase in cell toxicity. As depicted in Figure 3.1B, there is a significant decrease in cell viability, comparing –Dox with +Dox-treated cells. Interestingly, this observation is in accordance with the theory that postulates a toxic effect for α-syn overexpression in individuals with duplications/triplications of the locus containing SNCA (Lesage and Brice, 2009; Hatano *et al*., 2009).

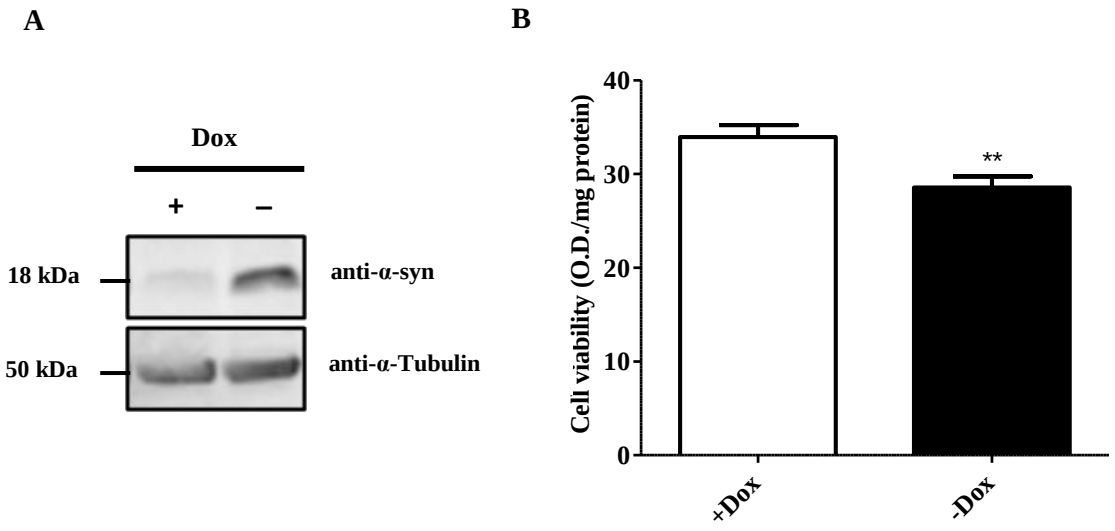


Figure 3.1 - Assessment of SH-SY5Y neuroblastoma cell viability in a basal and pathological conditions, respectively, in the presence (+Dox) or absence (–Dox) of Dox. A) The expression levels of α-syn (18 kDa), in the presence or absence of Dox, were analyzed by Western Blotting in order to assess the conditionally WT α-syn expression system in SH-SY5Y human neuroblastoma cells. Total protein extracts were evaluated by Western Blotting using antibodies against α-syn and α-tubulin. **B)** Evaluation of cell reducing capacity through Alamar Blue reducing essay, in the presence or absence of Dox. Data, expressed in absolute values (33.944±1.268 O.D./mg protein for +Dox condition and 28.568±1.173 O.D./mg protein for –Dox condition), are the mean±SEM of eighteen distinct experiments, run in triplicates. Statistical analysis was performed by Student's t-test: $^{**}P < 0.01$ when compared to +Dox-treated cells.

In order to evaluate the potential cytotoxic effect of the different kinase inhibitors, a screening of concentrations for each inhibitor was performed, during 24 hours of incubation. Our defined range of concentrations included some concentrations indicated by other researchers and other concentrations that stand below and above these values. The inhibitors were dissolved in DMSO 100%, and for all the cases the percentage of DMSO present in the cell culture medium never exceeded 0.5%. The selected inhibitor concentration that will be applied in the subsequent experiments, should not give rise to a percentage of unviable cells superior to 20%.

In the case of PLK1 inhibitor, ON-01910, we established a range of concentrations from 0.05 to 5 µM (Gumireddy *et al*., 2005; Chapman *et al*., 2009; Oussenko *et al*., 2009; Tan *et al*., 2009; Reddy *et al*., 2011; Chapman *et al*., 2012). By the analysis of the graph of PLK1 inhibitor, the compound ON-01910 (Fig. 3.2A) revealed maintained cell viability in the presence of 0.167% DMSO used in cell culture medium. Each one of the concentrations tested of ON-01910 (0.05-5 µM) caused a decrease in cell viability higher than 20%; in the case of 0.05 µM ON-01910 the percentage of cell viability was ~55% and ~66%, respectively, for

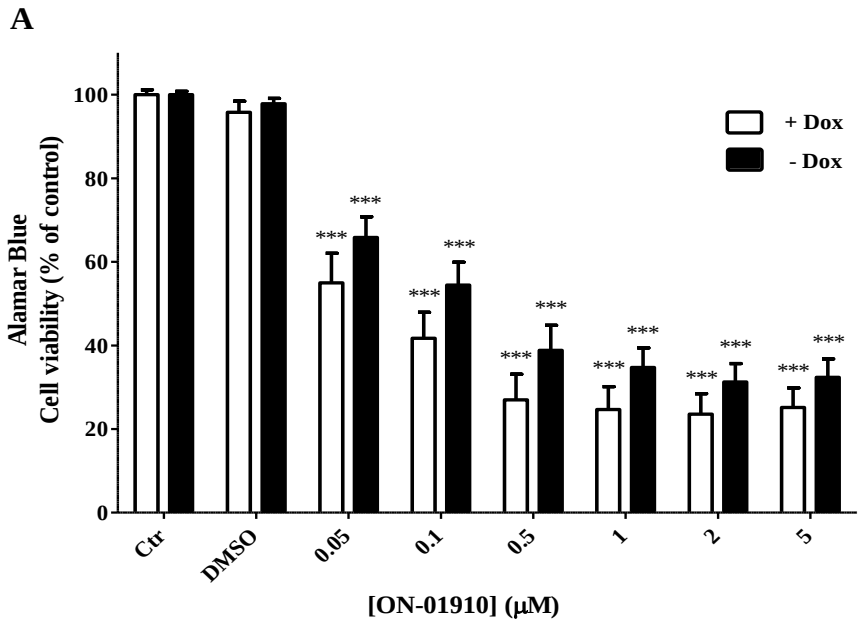
+Dox and –Dox. According to these results we decided to do not use this kinase inhibitor for the subsequent experiments. For PLK2 inhibitor, BI2536, a range of concentrations was defined (0.01-5 μM) in order to test its toxic effect on cells (Lénárt *et al*., 2007; Steegmaier *et al*., 2007; Nappi *et al*., 2009; Grinshtein *et al*., 2011; Watt *et al*., 2011; Waxman and Giasson , 2011). For BI2536 (Fig. 3.2B), the results were similar to those aforementioned for Fig. 3.2A, when testing the ranged concentrations. In the presence of 0.01 μM BI2536, we observed ~68% and ~80% cell viability, respectively, for +Dox and –Dox. For the remaining concentrations, the percentage values were below to those already referred, therefore this inhibitor was also discarded in subsequent experiments.

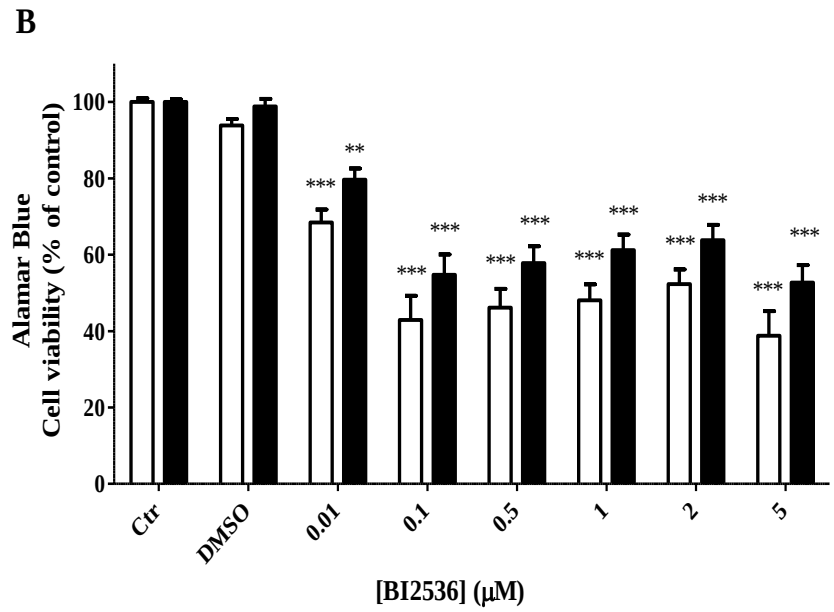
For the specific case of CK-1 inhibitor, D4476, we defined a range of concentrations between 0.01 μM and 100 μM (Rena *et al*., 2004; Bain *et al*., 2007; Tillement *et al*., 2008; Huart *et al*., 2009; Waxman and Giasson, 2011). Regarding CK-1 inhibitor (Fig. 3.2 C), and the correspondent DMSO control, we observed slightly lower cell viability when compared to the two former inhibitors. DMSO (at 0.333%) slightly reduced cell viability, namely ~90% for +Dox and ~95% for –Dox, which were within the acceptable range of cell viability. The concentration of 10 μM led to a cell viability percentage very close to the threshold that defines a toxic concentration. CK-1 inhibitor with a concentration of 100 μM conduced to a situation of almost none viable cells. According to the data in the graph, one of the first three concentrations could have been selected for subsequent experiments, however, we chose the lower one, because it had the highest viability values, namely ~91% for +Dox and ~97% for –Dox. Relatively to CK-2 inhibitor, CX-4945, we have established a concentration range of 0.01 to 250 μM (Drygin *et al*., 2009 ; Siddiqui-Jain *et al*., 2010; Pierre *et al*., 2011a; Pierre *et al*., 2011b; Bliesath *et al*., 2012; Siddiqui-Jain *et al*., 2012). Regarding CK-2 inhibitor (Figure 3.2D), DMSO (0.5%) in the cell culture medium induced ~90% of cell viability in both +Dox and –Dox condition. CX-4945 (at 0.01 μM or 0.1 μM) was within the concentrations for being chosen, and thus the lower concentration, exhibiting ~91% and ~89% cell viability for +Dox and –Dox, respectively, was selected.

In the particular case of LRRK2 inhibitor, LRRK2-IN-1, we established a range of concentrations from 0.05 to 10 μM (Deng *et al*., 2011; Thévenet *et al*., 2011). Testing the dose-response curve for the LRRK2 inhibitor (Fig.3.2E), DMSO (0.167%) induced ~99% cell viability in both +Dox and –Dox. Analysis of cell viability in the presence of LRRK2-IN-1 concentrations of 0.05-10 μM allowed the selection of 0.1 μM, since it showed ~94% and ~95% of cell viability, respectively, in +Dox and –Dox.

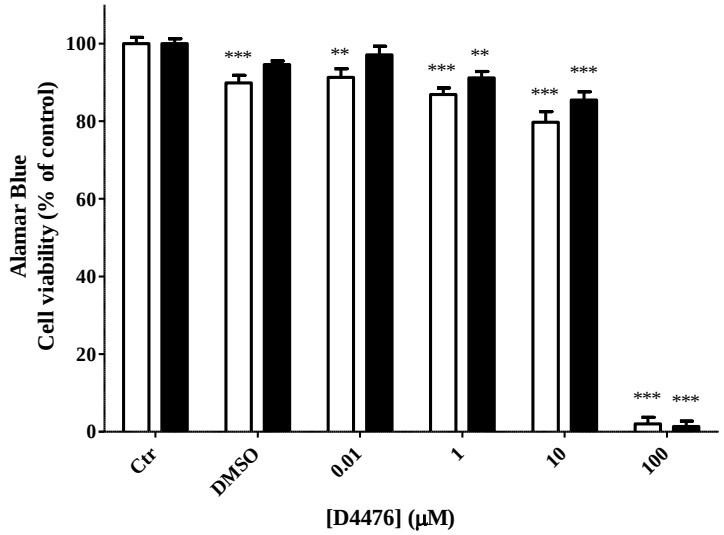
From the graphs of the kinase inhibitors, representing cell viability according to different concentrations (Fig. 3.2 A-E), IC_{50} values (inhibitor concentration that causes a decline in cell

activity by 50%) can be calculated. These IC_{50} values (Table 3.1) were calculated for all the kinase inhibitors taking into consideration the presence or absence of Dox. All kinase inhibitors significantly inhibited cell viability at low micromolar concentrations in SH-SY5Y human neuroblastoma cells, with IC_{50} values ranging from 0.38±0.33 to 21.46±3.70 μM for +Dox and ranging from 0.64±0.49 to 24.36±2.70 μM for –Dox.

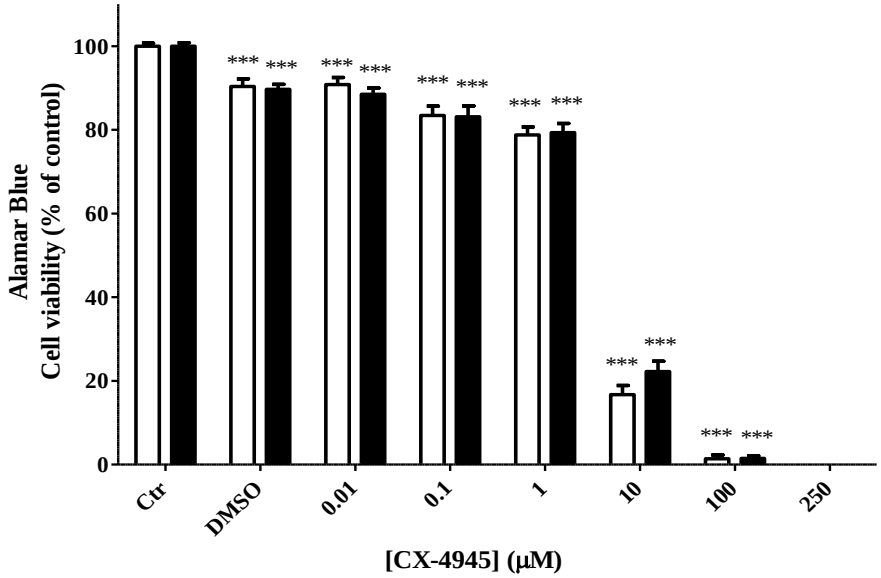




C


Alamar Blue
Cell viability (% of control)
100
80
60
40
20
0
Ctr
DMSO
0.01
1
10
100
[D4476] (μM)

D


Alamar Blue
Cell viability (% of control)
100
80
60
40
20
0
Ctr
DMSO
0.01
0.1
1
10
100
250
[CX-4945] (μM)

E

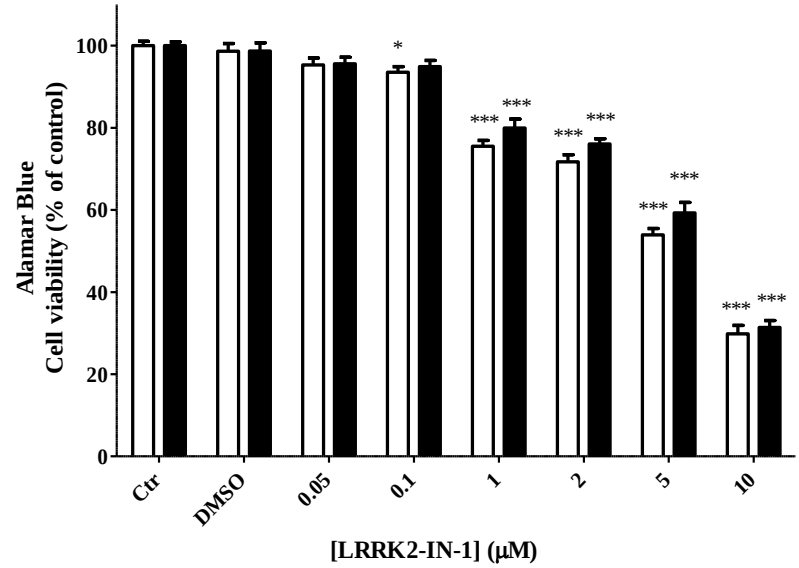

Alamar Blue
Cell viability (% of control)
100
80
60
40
20
0
Ctr
DMSO
0.05
0.1
1
2
5
10
[LRRK2-IN-1] (μM)

Figure 3.2 - Cell reducing capacity of SH-SY5Y neuroblastoma cells, in the presence of different kinase inhibitors, assessed by Alamar blue assay. Cells were incubated with or without Dox, and with a range of different inhibitor concentrations, for 24 h. Evaluation of cell reducing capacity through Alamar blue essay in the presence of: **A)** PLK1 inhibitor (ON-01910); **B)** PLK2 inhibitor (BI2536); **C)** CK-1 inhibitor (D4476); **D)** CK-2 inhibitor (CX-4945); **E)** LRRK2 inhibitor (LRRK2-IN-1). For all the cases the %DMSO≤0.5%. Data, expressed as a percentage of control, are the mean±SEM of four distinct experiments, run in triplicates, for PLK1, PLK2 and CK-2 inhibitors and three distinct experiments, run in triplicates, for CK-1 and LRRK2 inhibitors. Statistical analysis was performed by two-way ANOVA, followed by Bonferroni post test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ when compared to respective control (untreated) cells.

Table 3.1 - IC_{50} values of selective kinase inhibitors in SH-SY5Y human neuroblastoma cell line.

Kinase inhibitors	**IC_{50} values (μM)**	
	+Dox	**–Dox**
PLK1 (ON-01910)	0.38±0.33	0.64±0.49
PLK2 (BI2536)	1.69±1.42	3.83±2.47
CK-1 (D4476)	21.46±3.70	24.36±2.70
CK-2 (CX-4945)	2.72±0.26	3.12±0.40
LRRK2 (LRRK2-IN-1)	4.76±0.38	5.84±0.92

IC_{50} values were obtained by incubating SH-SY5Y cells with serial dilutions of the tested kinase inhibitors in the presence or absence of Dox, for 24 hours and assessing cell viability using the Alamar blue assay. Cells were treated with the kinase inhibitors in a range of concentrations of: ON-01910 (0.05-5 μM), BI2536 (0.01-5 μM), D4476 (0.01-100 μM), CX-4945 (0.01-250 μM) and LRRK2-IN-1 (0.05-10 μM). Values are shown as mean±SEM of four distinct experiments, run in triplicates, for PLK1, PLK2 and CK-2 inhibitors and three distinct experiments, run in triplicates, for CK-1 and LRRK2 inhibitors.

3.1.2. Effect of kinase inhibitors, Fe and GDNF on caspase-3 and LDH releaser

Caspase-3 is a critical executioner of apoptosis; it is an effector caspase responsible for the proteolytic cleavage of many downstream key proteins (Fernandes-Alnemri *et al*., 1994). During apoptosis caspase-3 is activated by upstream caspases through proteolytic processing, giving rise to activated p17 and p12 fragments (Nicholson *et al*., 1995). Caspase-3 activity is only detected in lysates of apoptotic cells, which means that the apoptotic process is clearly installed.

The previously selected inhibitors concentrations and GDNF (20 ng/ml) were thus subjected to analysis of caspase-3-like activity. According to data shown in Table 3.2, incubation for 24 hours with D4476 (0.01 µM), a CK-1 inhibitor, CX-4945 (0.01 µM), a CK-2 inhibitor, or LRRK2-IN-1 (0.1 µM), a LRRK2 inhibitor, or GDNF (20 ng/ml) in SH-SY5Y cells treated in the absence of Dox caused non-significant changes on caspase-3-like activity, when compared to control conditions.

Another way to quantify cell toxicity is through classical evaluation of plasma membrane integrity. Lactate dehydrogenase (LDH) is a stable cytosolic enzyme present in all cells and rapidly released into cell culture medium if any damage has occurred on plasma membrane. The amount of LDH released into the medium is proportional to the amount of unviable cells (e.g. Wolterbeek and van der Meer, 2005).

Ostrerova-Golts and coworkers (2000) have confirmed that $FeCl_2$ exposure led to iron-induced toxicity in untransfected BE-M17 cells, as assessed by MTT and LDH assay. Moreover, they showed that, overexpression of α-syn (WT, A53T and A30P) enhances iron-induced toxicity in BE-M17 transfected neuroblastoma cells. On the other hand, Takashi and colleagues (2007) found that $FeCl_2$ treatment at 1 mM significantly increased ROS production in a time-dependent manner in 3D5 neuronal cell line overexpressing human WT α-syn. Our group has recently shown that exposure to $FeSO_4$ (500 µM) for 2 hours evokes oxidative stress in SH-SY5Y neuroblastoma cell line (Perfeito *et al*., 2010). According to these findings, we decided to maintain the same concentration and incubation period for Fe, and incubate it with the selected kinase inhibitors or GDNF. Data in Table 3.3 show that incubation with Fe for 24 hours decreased (rather than increased) LDH release in +Dox-treated cells (36.15±0.71%), although there was a trend for an increase in LDH release after incubation with Fe for 2 hours (42.05±0.58%), when compared to +Dox control cells (40.19±0.80%). Stimulation with Fe for 2 hours showed a significant increase in LDH release when comparing with Fe exposure for 24 hours. Regarding –Dox condition, we observed a trend of increased (although not significant) in LDH release after 24 hours (43.78±2.74%) and 2 hours (42.77±1.75%) of Fe exposure, as

compared to untreated –Dox control cells (41.28±1.28%). In relation to the effects of the kinase inhibitors, we observed that D4476 decreased (38.41±0.75%), whereas CX-4945 (46.52±1.48%) increases LDH release, when comparing with the other compounds. However, such tendency remains relatively unchanged when these two inhibitors are compared with Fe exposure for 2 hours, in the absence of the inhibitors. Our results, although preliminary, indicate that there is no cell toxicity imposed by selected inhibitor concentrations, compared with the respective control cells, neither by LDH release nor by caspase-3-like activity assays. Notably, no significant changes in LDH release induced by GDNF in cells exposed for 2 hours with Fe have been found either (Table 3.3).

Table 3.2 - Assessment of caspase-3 activity in cell lysates in the presence or absence of Dox.

	Control	**D4476**	**CX-4945**	**LRRK2-IN-1**	**GDNF**
Caspase-3 activity (% of control) (+Dox)	100±2.51	---	---	---	---
Caspase-3 activity (% of control) (-Dox)	100±7.22	120.65±9.34	108.42±7.18	120.54±17.01	122.75±17.87

Cells were incubated with D4476 (0.01 µM), CX-4945 (0.01 µM), LRRK2-IN-1 (0.1 µM) or GDNF (20 ng/ml), for 24 h. Data, expressed as a percentage of control (considering 204229.600±5146.776 RFU/mg protein and 215894.000±37694.240 RFU/mg protein as 100%, respectively, for +Dox and –Dox condition), are the mean±SEM of two distinct experiments, run in triplicates.

Table 3.3 - Percentage of lactate dehydrogenase released into the cell culture medium in the presence or absence of Dox.

	Control	**Fe (24h)**	**Fe (2h)**	**D4476 + Fe (2h)**	**CX-4945 + Fe (2h)**	**LRRK2-IN-1 + Fe (2h)**	**GDNF + Fe (2h)**
% LDH released (+Dox)	40.19±0.80	36.15±0.71 t^{*}	42.05±0.58 $t^{\#\#}$	---	---	---	---
% LDH released (-Dox)	41.28±1.28	43.78±2.74	42.77±1.75	38.41±0.75 $t^{\S\S}$	46.52±1.48	41.62±0.76 $t^{\S}$; $t^{£}$	41.61±0.22 $t^{\S}$; $t^{£}$

Cells were incubated with 500µM $FeSO_4$ (Fe) for 24 h or 2 h. In the case of 2h of incubation with Fe, cells were incubated along with D4476 (0.01 µM), or CX-4945 (0.01 µM), or LRRK2-IN-1 (0.1 µM), or GDNF (20ng/ml), 30 min prior incubation with Fe. Data, expressed in percentage of lactate dehydrogenase released, are the mean±SEM of two distinct experiments, run in triplicates. Statistical analysis was performed by Student's t-test:

t^{*} $P < 0.05$ when compared to respective control (untreated) cells.

$t^{\#\#}$ $P < 0.01$ when compared to condition with Fe incubation during 24h.

$t^{\S}$ $P < 0.05$; $t^{\S\S}$ $P < 0.01$ when compared to condition with CX-4945 incubation along with Fe.

$t^{£}$ $P < 0.05$ when compared to condition with D4476 incubation along with Fe.

3.2. ASSESSMENT OF GDNF EFFECTS ON α-SYN PHOSPHORYLATION

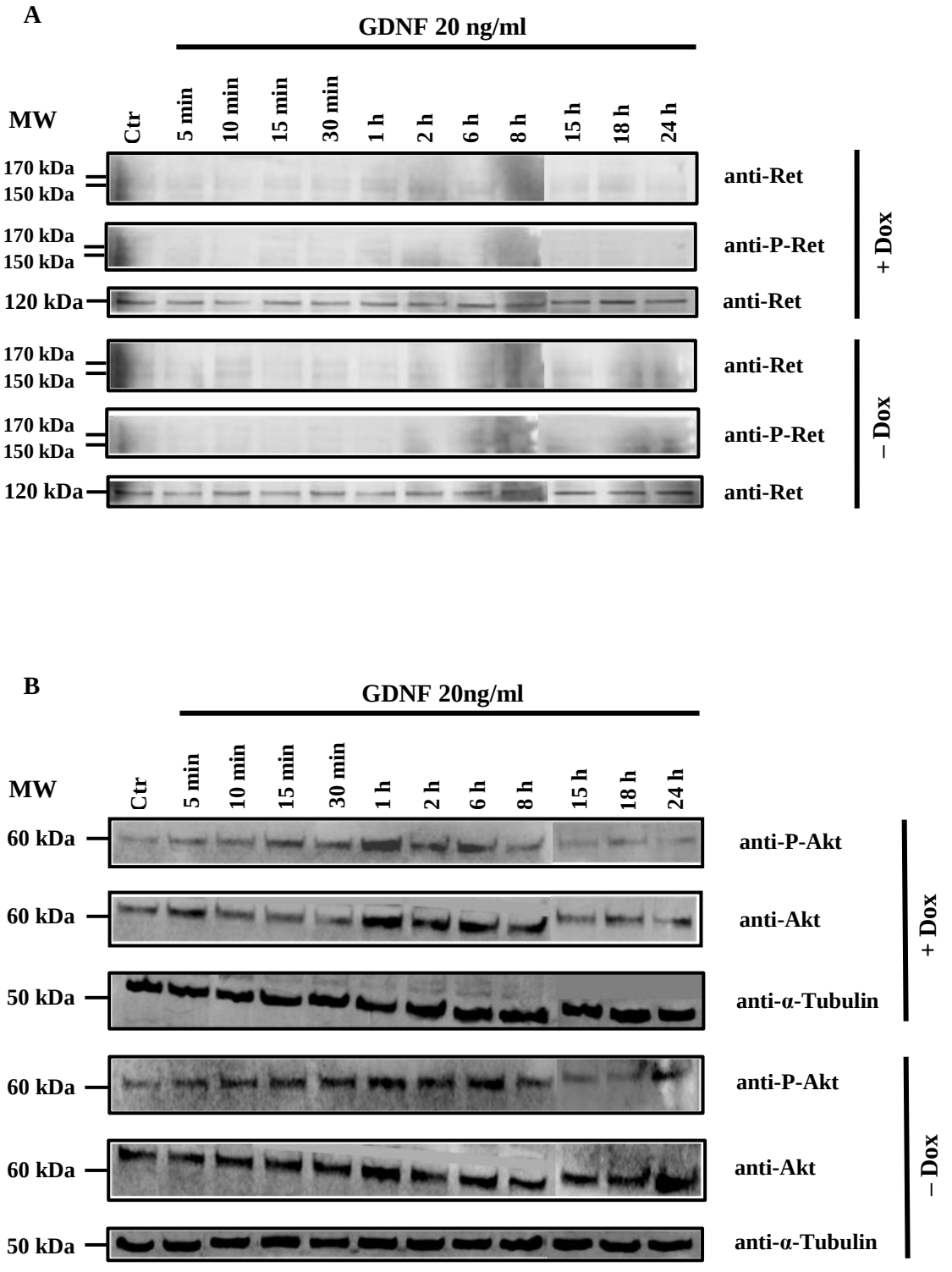
3.2.1. Time course of GDNF effects on Akt pathway activation

GDNF activates the PI3K/Akt pathway through activation of Ret tyrosine kinase receptor in several contexts (Pong *et al*., 1998; Trupp *et al*., 1999; Focke *et al*., 2001; Jin *et al*., 2002). Ret cannot directly bind GDNF but requires the presence of GFRα1 (Treanor et al., 1996); it is through this multicomponent receptor complex that GDNF exerts its effects. SH-SY5Y cell line was previously reported to express Ret mRNA and Ret protein, as well as GFRα1 mRNA and protein (Hirata and Kiuchi, 2003).

In an attempt to understand if our neuroblastoma cell line conditionally overexpressing α-syn was expressing Ret receptor and if this receptor was activated, we performed a western blotting analysis (Fig. 3.3A). Two fragments migrate at 170 and 150 kDa corresponding to the mature high glycosylated and immature low glycosylated full-length Ret proteins, respectively. However, an extra fragment at 120 kDa was also detected, corresponding to the non-glycosylated immature form of Ret protein, as described before (Takahashi *et al*., 1991; Takahashi *et al*., 1993). Either for the case of Ret or P-Ret the respective expression levels were

low, however the expression levels of the former was more intense than the later for both +Dox and –Dox condition. To examine if GDNF was exerting any effect upon cells, we further investigated GDNF-induced signaling on Akt pathway activation.

Due to small number of experiments there are significant deviations in the data, at some time points presented in the graph (Fig. 3.3C). Nevertheless, we observed a significant increase in Akt phosphorylation (on Ser473) in –Dox condition 10 minutes after stimulation with GDNF, when compared to +Dox GDNF-stimulated cells (1.42±0.27 and 0.84±0.04, respectively). This observation suggests the activation of Akt signaling pathway by GDNF, in a short period of time.


A
GDNF 20 ng/ml
MW
Ctr
5 min
10 min
15 min
30 min
1 h
2 h
6 h
8 h
15 h
18 h
24 h
170 kDa
150 kDa
120 kDa
anti-Ret
anti-P-Ret
anti-Ret
+ Dox
anti-Ret
anti-P-Ret
anti-Ret
– Dox
B
GDNF 20ng/ml
MW
Ctr
5 min
10 min
15 min
30 min
1 h
2 h
6 h
8 h
15 h
18 h
24 h
60 kDa
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anti-P-Akt
anti-Akt
anti-α-Tubulin
+ Dox
anti-P-Akt
anti-Akt
anti-α-Tubulin
– Dox

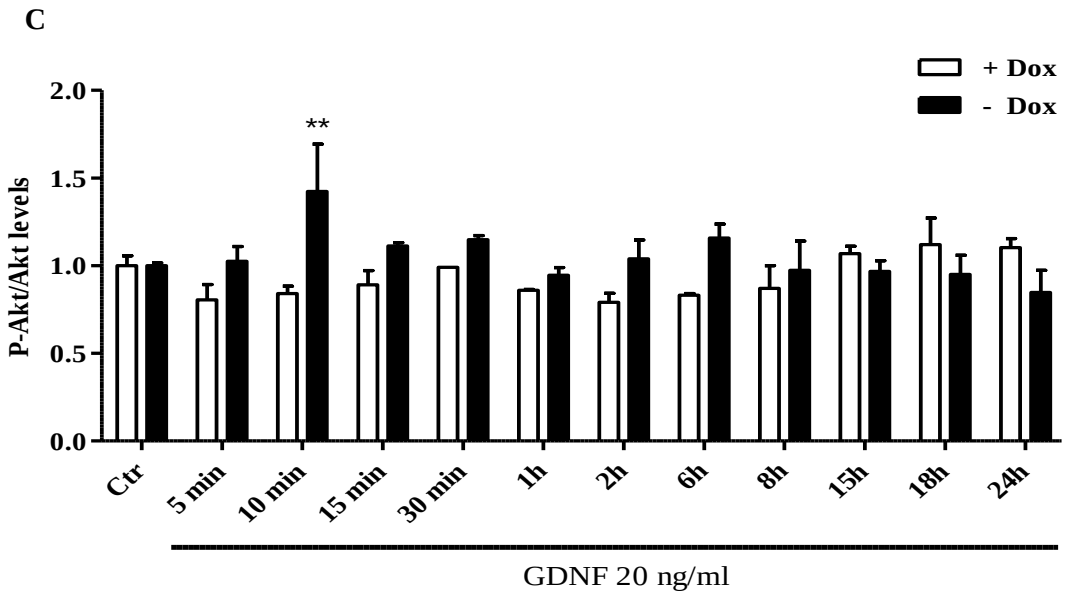

Figure 3.3 - Effect of GDNF on activation of Akt pathway in human SH-SY5Y neuroblastoma cells conditionally overexpressing α-syn. Cells were incubated with or without Dox along with GDNF (20 ng/ml), for the length of time specified above the blots and displayed on the graph. **A)** Western blots demonstrating the presence of imature (120 kDa) and mature (170 kDa) GDNF receptor (Ret) and its active phosphorylated form (P-Ret) in total protein extracts from SH-SY5Y neuroblastoma cells. The extracts were evaluated by anti-Ret and anti-P-Ret (Tyr905) immunoblotting (labeled on the *right*). **B)** The expression levels of P-Akt (60 kDa) were analyzed by Western Blotting. α-Tubulin served as loading control. The blots illustrate two-three independent experiments represented in **C)**. Data are the mean±SEM, normalized to Akt (60 kDa) and expressed as relative values of control. The average value obtained for control (untreated) cells was considered 1.0. Absolute values were 1.001±0.097 and 1.007±0.027, respectively, for +Dox and –Dox condition. Statistical analysis was performed by two-way ANOVA, followed by Bonferroni post test: ***P* <0.01 when compared to +Dox-treated cells.

3.2.2. Time course of GDNF effects on α-syn phosphorylation

GDNF has been linked for a whole series of phosphorylation cascades, which thus could also lead to α-syn phosphorylation. As can be seen in Figure 3.4B, there is a significant increase in α-syn phosphorylation at Ser129 in –Dox-treated cells after 2 hours of incubation with GDNF (20 ng/mL), as compared to +Dox-treated cells during the same stimulation period (1.47±0.37 and 0.83±0.18, respectively).

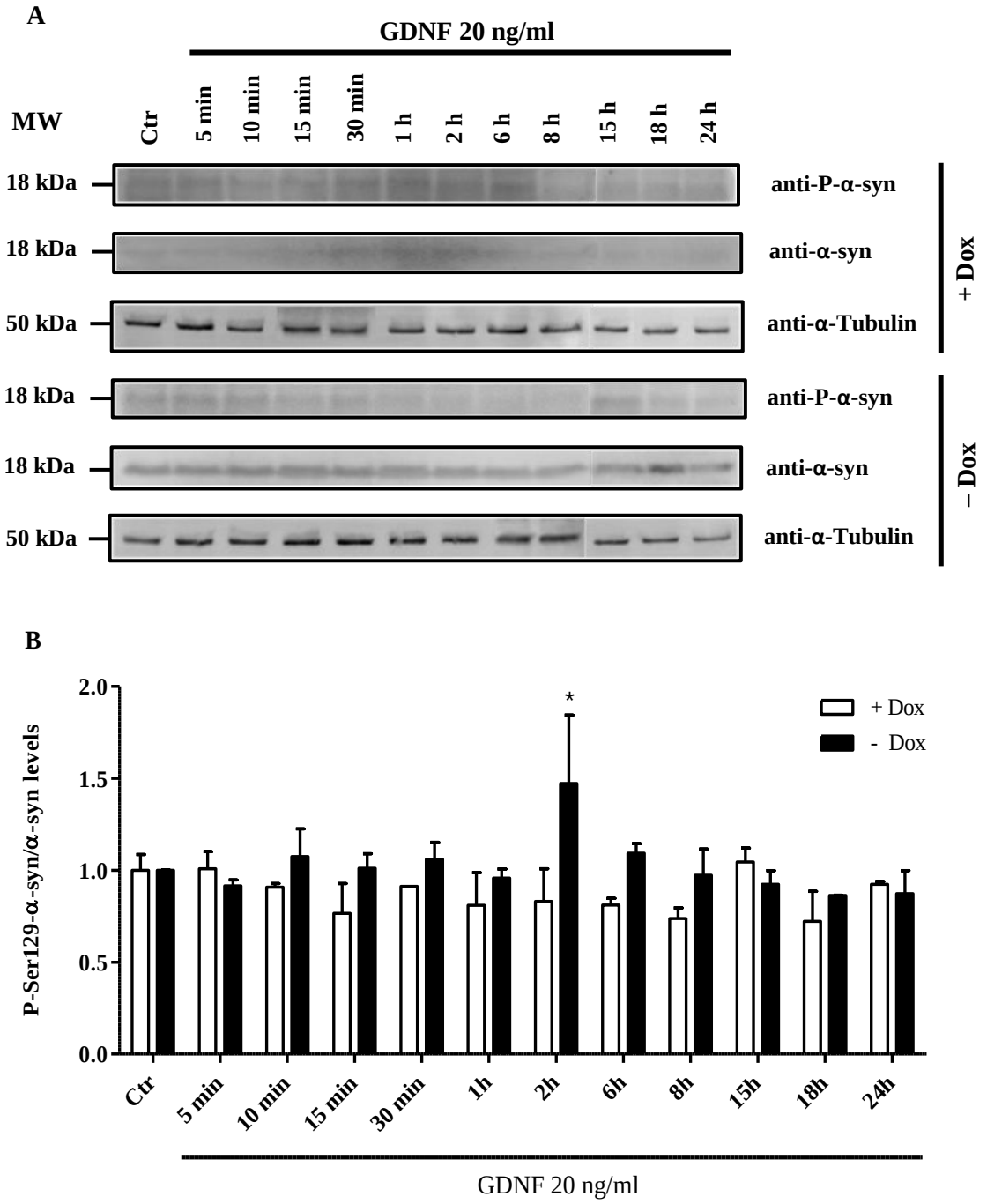

Figure 3.4 - Effect of GDNF incubation time on phosphorylation levels of α-syn in SH-SY5Y cells. Cells were incubated in the presence or absence of Dox along with GDNF (20 ng/ml), for the length of time specified above the blots and as displayed on the graph. **A)** Western blots demonstrating the expression of α-syn and it phosphorylation level, at different GDNF stimulation time points, performed with total protein extracts from SH-SY5Y neuroblastoma cells. The extracts were evaluated by anti-P-α-syn (Ser129) and anti-α-syn immunoblotting (labeled on the *right*). α-Tubulin served as loading control. The blots illustrate two to five independent experiments represented in the graph **B)**, showing the data (mean±SEM) normalized to α-syn (18 kDa) and expressed as relative values of control. The average value obtained for control (untreated) cells was considered 1.0. Absolute values were 1.327±0.114 and 0.920±0.001, respectively, for +Dox and –Dox condition. Statistical analysis was performed by two-way ANOVA, followed by Bonferroni post test: $*P < 0.05$ when compared to +Dox condition, during the same stimulation period (2h).

3.3. EVALUATION OF α-SYN PHOSPHORYLATION LEVELS

3.3.1. Effect of kinase inhibitors, Fe and GDNF on α-syn phosphorylation

Analysis of Figure 3.5B shows a significant increase (by about 50%) in α-syn (Ser129) phosphorylation levels following exposure to Fe (500 μM) for 2 hours, when comparing –Dox *versus* +Dox experimental conditions. Such findings seem to be in agreement with our initial hypothesis, according to which, in the presence of Fe the phosphorylation state of α-syn would be enhanced. Recently, our group has shown, in a similar approach, that a prolonged exposure (for 4 days) to Fe increased α-syn phosphorylation at Ser129 in a SH-SY5Y cell line transiently transfected with WT and mutant A53T α-syn (Perfeito *et al*., 2010).

Relatively to incubation with GDNF (20 ng/ml) for 30 minutes plus 2 hours similar results were obtained when compared to data depicted in Fig. 3.4B. These observations seem to be in accordance with our initial suggestion, for GDNF as an inductor of α-syn phosphorylation. Considering that GDNF has been largely described to be a neurotrophic, pro-survival growth factor for dopaminergic neurons (Airaksinen and Saarma, 2002 for review), we verified if GDNF affected α-syn (Ser129) phosphorylation before a noxious stimulus such as Fe exposure, known to cause oxidative stress in SH-SY5Y cells (Perfeito *et al*., 2010). By analyzing the effect of a preincubation with GDNF for 30 minutes, followed by stimulation with Fe for 2 hours, we found a trend for a decrease in both +Dox (0.45±0.14) and –Dox (0.87±0.13) condition, when compared, respectively, with Fe or GDNF incubations alone. These preliminary results suggest that although Fe and GDNF *per se* stimulate α-syn (Ser129) phosphorylation, the presence of the two stimuli inhibits α-syn phosphorylation, as neither of the stimuli exerted the previous observed effects.

We next tested the effect of the selected kinase inhibitors upon α-syn (Ser129) phosphorylation increased levels evoked by Fe. All the inhibitors tested demonstrated a general decrease in α-syn phosphorylation when compared with respective –Dox cells stimulated with Fe. Nevertheless, the CK-2 inhibitor (CX-4945) caused a more pronounced decline (+Dox, 0.44±0.10 and –Dox, 0.8±0.08) on α-syn phosphorylation, when compared to cells stimulated with Fe only (+Dox, 0.60±0.05 and –Dox, 1.19±0.20). For this reason, it seems to be the most promising inhibitor. These results, although preliminary and with no significant differences among groups, are in agreement with our first hypothesis for a potential protective role of selective kinase inhibitors under pathological conditions, herein modeled by the overexpression of α-syn.

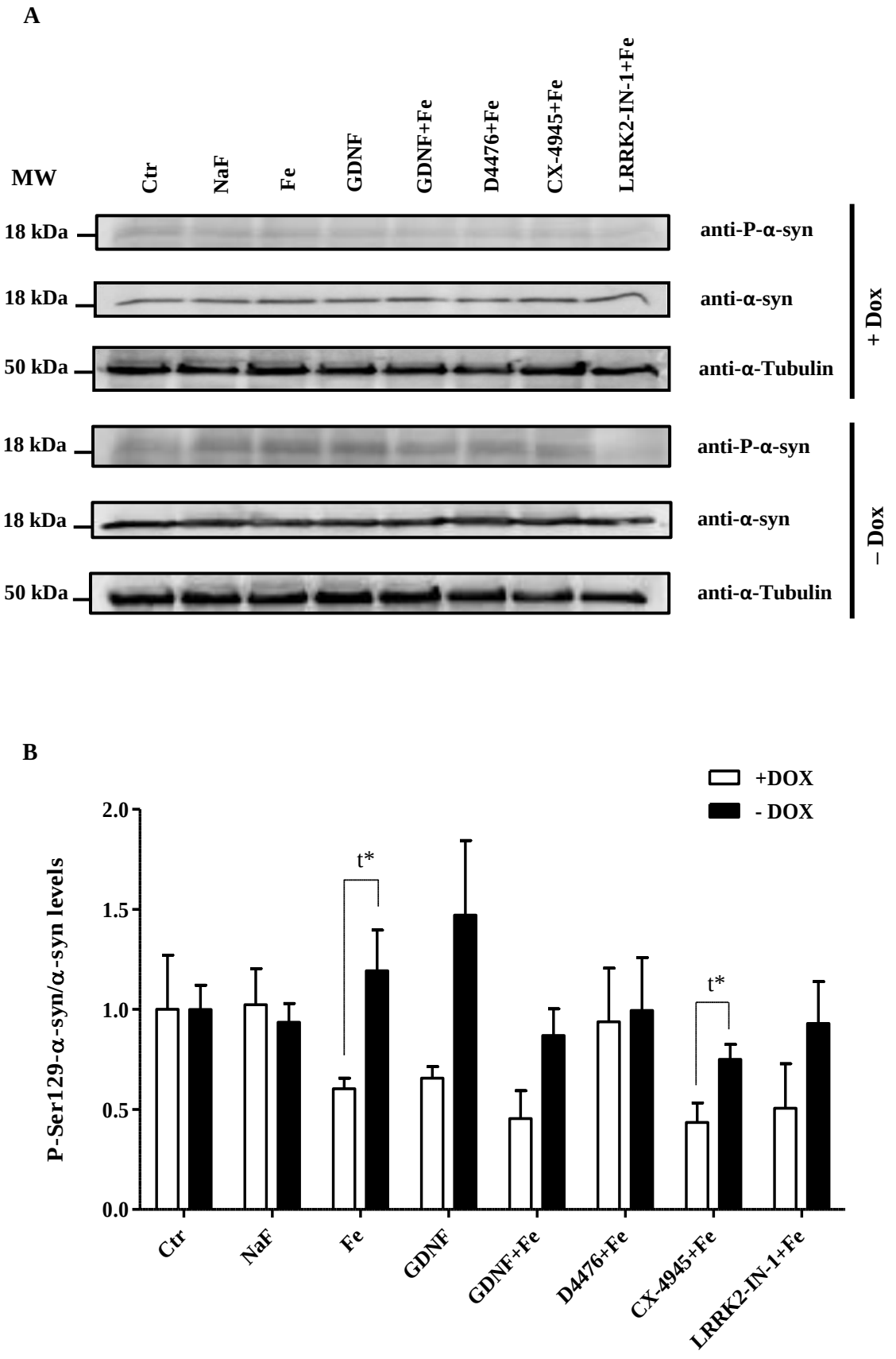
A
MW
Ctr
NaF
Fe
GDNF
GDNF+Fe
D4476+Fe
CX-4945+Fe
LRRK2-IN-1+Fe
18 kDa
anti-P-α-syn
18 kDa
anti-α-syn
50 kDa
anti-α-Tubulin
+ Dox
18 kDa
anti-P-α-syn
18 kDa
anti-α-syn
50 kDa
anti-α-Tubulin
– Dox
B
+DOX
- DOX
P-Ser129-α-syn/α-syn levels
2.0
1.5
1.0
0.5
0.0
t*
t*
Ctr
NaF
Fe
GDNF
GDNF+Fe
D4476+Fe
CX-4945+Fe
LRRK2-IN-1+Fe

Figure 3.5 - Effect of GDNF, Fe and selective kinase inhibitors on α-syn phosphorylation at Ser129. Cells were treated in the presence or absence of Dox, and depending on the conditions, exposed to Fe (500 µM), GDNF (20 ng/ml), D4476 (0.01 µM), CX-4945 (0.01 µM), or LRRK2-IN-1 (0.1 µM). Considering the incubation periods, Fe was incubated for 2 h and GDNF for 2 h and 30 min. In the case of double incubation, GDNF or inhibitors were added in the medium 30 min before incubation with Fe (2h). **A)** representative Western blots demonstrating the protein expression levels of α-syn and its phosphorylation at Ser129, performed with total protein extracts obtained from SH-SY5Y neuroblastoma cells. Membranes were probed with either specific antibodies to P-α-syn (Ser129) or α-syn. α-Tubulin was used as loading control. The blots illustrate five independent experiments represented in the graph **B)**, showing the data (mean±SEM) of phosphorylated form of α-syn normalized to α-syn (18 kDa) and expressed as relative values of control. The average value obtained for control (untreated) cells was considered 1.0. Absolute values were 1.970±0.535 and 1.163±0.141, respectively, for +Dox and –Dox condition. Statistical significance: $^{t*}P<0.05$ comparing +Dox and – Dox condition with the same stimulus (by Student's t-test).

CHAPTER 4 – DISCUSSION

Succinctly, our results demonstrated that α-syn overexpression triggered by the absence of Dox led to a decrease in cell viability when compared to cells incubated with Dox. We also showed that, with the exception of PLKs inhibitors, nanomolar concentrations of selected kinase inhibitors led to an acceptable percentage of viable cells. Moreover, assessment of plasma membrane integrity and caspase-3-like activity in cells exposed to the selected inhibitors, as well as $FeSO_4$ (Fe, 500μM) and GDNF (20 ng/ml) did not reveal significant cytotoxic effects upon the cells. We also were able to demonstrate that GDNF induced the early activation of PI-3K/Akt signaling pathway. Interestingly, Fe or GDNF exposure increased α-syn phosphorylation at Ser129. However, pre-incubation of GDNF prevented Fe-induced α-syn phosphorylation state. Finally, pre-incubation of cells with inhibitors of LRRK2 or CK-2 (in particular) followed by Fe exposure largely precluded Fe-induced stimulation of Ser129 α-syn phosphorylation.

4.1. CYTOTOXICITY

PD is characterized by the loss of mesencephalic dopaminergic neurons. Therefore, in this work we used a relatively homogeneous neuroblast-like cell line, known as SH-SY5Y, which provides an unrestrained supply of cells of human origin with comparable biochemical characteristics to human dopaminergic neurons. This cell line is useful as a model for dopaminergic neurons due to some particular characteristics: on one hand, SH-SY5Y cells possess the ability to synthesize dopamine and norepinephrine because the cells express key enzymes, like tyrosine and dopamine-β-hydroxylases (Oyarce and Fleming, 1991). On the other hand, these cells express dopamine receptors, as well as the dopamine transporter (DAT), a protein that is highly expressed in dopaminergic neurons only within the central nervous system (Takahashi *et al*., 1994); in addition, these cells have the capacity to express one or more neurofilament proteins (Ciccarone *et al*., 1989). Some compounds, like retinoic acid, are capable to induce differentiation, but confer tolerance to SH-SY5Y cells, and thus the role of toxicity or protection cannot be assessed in these differentiated cells (Cheung *et al*., 2009). This means that undifferentiated cells are an appropriate cell model for studying neurotoxicity or neuroprotection and hence become relevant in experimental PD research.

To determine if the Tet-Off gene expression system, present in our SH-SY5Y cell line conditionally overexpressing α-syn, was correctly working, we analyzed the total protein extracts by western blotting in order to verify the expression levels of α-syn, in the presence or absence of Dox. The results showed a tenuous basal expression of α-syn in the presence of Dox and a robust expression in its absence. The functioning of the Tet-Off gene expression system

was proved and the results are in agreement with what was previous published (Vekrellis *et al*., 2009).

In several species, toxic events can be promoted by expression of α-syn. These include organisms such as yeast, flies and worms, where no α-syn homologue is present, indicating that irrespective of its normal function, this protein can be toxic. Induction of toxicity by α-syn expression was observed *in vivo* using vertebrate organisms. Indeed, delivery of either WT or mutant α-syn through viral vectors directly to *substantia nigra* in mice, rats and non-human primates showed significant dopaminergic cell loss accompanied by α-syn deposition (Cookson, 2009, for review). Our cell line, besides being a good cell model of dopaminergic neurons it also conditionally overexpresses α-syn, mimicking the duplication/triplication of SNCA. It was documented that people with SNCA duplication have a brainstem-predominant PD phenotype (Chartier-Harlin *et al*., 2004), while triplication cases have a LB disease that involves several brain areas (Singleton *et al*., 2003; Fuchs *et al*., 2007). Measurements of α-syn levels in the blood of patients with SNCA triplication revealed a two-fold increase on α-syn protein levels, accompanied by increased levels and deposition of the protein in the brain cortex where LBs are found (Miller *et al*., 2004). Such genetic and biochemical evidences indicate that an increase in the levels of expression of WT α-syn is enough to cause neurodegeneration in PD. Furthermore, Iwata and coworkers (2001a) found that overexpression of WT or mutant forms of α-syn affects the cell viability of neuro2a cells, assessed in different perspectives. In the same study, the authors reported that WT, A30P and A53T α-syn inhibits MAPK (mitogen-activated protein kinase) signaling cascade and accelerates cell death, following serum reduction. Mutant and WT α-Syn overexpression attenuated not only the phosphorylation of classical MAPK ERK1-2 (extracellular signal-regulated kinase 1-2), but also the phosphorylation of p38 MAPK and SAPK/JNK (stress-activated protein kinase/c-Jun N-terminal kinase). Moreover, suppression of MAPK phosphorylation occurred as the levels of WT or mutant α-syn expression increased, as assessed in inducible α-syn cell lines (Iwata *et al*., 2001a). Overexpression of α-syn decreased MAPK phosphorylation as well as c-*fos* gene expression (Blackshear *et al*., 1987; Hibi *et al*., 1993; Derijard *et al*., 1994; Gould *et al*., 1995; Hodge *et al*., 1998). It was also reported that α-syn directly binds ERK2, forming a complex with Elk-1 (ETS domain-containing protein Elk-1, an ERK2 substrate) (Iwata *et al*., 2001b). Furthermore, α-syn is known to block PLD (phospholipase D) activity *in vitro* (Jenco *et al*., 1998), which is greatly related to ERK1/2 function (Wilkie *et al*., 1996; Ito *et al*., 1998). Thus, α-syn might regulate the MAPK pathway by decreasing the amount of available active MAPK. Considering this, α-syn may bind to a number of proteins in order to regulate signal transduction. Our results extend and reinforce the idea that α-syn overexpression *per se* may be toxic for the cells. Indeed, when we assessed the cell viability by the Alamar Blue assay in untreated cells, in the absence or presence of Dox, we

were able to observe a significant decrease in cell viability when Dox was absent, i.e. when α-syn overexpression was induced.

α-Syn may be modified post-translationally by truncation, nitration, ubiquitinylation and phosphorylation (Giasson *et al*., 2000; Fujiwara *et al*., 2002; Tofaris *et al*., 2003; Anderson *et al*., 2006). Such post-translational modifications have been explored as possible mediators of toxicity. In this work we focused on α-syn phosphorylation at Ser129. According to some authors phosphorylation at Ser129 is a α-syn modification that may play an important role in neurotoxicity and influence α-syn aggregation (Fujiwara *et al*., 2002; Anderson *et al*., 2006; Chen *et al*., 2009); however, this is still controversial as protein aggregates have been claimed to be less toxic than soluble oligomeric species (Wakamatsu *et al*., 2007; Gorbatyuk *et al*., 2008). One possible approach to understand α-syn phosphorylation is to identify the key kinase(s) that mediates the transference of a phosphate group to Ser129. Several kinases have been proposed as candidates for α-syn phosphorylation at this residue. Such kinases were identified in *in vitro* and cell culture-based studies. These include CK-1 and CK-2 (Okochi *et al*., 2000), LRRK2 (Qing *et al*., 2009a), and PLKs (Inglis *et al*., 2009; Mbefo *et al*., 2010). Taking into consideration that post-translational modifications, such as phosphorylation may play a pivotal role in α-syn neurotoxicity, we can use selective inhibitors against these kinases and hence reduce the phosphorylation levels of α-syn. This strategy leads to the idea that α-syn toxicity could be diminished, and that is true only if one considers that phosphorylation may play an important role in α-syn neurotoxicity, which is not fully understood or proven.

Based on these findings, we evaluated the role of five kinase inhibitors, namely ON-01910 (PLK1 inhibitor), BI2536 (PLK2 inhibitor), D4476 (CK-1inhibitor), CX-4945 (CK-2 inhibitor) and LRRK2-IN-1 (LRRK2 inhibitor). Almost all of these inhibitors have been used in cancer research as cytostatic or cytotoxic agents. Thus, before starting the experiments with these inhibitors it was mandatory to perform an intense research, in order to ascertain the range of concentrations and incubation periods in our cell model.

The PLK1 inhibitor used, ON-01910, is a cell-permeable non-ATP-competitive small molecule with a half-maximal inhibitory concentration (IC_{50}) of 9-10 nM, and is entering phase III clinical trials for myelodysplastic syndrome (Gumireddy *et al*., 2005; Reagan-Shaw and Ahmad, 2005; Jackson *et al*., 2007; Chapman *et al*., 2012). During mitosis, PLK1 participates in centrosome maturation and assembly of the mitotic spindle. PLK1 is also involved in exit from mitosis and the segregation of sister chromatids during anaphase. Such kinase might also play a critical role in cytokinesis through the phosphorylation of the mitotic kinesin-like protein 1 (MKLP1). For a normal regulation of cell-cycle processes the roles of PLK1 must be considered. It is involved in the entry to mitosis through the activation of cyclin-dependent

kinase 1 (CDK1) (Jackson *et al*., 2007 for review). PLK1 also participates on the regulation of tumour suppressors such as p53 and BRCA2 (Eckerdt *et al*., 2005). The inhibition of this kinase by small inhibitory molecules or siRNA has demonstrated to interfere with several steps of mitosis (Liu and Erikson, 2003; Sumara *et al*., 2004). Our data indicated that, ON-01910 treatment using a concentration of 0.05 μM decreased cell viability, when compared with the respective DMSO-treated control cells. Such concentration was able to induce a cytotoxic effect beyond the stipulated non-toxic limit (of 80%). The same effect was observed for the remaining concentrations, in a dose-dependent manner. Several evidences are in agreement with our results. ON-01910 was previously tested against 94 different cancer cell lines, and in all of these cells this compound was found to induce apoptosis with a GI50 (the concentration required to achieve 50% growth inhibition) that ranged between 50–200 nM. In this group of human cancer cells, ON-01910 was found to result in the induction of mitotic arrest characterized by abnormalities in spindle formation, leading to the apoptotic death (Gumireddy *et al*., 2005). In another study, using ON-01910 in HeLa cells, PARP cleavage was reported in cells treated for 24 hours, which became more pronounced after 48 hours of treatment, suggesting the progression of apoptosis in this cell line. In the same study, analysis of caspase-3 activity also demonstrated a huge upregulation of this activity in cells treated with 100 nM ON-01910 (Gumireddy *et al*., 2005). Another study with leukemic cells shows that ON-01910 activated the mitochondrial apoptosis pathway by detection of activated Bax and Bak (pro-apoptotic proteins) and decreasing Mcl-1 (antiapoptotic protein) expression, leading to mitochondrial depolarization and caspase-3 activation (Chapman *et al*., 2009). More recently, the same authors working on the same type of cell line demonstrated that ON-01910 (2 μM) was capable to induce apoptosis through two main mechanisms of action: PI3K/Akt inhibition and induction of ROS (in a dose- and time-dependent manner), resulting in an oxidative stress response. It was revealed that ON-01910 treatment induced a classic ROS triggered response pathway that involved c-Jun N-terminal kinases (JNK) activation, accumulation of c-Jun in the nucleus, and induction of activating transcription factor 3 (ATF3) and NOXA in all leukemic cells tested (Chapman *et al*., 2012). Another possible mechanism for cell-cycle-arrest and apoptotic death triggered by ON-01910 is revealed in a work done with MCL (mantle cell lymphoma) cells. In this work, a decrease of cyclin D1 levels was observed by blocking cyclin D1 translation through inhibition of the PI3K/Akt/mTOR/eIF4E-BP signaling pathway; the authors also observed an altered expression of Bcl-2 family proteins, induction of mitochondrial cytochrome c release and activation of caspases (Prasad *et al*., 2009). To reinforce the cytostatic and cytotoxic effects of ON-01910, a recent study performed with DU 145 (prostate cancer) cells revealed a block of their cell-cycle progression and increase in cell death in a dose-dependent manner (0.1-1.0 μM) (Reddy *et al*., 2011). Such aforementioned aspects could explain the lower cell viability observed in our range of concentrations of ON-01910 (0.05-5μM).

PLK2 inhibitor, BI2536, is a cell-permeable ATP-competitive inhibitor selective for PLK1, with an IC_{50} value of 0.83 nM; nevertheless it can also affects the activity of PLK2, with an IC_{50} value of 3.5 nM. The role of PLK2 on the cell cycle is still not well understood. During mitosis PLK2 is expressed and activated mainly during G1 and seems to interfere with G1 progression. PLK2 was shown to influence centriole duplication rather than centrosome segregation like PLK1. However, PLK2 activation is achieved through a mitotic checkpoint in a p53-dependent manner and thereby may prevent mitotic catastrophe after spindle damage (Reagan-Shaw and Ahmad, 2005 for review). Our results showed that a BI2536 concentration of 0.01 μM was sufficient to reach or to overcome the defined toxic threshold. Moreover, the remaining range of concentrations caused a more pronounced percentage of unviable cells, in a dose-dependent manner. It has been shown that BI2536 acts on cells through cell-cycle disruption and cell death via apoptosis. Indeed, BI2536 treatment (10, 30, and 100 nM) induces cell-cycle arrest and 10 nM BI2536 induced a gradual accumulation of cleaved PARP fragments, suggesting apoptosis induction (Grinshtein *et al.*, 2011). BI2536 (5-10 nM) caused cell-cycle arrest and detectable caspase-3 fragments (Nappi *et al*., 2009). Moreover incubation with BI2536 (60 nM) produced a strong signal for fragmented DNA and cleaved PARP (Steegmaier *et al.*, 2007). Incubation with this PLK1/PLK2 inhibitor at concentrations of 5 and 10 μM also induced cell-cycle arrest and apoptosis (Watt *et al*., 2011). Taken together, these findings indicate that low nanomolar concentrations of BI2536 induce cell-cycle arrest and cell death by apoptosis. On the basis of the results of these researchers, it can be speculated that the cells in our Alamar Blue assay underwent cell-cycle arrest and/or apoptosis.

D4476 was described as the more potent and selective cell-permeable CK-1 inhibitor; it acts as a potent, reversible and relatively specific ATP-competitive inhibitor of CK-1 with an IC_{50} value of 0.2 μM from *Schizosaccharomyces pombe* (Rena *et al*., 2004) and IC_{50} value of 0.3 μM for CK-1δ isoform (Bain *et al*., 2003; Rena *et al*., 2004). More recently, other authors working on A375 cells demonstrated the possible inhibitor effect of D4476 upon CK-1α isoform (Huart *et al*., 2009). Among the CK-1 substrates there are enzymes, cytoskeleton proteins, membrane-associated proteins, transcription factors, splice factors, receptors and cell-signaling proteins (Knippschild *et al*., 2005). It has been described that several CK-1 isoforms (α, δ and ε) phosphorylate p53 protein. This phosphorylation state is believed to attenuate the interaction between p53 and its inhibitory protein Murine double minute 2 (MDM2) and further stabilize the binding of the co-activator p300, resulting in p53 activation (Sakaguchi *et al*., 2000; MacLaine *et al*., 2008). Moreover, the cross-talk between p53 and MDM2 on different levels relies on the phosphorylation state not only of p53, but also of MDM2. Interestingly, it has been also shown that several CK-1 isoforms (δ and ε) phosphorylate MDM2, which are involved in the degradation of p53 (Winter *et al*., 2004). These data suggest a pivotal role of

CK-1 in the modulation of p53 on cell growth and genomic integrity. This could be one of the reasons that lead the cells to undergo cell-cycle arrest or cell death, when CK-1 is inhibited. In our results, D4476 incubation caused a dose-dependent decrease in cell survival, when compared to respective DMSO-treated (control) cells. These findings are in accordance with those reported by Huart and colleagues (2009), in a study performed on A375 melanoma cells with increasing concentrations of D4476 (10-40 μM). The authors demonstrated a possible cell-cycle arrest and apoptotic effect that increases with the concentration. Our results showed an increase in caspase-3-like activity (although not significant), with D4476 0.01 μM incubated for a period of 24 hours. It should be noted that the concentration we used for caspase-3 activity was 1000-fold lower than the minimal concentration used by the aforementioned authors. A possible explanation for the occurrence of the apoptotic event when cells are in the presence of D4476 may be due to the loss of E2F transcription factor 1 (E2F-1), which occurs in a dose-dependent manner (Huart *et al*., 2009). E2F-1is a member of E2F family of transcription factors, which plays an important role in proliferation and differentiation controlling the transcriptional activity of genes required for controlling G1 to S phase transition in cells (Dyson, 1998 for review). Relatively to LDH assay, 0.01 μM D4476 incubated with Fe did not alter the toxicity pattern compared with cells treated with Fe alone for 2 h. However, this inhibitor induced a lower LDH release when compared to CX-4945 (0.01 μM) and LRRK2-IN-1 (0.1 μM) in the same conditions.

Several ATP-competitive inhibitors of CK-2 have already been indicated in the literature, among them the commercially available TBB and DMAT have been the most widely used in cell-based assays. However, a relatively recent evaluation of their inhibitory properties performed against a large panel of kinases indicated that their selectivity profile was not as great as originally believed (Pagano *et al*., 2008). None the described CK-2 inhibitors have reached human clinical trials. However, more recently the first orally available inhibitor of CK-2, CX-4945, was discovered and is currently in clinical trials for the treatment of cancer. This small cell-permeable molecule is a potent, selective and ATP-competitive inhibitor of both isoforms of the CK-2 catalytic subunits, namely CK-2α and CK-2α′, with IC_{50} values of 1 nM (Siddiqui-Jain *et al*., 2010; Ferguson *et al*., 2011; Pierre *et al*., 2011a). CK-2 is ubiquitously expressed, constitutively active, and has been demonstrated to play a crucial role in cell cycle control, cellular proliferation and differentiation. Indeed, CK-2 becomes an integral part of regulation of many anti-apoptotic and pro-proliferative signaling cascades, including PI3K/Akt and Wnt signaling cascades, NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) transcription, and DNA damage response (Duncan and Litchfield, 2008; Guerra and Issinger, 2008 for review). Some findings suggest that CK-2 also phosphorylates pro-apoptotic proteins, protecting them from caspase-mediated cleavage, thereby acting as a suppressor of apoptosis

(Litchfield, 2003). A wide variety of cancer cells derived from tumors of different origin share abnormally high levels of CK-2 protein when compared with the respective normal cells (Tawfic *et al*., 2001). On the other hand, CK-2 has been associated to the regulation of EGFR activity as well as the downstream EGFR signaling pathways through multiple mechanisms (Pinna and Allende, 2009; Trembley *et al*., 2009). Another important role attributed to CK-2 is related with the phosphorylation and modulation of the activity of many members of the PI3K-Akt-mTOR pathway including PTEN (phosphatase and tensin homolog), p70S6K (70 kDa ribosomal protein S6 kinase 1), and Akt (Torres and Pulido, 2001; Di Maira *et al*., 2005; Panasyuk *et al*., 2006). Hsp90 (heat shock protein 90) and the Hsp90 co-chaperone Cdc37, which could form a complex with many members of the EGFR signaling pathway, are both directly phosphorylated by CK-2 (Miyata, 2009; Porter *et al*., 2010). Furthermore, this kinase is responsible for phosphorylating and regulating the activity and stability of several tumor suppressor proteins, oncogenes and transcriptional activators (Seldin and Leder, 1995; Landesman-Bollag *et al*., 2001; Litchfield, 2003). Recently, it was reported that CK-2 plays an important role in DNA repair mechanisms, either in single or double DNA strand breaks (Koch *et al*., 2004; Loizou *et al*., 2004; Becherel *et al*., 2010). Two of the best characterized CK-2-dependent DNA repair response substrates are XRCC1 (X-ray repair cross-complementing protein 1) and MDC1 (Mediator of DNA Damage Checkpoint protein1), which are indispensable elements, respectively, of the single strand break and double strand break repair machinery (Siddiqui-Jain *et al*., 2012). Our data regarding CX-4945 inhibitor showed a decrease in cell viability in a concentration-dependent manner. At 1 µM the inhibitor induced a cell survival profile inferior to the maximum acceptable percentage for cell viability (80%). The percentage of cell viability declined with increasing of inhibitor concentration, until a condition of none viable cells (CX-4945, 250 µM). The results provide some support to the cytostatic and cytotoxic effect of the inhibitor, already evidenced by many researchers. Indeed, CX-4945 (1 to 10 µM) treatment for 24 hours induced cell-cycle arrest on cancer cell lines. In the same study, treatment with the same inhibitor (3-100 µM) during the same period of time showed caspase 3/7 activation in a dose-dependent manner (Siddiqui-Jain *et al*., 2010). These findings were further supported by Pierre and colleagues (2011b). Very recently it was observed for the case of cancer cells stimulated with CX-4945 (10 µM), a percentage of viable cells similar to that found in our results using the same inhibitor concentration, although during a period of incubation of 96 hours. The same authors further observed a strong increase in caspase 3/7 activity after CX-4945 (10 µM) exposure during 24 hours (Bliesath *et al*., 2012). Another recent study performed in ovarian cancer cells showed an anti-proliferative effect of cells treated with 10 µM of CK-2 inhibitor during a period of incubation of 72 and 96 hours. Moreover, the same inhibitor concentration was able to induce cell-cycle arrest and increased caspase 3/7 activation, along with the appearance of cleaved PARP fragments, for 28 and 6 hours of stimulation,

respectively. Furthermore, CX-4945 incubation, again for the same concentration and for a period of 24 hours, prevented the activation of MDC1 and XRCC1 proteins and consequently suppressed the ability of cancer cells to repair DNA strand breaks (Siddiqui-Jain *et al*., 2012). We also performed an assay in order to evaluate caspase-3-like activity in SH-SY5Y cells, for a period of 24 hours and with a CX-4945 concentration of 0.01 µM. Our results showed a slight increase in caspase-3 activity; however, we must take into consideration the higher concentration used by the referred authors (1000-fold higher). Such difference in the results could also be explained, for example, by the fact that CX-4945 induced cell-cycle arrest in a different way on different cell lines, suggesting that cell-cycle regulation by CK-2 is cell-type dependent (Siddiqui-Jain *et al*., 2010). As already described in this thesis, CK-2 plays important roles in promoting pro-survival and anti-apoptotic pathways on cells and can interact with some key proteins in several signal cascades. Nevertheless, increasing attention has been paid to pro-survival PI3K/Akt/mTOR pathway. It has been postulated that attenuation of the signalling of this pathway may enhance the induction of apoptosis, since CX-4945 inhibits CK-2 it can promote apoptosis via PI3K/Akt/mTOR pathway. This could be, among other reasons, a possible explanation for the occurrence of the apoptotic event when cells are in the presence of CX-4945. Indeed, Di Maira and colleagues (2005) showed that phosphorylation of Akt on the CK-2 specific site (Ser129) hyperactivates Akt. In a further study, these authors showed that phosphorylation at this residue facilitates the interaction of Akt with Hsp90, which in turn stabilizes the phosphate at Thr308 on Akt (Di Maira *et al*., 2009). It was demonstrated, in cancer cell lines, that CX-4945 induced a robust dephosphorylation of Akt (Ser129) as well as in canonical regulatory sites, Thr308 and Ser473 (Siddiqui-Jain *et al*., 2010). Notwithstanding the direct effects on the phosphorylation of Akt, it was also demonstrated that CX-4945 treatment induced a decrease on phosphorylation of Cdc37 at the CK-2-specific site, Ser13 (Bliesath *et al*., 2012). Cdc37 is an Hsp90 co-chaperone and CK-2-dependent phosphorylation of this co-chaperone is necessary for efficient binding of Cdc37 to the respective client kinases and it is also essential for the recruitment of Hsp90 to these Cdc37-client kinase complexes (Miyata, 2009). Moreover, Hsp90 itself is also a direct CK-2 substrate. Akt it is a Cdc37 client kinase and the disruption of such chaperone-kinase complexes by CX-4945 should contribute to decrease the activation of PI3K/Akt/mTOR pathway leading to the suppression of pro-survival signaling and a further apoptotic event. Regarding LDH assay, 0.01 µM CX-4945 incubated with Fe induce a slight increase in LDH release (although not significant). Indeed, this inhibitor at this concentration did not induce a toxic effect as assessed by Alamar blue assay.

The LRRK2 inhibitor, LRRK2-IN-1, was described as the first selective and potent inhibitor of LRRK2. This is a cell-permeable compound that acts as a potent and selective ATP-competitive inhibitor of LRRK2, with an IC_{50} values of 13 nM and 6 nM for WT and G2019S

mutant kinase, respectively (Deng *et al*., 2011). The same authors established that LRRK2-IN-1 rapidly suppresses LRRK2 kinase activity leading to dephosphorylation of Ser910 and Ser935, loss of 14-3-3 binding and accumulation of LRRK2 within aggregate fibrillar structures. The normal physiological function of LRRK2 and its physiological substrates remains largely unknown (Li *et al*., 2010; Cookson, 2010). Nevertheless, some studies have been showing a few physiological substrates for LRRK2. It was reported that LRRK2 can efficiently phosphorylate ezrine, radixin, and moesin (ERM), proteins which anchor the actin cytoskeleton to the plasma membrane (Jaleel *et al*., 2007; Parisiadou *et al*., 2009). Moreover, it was revealed that LRRK2 phosphorylates 4E-BP protein, which modulates stress sensitivity and dopaminergic neuron survival in *Drosophila* (Imai *et al*., 2008). More recently, it was reported that FoxO is also phosphorylated by LRRK2 (Kanao *et al*., 2010). Our results on cell viability using LRRK2-IN-1 inhibitor show a decrease in cell viability in a dose-dependent manner. LRRK2-IN-1 at different concentrations caused a lower cell survival profile, when compared to respective DMSO-treated (control) cells. However, 1 µM induce a decrease in cell viability very close to the threshold that defines a toxic concentration, and cell viability subsequently declined with increasing inhibitor concentrations. This LRRK2 inhibitor was recently discovered and consequently only a few studies were performed so far. However, in a recent report, the authors did not observe any alteration on survival profile of the cells treated with LRRK2-IN-1 (10 µM), as assessed by Alamar Blue assay (Thévenet *et al*., 2011). These results do not support our observations; nevertheless it should be noted that the authors used a monocyte cell line, which suggests differential vulnerability of cell lines. Treatment of LRRK2-IN-1 (0.1 µM) for 24 hours showed a tendency for increased caspase-3-like activity. Several findings might explain this tendency for an increased activity of caspase-3. Kanao and colleagues (2010) have reported in human cells that FoxO is phosphorylared by LRRK2, which in turn upregulates the expression of Bim a pro-apoptotic protein. Several transcriptional targets of FoxO have been characterized, including molecules involved in cell cycle arrest, programmed cell death, oxidative stress and metabolism (van der Horst and Burgering, 2007). In an experiment carried out in LRRK2-deficient differentiated human mesencephalic neural progenitor cells, reduced number of dopaminergic neurons was observed due to apoptosis. Such cells also exhibited elevated cell cycle- and cell death-related markers. The same authors hypothesized that the absence of LRRK2 might impair the differentiation and/or survival of dopaminergic neurons and that this protein seems to be involved in cell-cycle regulation (Milosevic *et al*., 2009). Furthermore, gene array experiments in neuroblastoma cells also demonstrated that the absence of LRRK2 affects cell-cycle genes and p53 signaling cascades (Häbig *et al*., 2008). p53 activation can lead to either cell cycle arrest and DNA repair or apoptosis (Levine, 1997). In LRRK2-deficient dopaminergic cells, activation of p53 through phosphorylation was detected, which might influence cell death in these types of neurons (Nair *et al*., 2006). Impairment in autophagy-lysosomal pathway was

previously observed in LRRK2 knockout mice, which occurred concomitantly with the accumulation and aggregation of ubiquitinated proteins, which dramatically increases in apoptotic cell death, inflammatory responses and oxidative damage (Tong *et al*., 2010). Relatively to LDH assay, 0.1 μM LRRK2-IN-1 incubated with Fe induced no toxic effect compared with cells treated with Fe alone for 2 h. Further investigations will be required in order to clarify the cause of cell death in this particular SH-SY5Y cell line when treated with the selective kinase inhibitors used in this work.

Iron is known to catalyze oxidative damage, through production of ROS. Since Fe^{2+} can react with H_2O_2 generating the highly reactive hydroxyl free radical ($^{\bullet}OH$) through Fenton reaction, an increase in the levels of this metal will favor the production of ROS and oxidative stress (Bossy-Wetzel *et al*., 2004; Jomova and Valko, 2011). Relatively to Fe cytoxicity in dopaminergic cells, it is generally accepted that total Fe levels increase in the *substantia nigra* in PD, possibly leading to nigrostriatal neurodegeneration, as a result of its ability to generate ROS and cause nucleic acids and protein damage, as well as lipid peroxidation (Youdim *et al*., 1991; Jomova *et al*., 2010). Our results on cell toxicity, evaluated through the analysis of plasma membrane integrity, revealed a slight increase in the percentage of LDH released into the culture medium in –Dox cells (which model the duplication/triplication of SNCA gene) exposed to Fe for 2 h or 24 h, when compared with the respective control cells. Recently our group demonstrated that prolonged (for 4 days) and short (up to 2 h) incubations with iron led to an increase in the formation of endogenous peroxides in SH-SY5Y cells (Perfeito *et al*., 2010). A significant increase in the levels of ROS formation was demonstrated in SK-N-SH neuroblastoma cells when incubated with 1 mM ferrous iron (Li *et al*., 2011). In the same study, treatment with 100-300 μM ferrous iron for 24 h did not affect cell viability. However, using a concentration of 1 mM ferrous iron a significant decrease in cell viability was observed (Li *et al*., 2011). Nevertheless, the increase in LDH release was not as significant as expected. Some findings can contribute for a possible explanation. One of the critical roles of iron may be to participate in cellular defense response against the well-established capacity to induce oxidative damage. Indeed, when cells are iron-replete, but not in excess, aconitase enzyme is activated, resulting in elevated levels of intracellular L-glutamate. As a result, the production of GSH (L-γ-glutamyl-L-cysteinyl glycine or reduced glutathione) may be stimulated, although the presence of L-cysteine is also mandatory (Lall *et al*., 2008). As a potent antioxidant, GSH maintains enzymes and protein thiols in their reduced state and scavenges ROS species (Reddy, 1990; Gukasyan *et al*., 2003; Wu *et al*., 2004). Thereby the cellular oxidation state and potential to undergo oxidative damage can be reduced. Moreover, it was reported that SH-SY5Y neuroblastoma cells that survive an iron accumulation protocol evoke an adaptive response consisting of decreased synthesis of iron import transporter DMT1 (divalent metal transporter 1)

and increased synthesis of ferritin and ferroportin (Aguirre *et al*., 2005). Thus, cells can reduce the cytosolic content of Fe and avoid the well-known oxidative damage. Although speculative, these evidences could explain the small rather than large increase in LDH release.

The different kinase inhibitors were incubated along with Fe (double incubation) in order to assess their potential synergistic toxic effect, evaluated by LDH release. From the data we concluded that no significant differences occur between control (untreated) cells, cells treated with Fe (for 2 hours) or Fe plus kinase inhibitors, indicating no synergistic toxic effects.

In the case of GDNF stimulation our preliminary data showed no significant changes on caspase-3-like activity. Since the discovery of GDNF in 1993 (Lin *et al*., 1993) numerous cell culture experiments and rodent studies have demonstrated that it is active on different cell types in multiple tissues, acting as a particularly potent factor for survival and axonal growth of dopaminergic neurons. Moreover, several authors demonstrated that GDNF blocks the apoptotic event. The mechanisms by which GDNF exerts its effect are diverse, and some evidences highlighted that the rescue and repair of damaged neurons is a consequence of an anti-apoptotic action of GDNF. In fact, GDNF showed the ability to up-regulate Bcl-2 (B-cell lymphoma 2) and Bcl-X_L (B-cell lymphoma-extra-large) levels in rat mesencephalic neurons subjected to apoptosis, resulting in the attenuation of apoptotic event and consequently of caspase activation (Kitagawa *et al.*, 1998; Sawada *et al.*, 2000). Later, Ghribi and collaborators (2001) confirmed these anti-apoptotic properties of GDNF by working with lysates of microdissected rabbit hippocampus. A more profound analysis of this scientific report revealed that after a direct (intracisternal) injection of aluminum complexes into rabbit brain this caused mitochondrial cytochrome *c* release, Bax translocation into mitochondria, a decrease in Bcl-2 in both mitochondria and ER, activation of caspase-3 and DNA fragmentation. Co-administration of GDNF inhibited these Bcl-2 and Bax changes, upregulated Bcl-X_L, and strongly reduced caspase-3 activity, leading to a dramatic inhibition of apoptosis (Ghribi *et al*., 2001). A study performed in SK-N-SH neuroblastoma cells indicated that exogenous administration of GDNF inhibited both ethanol-induced apoptotic cell death associated with JNK cascade (McAlhany *et al*., 2000). Interestingly, expression of Ret was reported to induce apoptosis, which was blocked in presence of GDNF (Bordeaux *et al*., 2000). In a different approach, it was observed that addition of GDNF (100 ng/ml) had no effect on apoptosis in cultured glial cells, as assessed by caspase3/7 activity (Steinkamp *et al*., 2012). Our results indicated that GDNF did not induce the release of LDH. On the contrary, GDNF pre-incubation with Fe induced a slight reduction of LDH release into the culture medium, compared with incubation with Fe for 2 h. Although not significant these results are supported by the observation that GDNF exhibited a protective role against oxidative stress in cultured mesencephalic neurons and glial cells (Iwata-Ichikawa *et al.*, 1999). Moreover, Orth and colleagues (2000) reported no increase in trypan blue-positive cells

or LDH release into the supernatant in the presence of GDNF concentrations up to 500 ng/ml. Although performed on human adult mesangial cells (present in renal glomerulus) the authors used a concentration 25 times greater than the GDNF concentration (20 ng/ml) used in our study.

4.2. α-SYN PHOSPHORYLATION LEVELS

To evaluate if GDNF stimulates an intracellular signaling pathway in cells, we assessed the activation of Akt pathway through evaluation of Akt phosphorylation levels. GDNF activates the PI3K/Akt pathway through activation of Ret tyrosine kinase receptor in several contexts (Pong *et al*., 1998; Trupp *et al*., 1999; Focke *et al*., 2001; Jin *et al*., 2002). Ret cannot directly bind GDNF but requires the presence of GFRα1 (Treanor *et al*., 1996). Importantly, we were able to show that SH-SY5Y cells express Ret protein, in accordance with the prior detection of Ret mRNA and Ret protein as well as GFRα1 mRNA and protein (Hirata and Kiuchi, 2003). Our western blot results revealed the existence of three specific Ret fragments (120, 150 and 170 kDa) in total cell lysates. Glycosylation of Ret occurs in the ER, and the molecular weight of this receptor increases from 120 kDa to 150 kDa (Takahashi *et al*., 1991; Takahashi *et al*., 1993). Further glycosylation occurs in the Golgi apparatus and the molecular weight of mature Ret is approximately 170 kDa (Takahashi *et al*., 1991; Takahashi *et al*., 1993). In this context, we further evaluated the active form of Akt; we demonstrated an early increase of Akt phosphorylation at Ser473, which is indicative of the activation state of Akt (Kovacic *et al*., 2003), 10 minutes after stimulation with GDNF in –Dox cells. Other authors showed that GDNF stimulates Akt phosphorylation at different time points in different cell types and with different GDNF concentrations (Mograbi *et al*., 2001; Paratcha *et al*., 2001; Hauck *et al*., 2006). GDNF concentrations used by these authors was significantly higher (100 ng/ml and 50ng/ml) than the concentration of GDNF used in the present study (20 ng/ml). These findings seem to indicate that the time point for GDNF-induced phosphorylation of downstream substrates is cell- and dose-dependent. Lack of sustained increase in Akt phosphorylation observed in our cells might be explained by the expression of high levels of immature Ret; indeed, we observed a relatively strong expression of non-glycosylated immature Ret form (120 kDa), when compared with 150 and 170 kDa. Therefore, we may hypothesize that the absence of a functional mature Ret abrogates the ability of GDNF to trigger sustained intracellular signaling in our cells.

Taking into account that GDNF activates intracellular phosphorylation cascades, we further analyzed the role of GDNF on α-syn phosphorylation state at Ser129. Interestingly, we showed an increase on phosphorylation rate after 2 hours of GDNF incubation in the absence of the

stress stimuli (Fe) in cells overexpressing α-syn (-Dox), which is seen in independent experiments which data is depicted in Figs. 3.4B and 3.5B (a trend is shown in this last data). Although so far, and according to the scientific literature, no one has reported the influence of GDNF on α-syn phosphorylation levels, these data are worthwhile to be explored taking into account that GDNF protective effects are not always clear-cut (Kordower *et al*., 1999; Nutt *et al*., 2003).

Considering that GDNF has been largely described to be a neurotrophic, pro-survival growth factor for dopaminergic neurons (Airaksinen and Saarma, 2002 for review), we verified if GDNF affected α-syn (Ser129) phosphorylation before a noxious stimulus such as Fe exposure, known to cause oxidative stress in SH-SY5Y cells (Perfeito *et al*., 2010). As described previously, some authors have previously demonstrated that the levels of Ser129 phosphorylated α-syn are increased after treatment with ferrous iron, which causes cellular oxidative stress (Takahashi *et al*., 2007; Perfeito *et al*., 2010). Interestingly, we observed a significant increase in α-syn (Ser129) phosphorylation levels following Fe exposure comparing –Dox *vs* +Dox cells. We have also demonstrated a slight increase in Ser129 phosphorylated α-syn, by comparing control (untreated) cells with Fe-treated cells in –Dox condition. There are several evidences that may contribute to understand such finding. As described before in this thesis, elevated iron levels were observed in individual dopaminergic neurons of PD patients' brains as well as in the SN of PD models (Dexter *et al*., 1989; Good *et al*., 1992; Martin *et al*., 2008; Oakley *et al*., 2007; Riederer *et al*., 1989; Zecca *et al*., 2005). Elevated levels of iron were also found in the LBs of the parkinsonian SN (Hirsch *et al*., 1991). Neuropathological studies revealed that synucleinopathies are in general associated with increased levels of iron, which is in accordance with a pathological link between iron and α-syn (Duda *et al*., 2000). It is considered that oxidative stress contributes to the cascade of the neurodegeneration, resulting from enhanced levels of redox-active iron within the SN (Berg and Hochstrasser, 2006; Bush, 2000). An *in vitro* study showed that Fe could accelerate purified α-syn to aggregate rapidly (Golts *et al*., 2002). Exposure of cells to iron together with dopamine or hydrogen peroxide (other free radical generators), was able to induce α-syn aggregation (Ostrerova-Golts *et al*., 2000). Concordantly, Li and coworkers (2011) suggested that intracellular α-syn aggregation induced by Fe is partially dependent on oxidative stress. Increasing evidence suggest that phosphorylation may play a critical role in modulating α-syn aggregation, LBs formation and toxicity (Fujiwara *et al*., 2002 ;Anderson *et al*., 2006). Phosphorylated proteins, namely phosphorylated (Ser129) α-syn appear to be more prone to aggregation *in vitro* and *in vivo* (Fujiwara *et al*., 2002; Gorbatyuk, *et al*., 2008). As described before, it has been demonstrated that α-syn deposited in LBs is highly phosphorylated at Ser129 (Kahle *et al*., 2000; Okochi *et al*., 2000; Fujiwara *et al*., 2002; Anderson *et al*., 2006). Our group has recently shown that α-

syn phosphorylation at Ser129 is induced in a SH-SY5Y cell line by oxidative stress exerted by prolonged exposure (for 4 days) to Fe (Perfeito *et al*., 2010). Similar results were obtained by Takahashi and colleagues (2007).

Following pre-incubation of GDNF and exposure to Fe, we observed a reduction of α-syn (Ser129) phosphorylation, compared with Fe alone. These data suggest that GDNF is important to reduce α-syn phosphorylation induced by oxidative stress. As described before in this work, GDNF was shown to exhibit a protective role against oxidative stress in cultured neurons and glial cells (Iwata-Ichikawa *et al.*, 1999). Such findings seem to indicate that GDNF could reduce the potential oxidative stress induced by Fe and consequently reduce α-syn phosphorylation rate and potentially its aggregation. Thus, GDNF could protect cells from the potential toxic effect of Ser129 phosphorylated α-syn, although this was not proven in the present study. Unpublished data from our group (Perfeito *et al*.) showed that Fe^{2+}-induced α-syn (Ser129)phosphorylation occurred concomitantly with a slight but significant increase in propidium iodide labeling in SH-SY5Y cells, suggesting that some cells have undergone loss of plasma membrane integrity and thus necrotic cell death. These data suggest a correlation between Fe^{2+}-induced α-syn (Ser129) phosphorylation and cell death. Moreover, it was previously reported that SH-SY5Y neuroblastoma cells survived to an accumulation of iron, such survival response are related with decreased synthesis of DMT1 and increased synthesis of ferritin and ferroportin (Aguirre *et al*., 2005). Thus, cells can reduce the cytosolic content of Fe and avoid oxidative damage. Such finding along with the idea that GDNF exhibit a protective role on neurons against oxidative stress, suggests a possible preventive effect of GDNF against the noxious effects induced by Fe, i.e., herein defined by a rise in α-syn phosphorylation.

Phosphorylation of α-syn at Ser129 is characteristic of PD and related α-synucleinopathies. Unraveling the role of phosphorylation in modulating the physiological and pathogenic activities of α-syn requires identification of the kinases and phosphatases that may regulate its phosphorylation. Kinase inhibitors have been used for understanding the physiological roles of their targets. Thus, by using selective kinase inhibitors, we evaluated the role of some kinases in regulating α-syn phosphorylation.

In this study we used conditionally WT α-syn overexpressing cells under Fe exposure as an experimental model to study α-syn phosphorylation. A similar strategy was previously used by Takahashi and collaborators (2007). We focused on WT α-syn, because genomic duplication/triplication of WT α-syn was previously linked to familial forms of PD (Singleton *et al*., 2003). Moreover, environmental factors, including oxidative stress, are considered to play a critical role on PD pathogenesis (Sherer *et al*., 2002). α-Syn can undergo several post-translational modifications (Dev *et al*., 2003). Ser129 phosphorylation is thought to be one of

the most important events among such modifications (Fujiwara *et al*., 2002; Kahle *et al*., 2002; Takahashi *et al*., 2003; Anderson *et al*., 2006), because immunohistochemical and biochemical studies have revealed that almost 90% of α-syn in LB is phosphorylated at Ser129 (Fujiwara *et al*., 2002; Anderson *et al*., 2006) and that the Ser129 phosphorylation is closely associated with aggregate assembly in cellular models (Smith *et al*., 2005b). It was also suggested that α-syn phosphorylation at Ser129 plays a key role in α-syn-related cell death; one of the cell culture-based study was performed on α-syn-overexpressing SH-SY5Y cells (Sugeno *et al*., 2008). However other reports have suggested no link between α-syn phosphorylation at Ser129 and cell toxicity; instead the authors suggested that α-syn phosphorylation at Ser129 may protect nigral dopaminergic neurons from neurodegeneration. Studies performed in a rat model of PD, revealed that overexpression of α-syn mutant S129A (which cannot be phosphorylated) was toxic for dopaminergic neurons, overexpression of α-syn mutant S129D (which mimics constitutive phosphorylation) produced no obvious toxicity and overexpression of WT α-syn revealed an intermediate toxic effect (Gorbatyuk *et al*., 2008). Moreover, accumulation of A53T mutant α-syn phosphorylated on Ser129 was not associated with toxicity (Wakamatsu *et al*., 2007). These findings led us to speculate that if Fe-induced increased levels of Ser129 α-syn phosphorylation can be reduced by pre-incubation with inhibitors molecules, therefore the respective kinase might be involved in α-syn phosphorylation. Moreover, such kinase inhibition can even be protective for dopaminergic neurons, if we consider that Ser129 α-syn phosphorylation plays an important role in the fibrillogenesis, LB formation, and neurotoxicity, as already mentioned in this study. Thus, we evaluated the potential role of three different kinases (CK-1, CK-2 and LRRK2) on α-syn phosphorylation, by using selective inhibitor compounds, under non-toxic concentrations. *In vitro* and *in vivo* studies have shown that both soluble and fibrillized α-syn can be a substrate for CK-1, at Ser129 (Okochi *et al*., 2000; Waxman and Giasson, 2008). Inhibition of CK-1 with D4476 demonstrated no major reduction on α-syn phosphorylation. Indeed, a previous study indicated that inhibition of CK-1 (using D4476) had no effect on α-syn phosphorylation at Ser129 (Waxman and Giasson, 2011). Nevertheless, Okochi and colleagues (2000) reported that α-syn phosphorylation at Ser129 was reduced *in vivo* upon inhibition of CK-1; however such kinase was inhibited with a different molecule. Despite the fact that some authors recommend the use of D4476 to inhibit CK-1 isoforms in cell-based experiments (Bain *et al*., 2007), these findings seems to indicate that D4476 does not significantly inhibit CK-1 in our PD cell model.

So far, there is only one published *in vitro* study demonstrating that both WT and G2019S mutant LRRK2 and its kinase domain-containing fragments have a significant capacity to phosphorylate α-syn at Ser129 (Qing *et al*., 2009a). However, other studies in cell cultures and animal models, in which WT or PD related G2019S LRRK2 was overexpressed, failed to

identify any increase on Ser129 phosphorylated α-syn (Liu *et al*., 2012). Our results show a tendency for a slight reduction of α-syn phosphorylation at this specific residue after LRRK2 inhibition. Because the ability of LRRK2 to phosphorylate α-syn is still quite controversial, more studies will be required to clarify the role of LRRK2 on phosphorylation of α-syn, and to elucidate the efficacy of LRRK2-IN-1 in cell culture-based experiments.

In vitro and *in vivo* studies have shown that both soluble and fibrillized α-syn can be a substrate for CK-2 at Ser129 (Okochi *et al*., 2000; Pronin *et al*., 2000; Takahashi *et al*., 2007; Waxman and Giasson, 2008; Qing *et al*., 2009a). Studies in cellular models demonstrated that activation or overexpression of CK-2 can strongly increase the phosphorylation of α-syn at Ser129 (Waxman and Giasson, 2008). Moreover, treatment with selective inhibitors of CK-2 significantly reduced Ser129 α-syn phosphorylation (Okochi *et al*., 2000; Takahashi *et al*., 2007; Sugeno *et al*., 2008; Waxman and Giasson, 2008; Waxman and Giasson, 2011). Takahashi and coworkers (2007) reported that cells exposed to $FeCl_2$ promoted accumulation of Ser129 phosphorylated α-syn and CK-2 protein. They postulated that α-syn phosphorylation caused by $FeCl_2$ was due to CK-2 upregulation. According to these authors, $FeCl_2$ exposure evokes ROS production and consequently oxidative stress, which in turn upregulates CK-2 that increases Ser129 α-syn phosphorylation. Altogether, previous published data suggest that CK-2 is a kinase important for α-syn phosphorylation induced by oxidative stress (Smith *et al*., 2005; Takahashi *et al*., 2007). Importantly, our results are in agreement with these findings indicating that CK-2 plays a crucial role in α-syn phosphorylation caused by Fe exposure. We demonstrated that CX-4945 inhibited CK-2-mediated phosphorylation of α-syn. Indeed, among the three kinase inhibitors tested to evaluate the role of their targets on α-syn phosphorylation, we believe that our results allow to conclude that CK-2 is the most effective kinase on α-syn phosphorylation at Ser129 in SH-SY5Y cells subjected to the noxious Fe stimulus, since inhibition of this kinase led to a more pronounced decrease in α-syn phosphorylation levels. This view is supported by the results from previous studies (Okochi *et al*., 2000; Smith *et al*., 2005; Ishii *et al*., 2007; Takahashi *et al*., 2007). Moreover, as described previously, our results demonstrate that α-syn phosphorylation was increased in –Dox condition cultures with Fe stimulation. Such results might be explained by α-syn overexpression and oxidative stress triggered by Fe, that led to an increase in activity/levels of CK-2 protein (Takahashi *et al*., 2007). Fe incubation did not increase CK-2 mRNA levels, thus its influence on CK-2 is likely due to suppression of protein degradation (Takahashi *et al*., 2007).

Considering this part of the study, more studies will be required to perceive what is the role of CK-2 and other potential kinases in the context of α-syn (Ser129) phosphorylation and in PD pathogenesis, providing valuable insights for the development of novel PD therapies.

CHAPTER 5 – CONCLUSIONS AND FUTURE STUDIES

In conclusion, this work allowed us to better understand the potential kinases involved in α-syn (Ser129) phosphorylation state as well as the possible protective role of GDNF as an important factor able to reduce α-syn phosphorylation induced by oxidative stress in the context of PD. Studies were performed in human SH-SY5Y neuroblastoma cell line overexpressing α-syn in a Tet-Off regulated manner (in the absence of Dox), allowing the study of α-syn pathogenesis in PD. We defined nanomolar non-toxic concentrations of kinase inhibitors, which were used in this work to modulate oxidative stress-induced α-syn (Ser129) phosphorylation. Indeed, high concentrations were toxic and were linked to cell cycle arrest and apoptosis in a large number of cells. We showed that Fe (an oxidative stress inducer) exposure in –Dox cells can provoke an increase in α-syn phosphorylation at Ser129. Moreover, under non-stressed conditions (in the absence of Fe), GDNF can also induce α-syn (Ser129) phosphorylation. However, in the presence of Fe, this neurotrophic factor appears to contribute for the prevention of neurotoxicity, by both reducing α-syn phosphorylation imposed by Fe and possibly reducing oxidative stress. The effect of GDNF in cells overexpressing α-syn subjected to oxidative stress appears to occur through the early activation of PI3K/Akt signaling pathways, most probably by activation of the RET protein. Moreover, we showed that LRRK2 and CK-2 inhibitors can reduce the levels of Seri129 phosphorylated α-syn. Nevertheless, CK-2 seems to be the major and mostly accepted kinase able to promote the transfer of a phosphate group to Ser129 of α-syn. By having CK-2 as the most promising therapeutic target and GDNF as a possible neuroprotective agent against increased α-syn phosphorylation induced by oxidative stress, this study took a step forward in the search for new therapeutic targets and control of PD cellular pathology.

There are some key aspects that should be taking into consideration in future studies that may either reinforce our data or achieve new complementary findings. On the one hand, and taking into account both the toxicity of the inhibitors tested and the relevant role of kinase pathways, it is important to prove that some effects were indeed due to a selective kinases; in this regard, the role of certain kinases can be addressed through siRNAs. Moreover, it would be important to determine the role of such kinases on α-syn phosphorylation at Ser87, another potential regulated residue. Indeed, it would be interesting to explore whether Ser87 and/or Ser129 phosphorylated α-syn have implications in the formation of LB-like α-syn aggregates and toxicity in cells overexpressing α-syn, as model of PD pathogenesis. On the other hand, in order to prove if GDNF exerts protective effect upon α-syn phosphorylation, transfected cells for RET and GFRα1 receptors might be considered. In addition, analysis of activation of Erk signaling pathways and test of different concentrations of GDNF are also important, since GDNF effect is dose and cell type-dependent. Different Fe concentrations and exposure to different oxidative stress stimuli, such as hydrogen peroxide or mitochondrial inhibitors (e.g.

rotenone, a selective inhibitor of complex I) should be also considered. These experiments might be carried out in differentiated SH-SY5Y cells overexpressing α-syn and cells expressing mutant forms of α-syn (e.g. A53T) to mimic other PD familial forms. Last but not least, in future studies modulation of LRRK2 or CK-2 activity or expression (using selective inhibitors and/or siRNAs) might be tested in *in vivo* PD models (e.g. PD transgenic mice or mice treated with MPTP), in order to validate their beneficial effects.

CHAPTER 6 – BIBLIOGRAPHY

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