Sara Patrícia Ferreira Moreira

Licenciada em Biologia

Nrf2 activation by TUDCA in experimental models of Parkinson's disease

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientador: Margarida Castro-Caldas, PhD
Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa

Co-orientador: Maria João Gama, PhD
Faculdade de Farmácia, Universidade de Lisboa

Presidente: Prof. Doutora Ilda Sanches
Arguente: Doutora Sara Xapellí
Vogal: Prof. Doutora Margarida Castro-Caldas

FACULDADE DE CIÊNCIAS E TECNOLOGIA
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To my family

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ABSTRACT

Parkinson's disease (PD) is a progressive neurological disorder, mainly characterized by the loss of dopaminergic neurons in the substantia nigra *pars compacta*. Although the cause of PD remains elusive, several lines of evidence implicate mitochondrial dysfunction and oxidative stress as possible mechanisms by which cell death occurs in this disease.

Under oxidative stress, the master regulator of cellular redox status, nuclear factor erythroid 2 related factor 2 (Nrf2), is responsible for activating the transcription of several cytoprotective enzymes, namely glutathione peroxidase 1 (Gpx1), heme oxygenase-1 (HO-1) and superoxide dismutase 2 (SOD2), being a promising target to limit reactive oxygen species (ROS)-mediated damage in PD.

In this work, we aim to evaluate the ability of tauroursodeoxycholic acid (TUDCA) to modulate, not only the Nrf2 pathway and the expression of the Nrf2 stabilizer, DJ-1, but also the cellular redox status, in both animal and cellular models of PD, using twelve-week-old C57BL/6 male mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and the human neuroblastoma cell line, SH-SY5Y, treated with 1-methyl-4-phenylpyridinium (MPP^+).

Our Western blot results, together with quantitative real time polymerase chain reaction, demonstrate that TUDCA treatment increases DJ-1, Nrf2, Gpx1, HO-1 and SOD2 expression, in mice striatum and midbrain. Moreover, enzymatic assays also reveal that TUDCA treatment enhances Gpx biological activity, in mice. In SH-SY5Y cells, we demonstrate by immunocytochemistry that TUDCA induces Nrf2 nuclear translocation, with the consequent increase in HO-1 mRNA levels. Additionally, TUDCA also attenuates both MPP^+-induced ROS production and lipid peroxidation, in this cell line.

Together, our results suggest that TUDCA is a promising agent to limit ROS-mediated damage, in different models of PD acting, at least in part, through modulation of the Nrf2 signaling pathway, and therefore, should be considered a promising therapeutic agent to be implemented in PD.

Keywords: Nrf2 signaling pathway, DJ-1, ROS production, lipid peroxidation, MPTP/MPP^+, beneficial effects of TUDCA

RESUMO

A doença de Parkinson (DP) é uma doença neurodegenerativa progressiva, caracterizada principalmente pela perda de neurónios dopaminérgicos na *substantia nigra pars compacta* (SNpc). Apesar da causa da DP permanecer indefinida, várias evidências implicam a disfunção mitocondrial e o *stress* oxidativo, como possíveis mecanismos responsáveis pela morte celular nesta doença.

Sob *stress* oxidativo, o regulador do estado redox celular, *nuclear factor erythroid 2 related factor 2* (Nrf2), é responsável por ativar a transcrição de diversas enzimas protetoras, como a glutationa peroxidase 1 (Gpx1), a heme oxigenase-1 (HO-1) e a superóxido dismutase 2 (SOD2), sendo um alvo promissor na limitação dos danos mediados pelas espécies reativas de oxigénio (ROS) na DP.

Assim, o nosso objetivo é avaliar a capacidade do ácido tauroursodesoxicólico (TUDCA), tanto na modulação da via do Nrf2 e na expressão da DJ-1, como no estado redox celular, em modelos animais e celulares da DP, utilizando murganhos macho C57BL/6 com 12 semanas tratados com 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP), e a linha celular SH-SY5Y tratada com 1-metil-4-fenilpiridina (MPP^+).

Os resultados obtidos por *Western blot*, juntamente com a análise por reação em cadeia da polimerase por método quantitativo (qRT-PCR) demonstram que o tratamento com TUDCA aumenta a expressão da DJ-1, Nrf2, Gpx1, HO-1 e SOD2, tanto no estriado como no *midrain*. Além disso, ensaios enzimáticos também revelam que o tratamento com TUDCA aumenta a atividade biológica da Gpx nos murganhos. Nas células SH-SY5Y demonstramos, por imunocitoquímica, que o TUDCA induz a translocação nuclear do Nrf2, com o consequente aumento nos níveis de mRNA da HO-1. Adicionalmente, o TUDCA atenua a produção das ROS e a peroxidação lipídica, induzidas pelo MPP^+, nesta linha celular.

Estes resultados sugerem que o TUDCA é um agente promissor na limitação dos danos induzidos pelas ROS em diferentes modelos da DP, atuando em parte, através da modulação da via do Nrf2 e, por isso, poderá ser considerado um agente terapêutico promissor a ser implementado na DP.

Palavras-chave: Via de sinalização do Nrf2, DJ-1, produção de ROS, peroxidação lipídica, MPTP/MPP^+, efeitos benéficos do TUDCA

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ABBREVIATIONS

6-OHDA	6-hydroxydopamine
ADP	Adenosine diphosphate
ARE	Antioxidant response element
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BSA	Bovine serum albumin
BTB	Broad complex, tramtrack, bric-a-brac domain
cDNA	Complementary DNA
CNS	Central nervous system
Cys	Cysteine
DA	Dopamine
DAT	Dopamine transporter
DCF-DA	2', 7'-dichlorofluorescein diacetate
DGR	Double glycine repeat domain
DNA	Deoxyribonucleic acid
ETC	Electron transport chain
FAD	Flavin adenine dinucleotide
$FADH_2$	Flavin adenine dinucleotide (reduced form)
FBS	Fetal bovine serum
Gpx	Glutathione peroxidase
GSH	Glutathione
GUDCA	Glycoursodeoxycholic acid
H_2O_2	Hydrogen peroxide
HNE	4-hydroxyl-2-nonenal
HO	Heme oxygenase
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
i.p	Intra-peritoneally
IVR	Intervening region
JNK	c-Jun N-terminal kinase
Keap1	Kelch-like ECH associated protein 1
LAA	Linoleamide alkyne

LB	Lewy bodies
LRRK2	Leucine-rich repeat kinase 2
Maf	Musculo-aponeurotic fibrosarcoma
MAO-B	Monoamine oxidase B
MEM	Minimum essential medium
$MPDP^+$	1-methyl-4-phenyl-2,3-dihydropyridium
MPP^+	1-methyl-4-phenylpyridinium
MPPP	1-methyl-4-phenyl-4-propionpiperidine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate
NEAA	Non-essential amino acids
Neh	Nrf2-ECH homologies
NQOs	NADPH: quinone oxireductases
Nrf2	Nuclear factor erythroid 2 related factor 2
O_2^-	Superoxide anion
PARK7	Parkinson protein 7
PBS	Phosphate buffered saline
PD	Parkinson's disease
PFA	Paraformaldehyde
PINK1	Phosphatase and tensin (PTEN)-induced putative kinase1
POLG1	Polymerase γ 1
Prx	Peroxiredoxins
PVDF	Polyvinyl difluoride
qRT-PCR	Quantitative real time polymerase chain reaction
Rbx1	Ring-box protein 1
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate

SDS-PAGE	Sodium dodecyl sulphate-polyacrilamide gel electrophoresis
SNpc	Substantia nigra *pars compacta*
SOD	Superoxide dismutase
TBS-T	Tris-buffered saline-Tween 20
TFAM	Mitochondrial transcriptional factor A
Trx	Thioredoxins
TUDCA	Tauroursodeoxycholic acid
UDCA	Ursodeoxycholic acid
VMAT	Vesicular monoamine transporter

I. Introduction

1. Parkinson's disease

Parkinson's disease (PD), is the second most common neurodegenerative disorder, after Alzheimer's disease, and it was first reported by James Parkinson, in 1817 (Parkinson, 1817). PD is a severe progressive neurological disorder characterized, not only by the loss of dopaminergic neurons in the substantia nigra *pars compacta* (SNpc) and the consequent depletion of the neurotransmitter dopamine (DA) in the striatum, but also by the presence of intracytoplasmic inclusions of aggregated proteins designated by Lewy bodies (LB), formed mainly by α-synuclein and ubiquitin (Przedborski, 2005; Nagatsu and Sawada, 2006; Thomas and Beal, 2007).

Although the cause of PD remains elusive, several lines of evidence implicate that dopaminergic cell loss is associated with different mechanisms of cell damage, all of which may be interconnected. In fact, mitochondrial dysfunction and the consequent oxidative stress, inflammation, protein aggregation, excitotoxicity, impairment of both ubiquitin system and calcium homeostasis, and finally apoptosis have all been reported in PD patients' brains (Jenner, 1999; Lev *et al*., 2003; Keane *et al.*, 2011).

Despite the intensive research in PD field, it still remains unclear whether the disease results from either environmental factors, genetic causes or a combination of both. Therefore, PD is considered to have a multifactorial etiology, including genetic factors (designated by familial PD), in about 5% of PD cases, and environmental factors (referred to as sporadic PD), in the remaining 95% of PD cases (Kurth and Kurth, 1999; Lev *et al*., 2003; Martins *et al*., 2013). The familial form of PD comprises the autosomal dominant forms, involving possibly gain-of-function mutations in *α-synuclein* and *leucine-rich repeat kinase 2* (LRRK2) genes, and the autosomal recessive forms, involving presumably loss-of-function mutations in *parkin*, *phosphatase and tensin* (PTEN)-*induced putative kinase 1* (PINK1) and *DJ-1* genes (Krüger, 2004; Lesage and Brice, 2012). In turn, the sporadic form of PD assumes that the progressive nigral cell loss, characteristic of the disease, results from either chronic or limited exposure to environmental dopaminergic neurotoxins (Dauer and Przedborski, 2003). Remarkably, in the last few

years, sporadic PD has gained a large genetic influence in its etiopathogenesis (Moon and Paek, 2015). For instance, numerous studies have demonstrated that several polymorphisms are responsible for conferring increased susceptibility to sporadic PD (Vilar *et al*., 2007*;* Lesage and Brice, 2012; De Rosa *et al*., 2015). Therefore, all of those factors account for the multifactorial etiology of this complex disease.

1.1. Clinical and neuropathological features of PD

The prevalence of PD is approximately 0.3% of the entire population, affecting about 8 to 18 out of 100.000 people per year (Massano and Bahtia, 2012). PD affects more than 1% of people older than 60 years, and since PD incidence increases with age, it is estimated an increase of 3% in the population over 80 years (Massano and Bahtia, 2012; Andalib *et al*., 2014). In addition, the mean age of onset of the disease is roughly 60 years; however in about 10% of PD cases, the onset occurs earlier, between 20 and 50 years of age, being classified as young onset (Dexter and Jenner, 2013). Interestingly, Schrag and collaborators (2000), among others, demonstrated that PD affects more men than women, probably due to the protective effects of estrogen (Dluzen, 2000).

The main neuropathological feature of PD is the loss of midbrain dopaminergic neurons within SNpc. These dopaminergic neurons, whose cell bodies are located in SNpc, send projections to the caudate and putamen nucleus, in the striatum, creating the nigrostriatal pathway **(**Figure I.1**)**, which is essential for a normal motor function and voluntary movement control (Speciale, 2002; Martins *et al.*, 2013). Therefore, the loss of these nigral neurons, normally enriched in neuromelanin, results in complex changes in the brain's motor system and also in a profound striatal dopamine loss and SNpc depigmentation (Dauer and Przedborski, 2003; Smeyne and Jackson-Lewis, 2005) **(**Figure I.1 – B**)**. Together, all these alterations cause the motor deficits characteristics of PD.

Unfortunately, it is well known, that relevant clinical symptoms do not fully develop until there is a loss of about 60% of SNpc cells and 70% of dopamine response (Smeyne and Jackson-Lewis, 2005). Thus, the main symptoms of the disease can be divided in two categories: i) the motor symptoms, including tremor which occurs at rest but decreases with voluntary movement, rigidity (stiffness), slowness of movement known as bradykinesia, and postural instability; and ii) the non-motor symptoms, including depression and cognitive decline (Dickson, 2012; Massano and Bahtia, 2012; Dexter and Jenner, 2013). Other important motor and non-motor symptoms are described in Table I.1.

Since PD is characterized by numerous symptoms that are, not only linked to different stages of its progression, but are also common to other movement disorders, the identification of this pathology at an early stage is very difficult (Gaki and Papavassiliou, 2014). Therefore, it is important to emphasize that despite the diagnosis of PD is made on clinical grounds, the definite diagnosis requires the identification of both nigral neurons loss and LB formation that cannot be confirmed with any of the

available tests, during the patient's life. Therefore, the final diagnosis of this disease is only confirmed through brain autopsy (Massano and Bahtia, 2012).

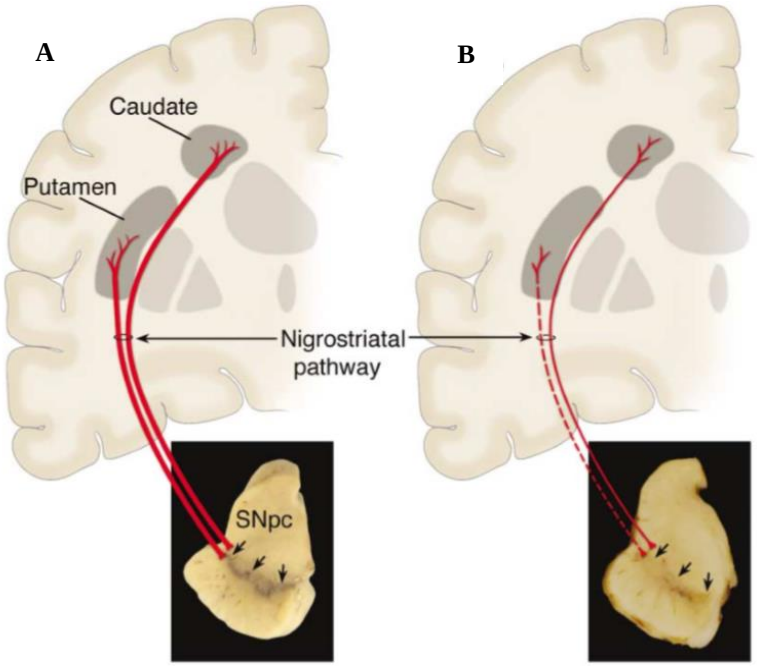


Figure I.1 – Schematic representation of Parkinson's disease neuropathology. A – *Normal nigrostriatal pathway*. This nigrostriatal pathway is composed of midbrain dopaminergic neurons, whose cell bodies are located in substantia nigra *pars compacta* (SNpc) (indicated by the arrows). These neurons send projections, represented by the thick red lines, to the striatum (caudate and putamen nucleus). In the picture it is evident the normal pigmentation of SNpc, produced by neuromelanin within the dopaminergic neurons. **B** – *Diseased nigrostriatal pathway*. The degeneration of the nigrostriatal pathway is marked by a sharp loss of the dopaminergic neurons that project to the striatum, represented by the dashed thin red line. It is also possible to see the characteristic depigmentation, caused by the loss of the dark-brown pigment, neuromelanin, in the SNpc. *In Dauer and Przedborski (2003).*

Table I.1 – Clinical features of Parkinson's disease. *Sources: Dauer and Przedborski, Dickson, 2012; Massano and Bahtia, 2012; Dexter and Jenner, 2013*

Motor Symptoms	Non-motor Symptoms
Resting tremors	Depression
Postural instability	
Drooling	
Rigidity (increased resistance to passive movement)	Cognitive decline
Bradykinesia (slowness of movement)	
Hypokinesia (reduction in movement amplitude)	
Akinesia (absence of normal unconscious movements)	Sleep disturbance
Hypomimia (absence of normal facial expression)	
Hypophonia (decreased voice volume)	
Micrographia (decreased size of handwriting)	Dementia
Decreased speed of handwriting	

1.2. Mitochondrial dysfunction and oxidative stress in PD

Mitochondria are considered unique and important cellular organelles, since they have their own deoxyribonucleic acid (DNA), and function to produce cellular energy, in the form of adenosine triphosphate (ATP), through a mechanism called oxidative phosphorylation (Keane *et al.*, 2011). Besides their main function as energy producers, mitochondria also play important roles in the regulation of cell death via apoptosis, and are implicated in the control of cell growth and division (Keane *et al.*, 2011; Moon and Paek, 2015).

Mitochondrial DNA (mtDNA) is also known by its increased vulnerability to damages, probably due to a less efficient DNA repair mechanism, and also due to an absence of a protective histone coating (Schapira *et al*., 1990; Schapira, 1994; Winklhofer and Haass, 2010). Since mtDNA is located close to the electron transport chain (ETC) it is particularly susceptible to suffer damage from free radicals produced during oxidative phosphorylation (Keane *et al.*, 2011). Protein mutations, caused by oxidative stress, lead to respiratory chain dysfunctions and/or alterations on the processes of mitochondrial replication, transcription or translation (Finsterer, 2006). Importantly, several mutations in mtDNA have been linked with PD, namely in the mitochondrial transcriptional factor A (TFAM), a regulator of mtDNA transcription, in the mitochondrial DNA polymerase γ 1 (POLG1), an enzyme involved in the synthesis of mtDNA, and in genes that encode for proteins that constitute the ETC (Ekstrand *et al*., 2007; Keane *et al*., 2011; Moon and Paek, 2015).

To accomplish the generation of cellular energy, the transport of electrons from reduced nicotinamide adenine dinucleotide (NADH) or reduced flavin adenine dinucleotide ($FADH_2$) oxidation, are passed along the complexes of ETC, located in the inner mitochondrial membrane, until they reduce oxygen to water at complex IV (Winklhofer and Haass, 2010). This transport of electrons, generates proton movement from the mitochondrial matrix to the intermembrane space, creating an electrochemical gradient resulting in ATP production from adenosine diphosphate (ADP), through the ATP synthase (complex V) (Winklhofer and Haass, 2010; Keane *et al.*, 2011) (Figure I.2).

During the process of oxidative phosphorylation, electrons can leak from the ETC, specifically from complex I and III, and react with molecular oxygen to form reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and nitric oxide that can produce oxidative damage by reacting with DNA, lipids and proteins (Kirkinezos and Moraes, 2001). Under normal physiological conditions, the cell is endowed with a free radical scavenging system, capable of clearing ROS from mitochondria, preventing the damage of cellular and mitochondrial structures (Betarbet *et al*., 2002). These scavenging systems include the antioxidant, glutathione (GSH), and the enzymes glutathione peroxidase (Gpx) and superoxide dismutase (SOD) (Betarbet *et al*., 2002).

One point that has relevance to be addressed is the fact that the brain is considered the organ that is more vulnerable to oxidative stress and oxidative damage (Gaki and Papavassiliou, 2014). For example, on one hand the brain consumes more oxygen than any other organ, on the other hand, the

brain contains a relatively low level of antioxidant enzymes, compared to other tissues, as well as high amounts of phospholipids, which are vulnerable to oxidative changes (Dias *et al*., 2013; Gaki and Papavassiliou, 2014). Therefore, mitochondrial dysfunction, characterized by mitochondrial complex I impairment, together with the consequent rise in the leakage of electrons from the ETC, results in an increase in ROS generation, which may overwhelm the endogenous antioxidant mechanisms of the cell (Betarbet *et* al., 2002; Moon and Paek, 2015). Importantly, it has been well documented that several oxidative stress-related changes have been detected in PD patients' brains (Jenner, 1998). Specifically, different *postmortem* analysis revealed decreased GSH levels in SNpc (Perry *et al*., 1982; Perry and Yong, 1986), increased levels of byproducts of lipid peroxidation, like 4-hydroxyl-2-nonenal (HNE), as described by Yoritaka and colleagues (1996), carbonyl modifications of soluble proteins, described by Floor and Wetzel (1998), and also DNA and ribonucleic acid (RNA) oxidation products (Alam *et al*., 1997; Zhang *et al*., 1999).

In addition, the characteristic neuronal cell death in PD may occur through severe oxidative mtDNA damage, protein oxidation and lipid peroxidation, and also through redox signaling pathways perturbation, due to an increase in mitochondrial ROS formation and/or defective ROS scavengers (Winklhofer and Haass, 2010; Keane *et al*., 2011). Although the exact mechanisms leading to neuronal death in PD remain elusive, it is believed that besides oxidative stress, apoptosis, p53, c-Jun N-terminal kinase (JNK), as well as inflammation contribute to the process (Lev *et al*., 2003; Winklhofer and Haass, 2010; Castro-Caldas *et al*., 2012*a*).

Finally, and despite the importance of an imbalanced ROS production and antioxidant levels in the brain, as possible causes of oxidative stress in PD, there are other significant causes, with diminished relevance for this thesis, such as: i) oxygen metabolism in the brain; ii) DA synthesis and dopaminergic neurons; iii) metal concentration; iv) calcium influx and v) gene mutation (Gaki and Papavassiliou, 2014).

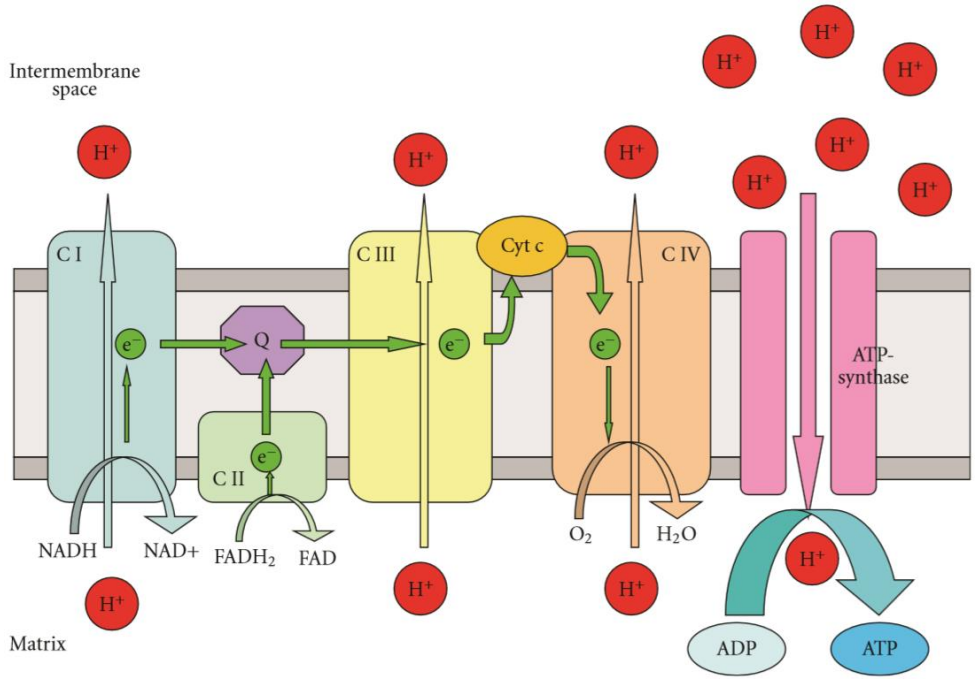


Figure I.2 – Simplified scheme of the electron transport chain. The electrons (e^-) generated by the conversion of NADH to NAD^+ (C I) or $FADH_2$ to FAD (C II), are passed through ubiquinone (Q), complex III (C III), cytochrome c (Cyt c) and complex IV (C IV), where the electrons are used to reduce oxygen (O_2) to water (H_2O). This electron transportation along the ETC, generates proton movement (H^+) creating an electrochemical gradient that culminates in ATP formation from ADP, by ATP-synthase. ADP – Adenosine diphosphate; ATP – Adenosine triphosphate; C I – Complex I; C II – Complex II; ETC – Electron transport chain; FAD – Flavin adenine dinucleotide; $FADH_2$ – Reduced flavin adenine dinucleotide; NAD^+ – Nicotinamide adenine dinucleotide; NADH – Reduced nicotinamide adenine dinucleotide. *In Keane et al. (2011).*

1.2.1. Role of DJ-1 in oxidative stress

DJ-1, a small homodimeric protein, ubiquitously expressed and encoded by *Parkinson protein 7* (PARK7) gene, was initially identified as an oncogene, by Nagakubo and co-workers (1997), with crucial roles in cancer and male infertility (Thomas and Beal, 2007). Loss of function mutations in DJ-1 lead to autosomal recessive early-onset of familial PD, although the underlying mechanisms remain unknown (Bonifati *et al*., 2003). Importantly, several studies involving mouse models lacking DJ-1 demonstrated that the absence of this protein results in age-dependent motor deficits, hypokinesia, dopaminergic dysfunction and increased susceptibility to oxidative insults (Chen *et al*., 2005; Goldberg *et al*., 2005; Kim *et al*., 2005), revealing the neuroprotective role of DJ-1 in PD. Moreover, DJ-1 is considered a multifunctional protein that encompasses functions, such as chaperone, antioxidant, autophagy modulator, and transcriptional regulator (Im *et al*., 2012; Milani *et al*., 2013).

Under normal physiological conditions, DJ-1 has a predominantly cytoplasmic localization, however, under oxidative stress situations, this protein can be recruited either to the mitochondria or to the nucleus, where it functions as a ROS scavenger, by undergoing self-oxidation, oxidizing in particular

its cysteine residue 106 (Canet-Avilés *et al*., 2004; Milani *et al*., 2013). Notably, it was showed by Choi and colleagues (2006) that this oxidized form of DJ-1 is present in sporadic PD patients' brains. Additionally, it is well documented that the overexpression of this protein allows cell protection, against oxidative stress, induced either by H_2O_2 or neurotoxin-based models of PD (Im *et al*., 2012), which will be further described in this thesis.

Summarizing, DJ-1 acts as a sensor of cellular redox status and therefore, it is considered a signaling molecule that responds to oxidative stress. Taken together the previous information, it is possible to conclude that DJ-1 has an essential role in cell protection, preventing ROS-mediated damage.

2. The Nrf2-Keap1 signaling pathway

To maintain a proper physiological redox balance, cells are endowed with a wide variety of endogenous antioxidant enzymes to lessen the levels of ROS production and the consequent oxidative stress (de Vries *et al*., 2008; Tufekci *et al*., 2011). Crucially, the expression of several of these cytoprotective enzymes is activated, upon ROS exposure, by the transcription factor, nuclear factor erythroid 2 related factor 2 (Nrf2), the master regulator of cellular redox status (de Vries *et al*., 2008).

Nrf2 belongs to the basic leucine zipper transcription factor family, which is characterized by the requirement of a heterodimeric formation, with small musculo-aponeurotic fibrosarcoma (Maf) proteins, for DNA binding, as described by Itoh and collaborators (1997). Structurally, Nrf2 is formed by six functional domains, each one of them with specific functions, designated by Nrf2-ECH homologies (Neh1-6) (Tong *et al*., 2006).

Kelch-like ECH associated protein 1 (Keap1), in turn, is constituted by three functional domains, well documented by Tong and co-workers (2006), designated by Broad complex, Tramtrack, and Bric-a-brac (BTB) domain, an intervening region (IVR) and a Kelch domain, also designated by double glycine repeat (DGR) domain. Keap1 is an endogenous negative regulator of the Nrf2 pathway by forming a Keap1/Nrf2 complex, holding the transcription factor in the cytosol, with actin filaments, thus preventing it to function as a transcription factor in the nucleus (Tufeckci *et al*., 2011; Williamson *et al*., 2012). To form this cytoplasmic complex, Keap1 has to form a homodimer, wherein each dimer binds one molecule of Nrf2, in the DLG and ETGE motifs, via its two DGR domains (Tong *et al*., 2007). In addition, Nrf2 regulation involves interactions between the conserved motifs DLG (week affinity for Keap1) and ETGE (high affinity for Keap1), within the Neh2 domain (responsible for cellular stress response regulation), and also the DGR domain on Keap1 (Tufekci *et al*., 2011).

Under normal physiological conditions, the cytoplasmic Keap1/Nrf2 complex is connected, by the BTB domain in Keap1, to a functional E3 ubiquitin ligase complex (Ring-box protein 1 – Rbx1) through an adaptor protein, designated by Cullin3 (Zhang *et al*., 2004; Zhang *et al*., 2013). This E3 ubiquitin

ligase complex is responsible for the poly-ubiquitination and the consequent rapid degradation of Nrf2, by the 26S proteasome (Zhang *et al*., 2004; Williamson *et al*., 2012).

On the other hand, under oxidative stress situations, the high reactive cysteine residues of Keap1 (Cys273 and Cys288 in the IVR domain; Cys151 in the BTB domain) are oxidized, affecting Keap1 conformation and causing its dissociation from the DLG motif of Nrf2, preventing its degradation (Zhang and Hannink, 2003; Yamamoto *et al*., 2008; Williamson *et al*., 2012). These events allow Nrf2 stabilization and subsequent translocation into the nucleus, where Nrf2 forms a heterodimer with a Maf protein, through its Neh1 domain and subsequently binds the antioxidant response element (ARE), located in the promoter or enhancer regions of antioxidant and cytoprotective genes, activating their expression (Williamson *et al*., 2012; Zhang *et al*., 2013). The schematic representation of Nrf2 regulation by Keap1, under normal conditions and oxidative stress, is illustrated in Figure I.3.

It is noteworthy that once the cellular redox homeostasis is restored, Nrf2 is transported out of the nucleus to the cytoplasm, where it is poly-ubiquitinated and subsequently degraded (de Vries *et al*., 2008).

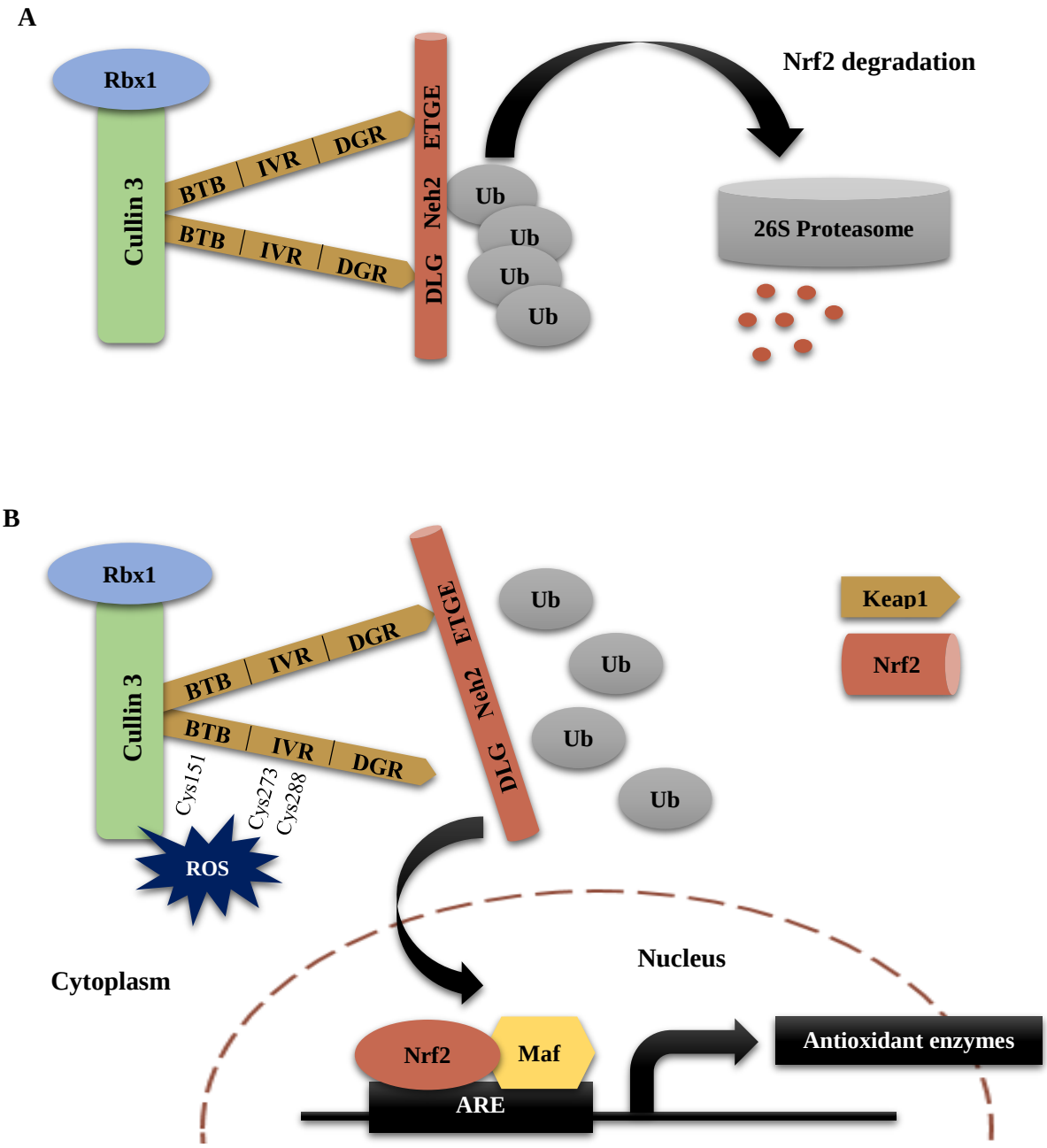


Figure I.3 – Illustrative representation of Nrf2 regulation by Keap1. Under normal physiological conditions (A), Keap1 forms a dimer that binds to Nrf2, in the DLG and ETGE motifs, through its two DGR domains. The formation of Keap1/Nrf2 complex, retains Nrf2 in the cytoplasm preventing its translocation to the nucleus and therefore the activation of antioxidant enzymes. In addition, since Nrf2 is not activated, the complex Keap1/Nrf2 binds to Rbx1, through the adaptor protein Cullin3. The formation of the complex Rbx1/Cullin3/Keap1/Nrf2 permits Nrf2 ubiquitination and proteasomal degradation. Under oxidative stress situations (B), cysteine residues of Keap1 are oxidized, upon ROS exposure, allowing the dissociation of Nrf2 from the complex Rbx1/Cullin3/Keap1. This dissociation allows Nrf2 stabilization and nuclear translocation, where it binds to Maf protein and subsequently to ARE, initiating the transcription of antioxidant and cytoprotective genes. ARE – Antioxidant response element; BTB – Broad complex, tramtrack, and bric-a-brac domain; Cys – Cysteine; DGR – Double glycine repeat domain; IVR – Intervening region; Keap1 – Kelch-like ECH associated protein 1; Maf – Musculo-aponeurotic fibrosarcoma; Neh2 – Nrf2-ECH homology 2; Nrf2 – Nuclear factor erythroid 2 related factor 2; Rbx1 – Ring-box protein 1; ROS – Reactive oxygen species; Ub – Ubiquitin. *Adapted from Zhang et al. (2013).*

2.1. Role of DJ-1 in Nrf2 regulation

As previously described in section *"Role of DJ-1 in oxidative stress"*, the redox-sensitive protein, DJ-1, is responsible for the activation of antioxidant defenses, upon exposure to oxidative stress. Interestingly, it was recently demonstrated that the antioxidant response of DJ-1 may also result from the activation of the Nrf2 pathway (Im *et al*., 2012). The evidence of the existence of this link between DJ-1 and Nrf2 emerged from studies involving primary cell lines, from both human and mouse species, and also DJ-1-deficient patients. Studies regarding primary cell lines demonstrated that knockdown and knockout of DJ-1 caused a decrease in Nrf2 expression and stability, respectively, along with an increase of Nrf2 degradation and consequently, a decrease in the expression of downstream antioxidant enzymes (Clements *et al*., 2006; Im *et al*., 2012). Another study carried out by Gan and colleagues (2010) demonstrated that messenger RNA (mRNA) levels of Nrf2 were decreased in DJ-1 knockout mice, when compared with wild-type mice. On the other hand, studies involving DJ-1 overexpression demonstrated a significant increase in Nrf2 stabilization and subsequently, diminished Nrf2 ubiquitination (Clements *et al*., 2006). Moreover, DJ-1-deficient patients showed diminished expression of the cytoprotective genes, accompanied by an increase in the oxidative stress levels (Zhang *et al*., 2013).

Together, these studies suggest that DJ-1 stabilizes Nrf2, either by disrupting Keap1/Nrf2 complex and/or by preventing its interaction with Keap1, thus reducing Nrf2 ubiquitination and consequent degradation.

2.2. Involvement of Nrf2 dysregulation in the pathogenesis of PD

In the last few years, several studies, including *postmortem* studies from PD patients' brains, and studies involving toxin-based animal models, have implicated the involvement of Nrf2 dysregulation in the pathogenesis of PD (Tufekci *et al*., 2011). In fact, studies regarding *postmortem* data from PD patients' brains revealed that in the nucleus of SNpc neurons, Nrf2, as well as its downstream targets levels are enhanced, suggesting an increased activation of this transcription factor (Ramsey *et al*., 2007; Wang *et al*., 2014). Importantly, these increments observed may be a compensatory response of the cell to increase the levels of antioxidant cytoprotective enzymes, in response to oxidative toxicity (Zhang *et al*., 2013). Additionally, it was showed, in studies using neurotoxin-based animal models of PD that Nrf2 knockout mice displayed increased susceptibility to different neurotoxins, decreased levels of dopamine transporters (DAT) in the striatum, and increased dopaminergic neurons depletion (Burton *et al*., 2006; Jakel *et al*., 2007; Chen *et al*., 2009).

Taking together these observations, it can be suggested that Nrf2 is a promising candidate to limit oxidative stress-mediated damage, and therefore it could be used as a target for therapeutic strategies in the pathogenesis of PD (de Vries *et al*., 2008).

2.3. Downstream targets of Nrf2

The activation of the Nrf2 pathway induces the transcription of several endogenous antioxidant, detoxification, GSH synthesis enzymes, heat shock proteins, among others (Trachootham *et al*., 2008; de Vries *et al*., 2008). Between the different Nrf2 downstream targets, the most important enzymes, whose expression is activated by this transcription factor are SOD, catalase, Gpx, peroxiredoxins (Prx), nicotinamide adenine dinucleotide phosphate (NADPH): quinone oxidoreductases (NQOs), GSH and its synthesis enzymes, heme oxygenases (HO) and thioredoxins (Trx) (de Vries *et al*., 2008). Here we will focus our attention, more specifically in Gpx1, the inducible form of HO and mitochondrial SOD.

The overall scheme showing the importance of both Nrf2 and DJ-1 neuroprotective roles in PD, is illustrated in Figure I.4.

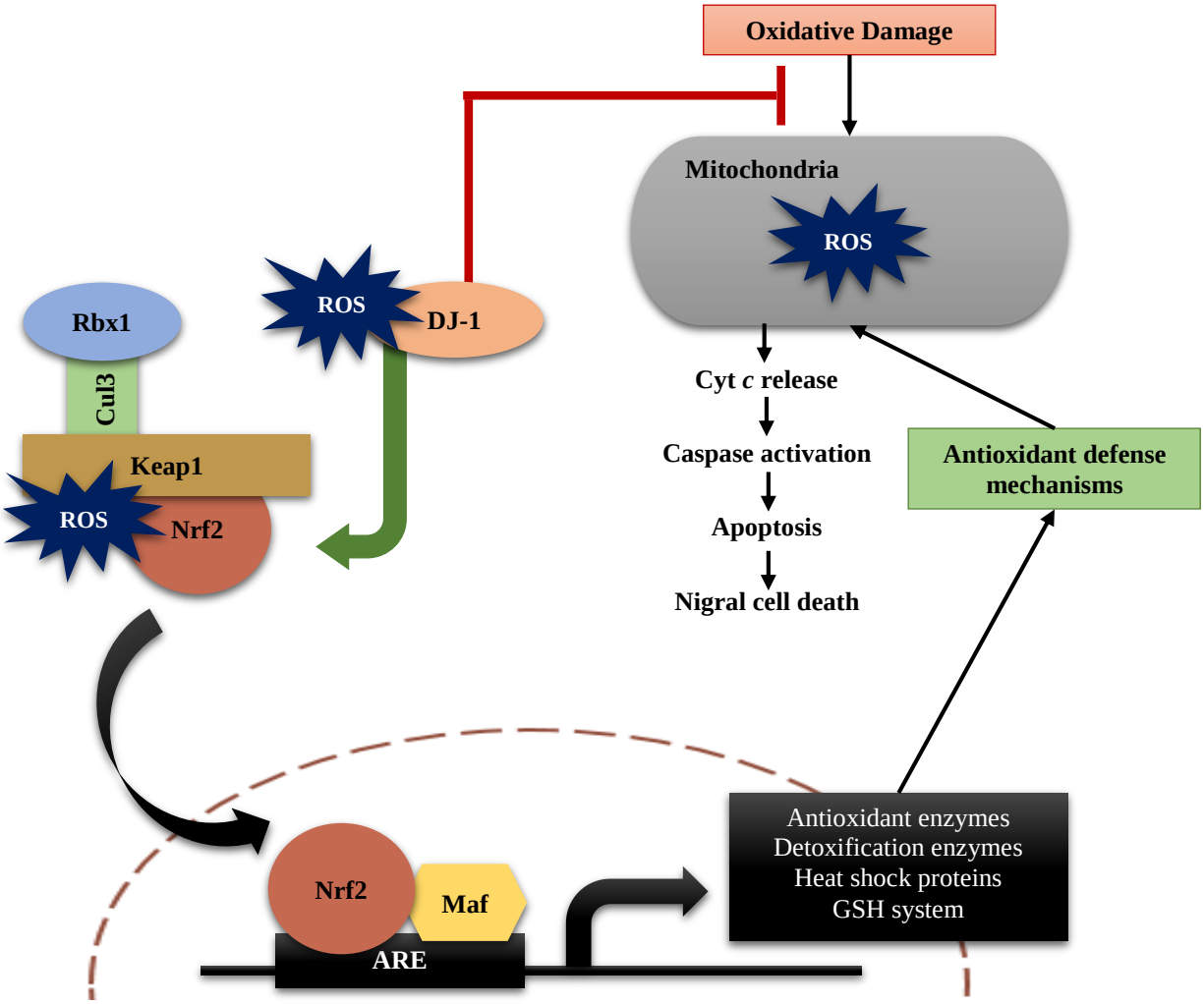


Figure I.4 – Schematic representation of the neuroprotective role of Nrf2 and DJ-1 in Parkinson's disease. Mitochondrial dysfunction, characterized by mitochondrial complex I impairment which in turn results in higher levels of ROS formation, has been deeply implicated in PD. Under normal physiological conditions, the master regulator of cellular redox status, Nrf2, is maintained in the cytoplasm through its interaction with the complex Keap1/Cul3/Rbx1. Therefore, if not activated, Nrf2 is ubiquitinated and consequently degraded. Upon ROS exposure, Keap1 is oxidized and its conformation is affected, allowing Nrf2 dissociation and subsequent translocation to the nucleus. In the nucleus, Nrf2 binds to a Maf protein forming a complex which in turn binds to ARE, located in the regulatory regions of antioxidant genes, thus activating their expression. The antioxidant proteins, including for instance SOD, Gpx, and the heat shock proteins HO, act quickly in the cell reducing the levels of free radicals, by degradation or conversion, generating more powerful antioxidants, thus reducing cell damage induced by oxidative stress. Finally, DJ-1, upon ROS exposure, has the ability to undergo self-oxidation, being recruited either to the mitochondria or to the nucleus, functioning as a ROS scavenger preventing, once again, oxidative damage. Another important role of DJ-1 is related to an increase in Nrf2 stabilization, by disrupting and/or preventing Keap1/Nrf2 complex, reducing Nrf2 ubiquitination and degradation, thus increasing its nuclear translocation. Thereby, DJ-1 is also responsible for causing the activation of antioxidant genes, culminating in an increase in cellular defenses, being responsible for protecting the cells against apoptotic death, induced by ROS. ARE – Antioxidant response element; Cul3 – Cullin3; Cyt *c* – Cytochrome *c*; Gpx – Glutathione peroxidase; GSH – Glutathione; HO – Heme oxygenase; Keap1 – Kelch-like ECH associated protein 1; Maf – Musculo-aponeurotic fibrosarcoma; Nrf2 – Nuclear factor erythroid 2 related factor 2; Rbx1 – Ring-box protein 1; ROS – Reactive oxygen species; SOD – Superoxide dismutase. *Adapted from Tufekci et al. (2011).*

2.3.1. Superoxide dismutases

The first line of defense against oxidative stress is provided by the metal containing SODs, such as cytosolic cooper, zinc superoxide dismutase (Cu, Zn-SOD or SOD1), mitochondrial manganese superoxide dismutase (MnSOD or SOD2) and extracellular superoxide dismutase (SOD3) (Johnson and Giulivi, 2005; de Vries *et al*., 2008). These enzymes are responsible, in general, for catalyzing the dismutation of the O_2^- to molecular oxygen and H_2O_2 (de Vries *et al*., 2008).

SOD1 is a cytoplasmic protein, mainly expressed in astrocytes and neurons (de Vries *et al*., 2008; Johnson and Giulivi, 2005). SOD3, in turn, is found in the extracellular matrix in most tissues, including the central nervous system (CNS) (Flynn and Melov, 2014). Therefore, both SOD1 and SOD3 are responsible for reducing the levels of superoxide in the extracellular and cytosolic environment, thus preventing CNS damage (de Vries *et al*., 2008; Flynn and Melov, 2014).

Finally, SOD2 is the most important isoform in the defense against oxidative stress (Flynn and Melov, 2014). This enzyme is mainly localized in neurons, within the mitochondrial matrix, which in turn is the major site of free radical generation, as described by Weisiger and Fridovich (1973). Consequently, SOD2 is characterized as a critical enzyme in the fight against mitochondrial dysfunction and oxidative stress, playing an important role in several neurodegenerative diseases, including PD (de Vries *et al*., 2008; Flynn and Melov, 2014).

2.3.2. Glutathione system

The GSH system is one of the most important antioxidant systems in the cell. GSH, a powerful antioxidant, scavenges alone or with different enzymes, several oxidative species such as NO, O_2^-, hydroxyl radicals, peroxynitrites among others, thus providing protection to the cell (Smeyne and Smeyne, 2013; Zhang *et al*., 2013).

The GSH system comprises numerous enzymes with specific functions. For instance, γ-glutamylcysteine ligase and glutathione synthetase are the enzymes responsible for GSH synthesis. In turn, glutathione reductase is responsible for recycling GSH, by converting oxidized GSH into reduced GSH. In addition, the enzymes Gpx and glutathione s-transferases are responsible for catalyzing the transfer of GSH to its substrates (Zhang *et al*., 2013).

Glutathione peroxidases are a group of 8 enzymes (Gpx1 to Gpx8) that play a crucial role in reducing H_2O_2 to water, as well as reducing the levels of oxidized lipids in the cell (Smeyne and Smeyne, 2013). Gpx1 is the most abundant member of the Gpx family and is characterized as a crucial antioxidant enzyme because it is responsible for preventing the detrimental accumulation of intracellular hydrogen peroxide (Lubos *et al*., 2011). Importantly, this enzyme is found in both neurons and glial cells, either in cytosol, nucleus, mitochondria or peroxisomal compartments (Trépanier *et al*., 1996; Power and Blumbergs, 2009).

Curiously, it was demonstrated by Wang and collaborators (2003) that the overexpression of these family members, under neurotoxic conditions, prevents neuron loss, and also hydrogen peroxide accumulation and lipid peroxidation.

2.3.3. Heme oxygenases

There are two isoforms of active HO, the inducible isoform, HO-1, and the constitutive isoform, HO-2 (Zhang *et al*., 2013). These two isoforms belong to the family of heat shock proteins and are responsible for protecting brain cells from oxidative stress (Wagener *et al*., 2003). HO enzymes are responsible for catalyzing the first step of heme catabolism, in other words, these enzymes are responsible for the degradation of intracellular heme (present in oxidases and peroxidases) into biliverdin, free iron and carbon monoxide (Wagener *et al*., 2003; Hung *et al*., 2008). Subsequently, biliverdin is converted to bilirubin, by biliverdin reductase (Wagener *et al*., 2003). Crucially, both biliverdin and bilirubin are powerful antioxidants that are capable to protect the brain from ischemic injury, as described by Deguchi and co-workers (2008), as well as perform anti-inflammatory actions (Hung *et al*., 2008). Not only biliverdin and bilirubin play important roles in cell defense, but also does carbon monoxide, for instance, this endogenous gaseous molecule plays essential roles in anti-apoptosis, anti-inflammation, anti-proliferation and in neurotransmission actions (Hung *et al*., 2008).

Ryter and colleagues (2006) described that HO enzymes can be found in several cell membranes, such as endoplasmic reticulum, nucleus and plasma membrane. HO-1 is considered a cellular stress response protein that is uniquely and rapidly expressed under oxidative stress and other harmful stimuli (Hung *et al*., 2008). HO-2, in turn, is expressed constitutively and does not respond to oxidative stress (Hung *et al*., 2008). Significantly, under oxidative stress situations, the synthesis of HO-1, in both neuronal and non-neuronal cells, increases. Thus, HO-1 performs a key role in stress response and therefore, it can be considered crucial in neuroprotection, firstly by degrading heme, and secondly by being responsible for the production of powerful antioxidants (Hung *et al*., 2008).

3. Experimental models of PD

Despite the years of research, very little is known about why and how the neurodegenerative mechanism of PD starts and evolves. Even so, in the last years, remarkable advances in the etiology and pathogenesis of PD have been made, thanks to the experimental models of the disease. Moreover, these models can be divided in two major categories: genetic models, which do not present the classic degeneration of nigral neurons, and neurotoxin-based models, which produce selective neuronal death, both *in vitro* and *in vivo* thus, being considered the most valuable and popular models in PD (Bové *et al*., 2005; Tieu, 2011). Although remarkable progress has been made in the study of the underlying

mechanisms that lead to the development of the pathophysiology of PD, much more needs to be done to fully understand which mechanisms are actually responsible for the severe neurodegeneration, characteristic of the disease, and what are the causes that culminate in these mechanisms.

3.1. Neurotoxin models of PD

An optimal model of PD should encompass all of the clinical and pathological features of the disease. In fact, the greater the similarity between a model and PD, the higher the predictive validity for clinical efficacy, as mentioned by Emborg (2004). Therefore, the neurotoxins currently available, should comprise both non-dopaminergic and dopaminergic systems, together with non-motor and motor symptoms, to be considered ideal models of PD (Tieu, 2011). Unfortunately, it is well known that, none of the available substances reproduces completely all the clinical and pathological features of the disease.

Currently, we can find among the neurotoxic chemicals used to induce dopaminergic neurodegeneration, 6-hydroxydopamine (6-OHDA), paraquat, rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Bové *et al*., 2005). Despite all the neurotoxins available, 6-OHDA and MPTP are the best characterized and most widely used agents, not only to study the molecular mechanisms leading to the neuropathology of the disease, but also to be used in the development of therapeutic strategies (Emborg, 2004). Nevertheless, the MPTP model, which does not exactly reproduces all the neuropathological features of PD (absence of LB, for instance), is clearly the most widely used neurotoxin as an experimental model of PD, due to the great similarity that individuals intoxicated with this substance, present with PD patients (Speciale, 2002; Dauer and Przedborski, 2003).

The discovery of the MPTP model occurred in California, in the early 1980's, when several drug users showed severe motor symptoms similar to those observed in PD (Langston *et al*., 1983). Further investigations revealed that these patients had injected a "street" preparation of 1-methyl-4-phenyl-4-propionpiperidine (MPPP), an analog of the narcotic meperidine, contaminated with MPTP (Langston *et al*., 1983). Later, it was discovered that the substance responsible for the severe clinical manifestations, observed in these patients, was MPTP (Langston *et al*., 1983). Crucially, *postmortem* studies in some of these patients revealed, like in PD, the loss of nigrostriatal structures (Langston *et al*., 1999).

Since the discovery of MPTP as an inducer of Parkinsonism, a massive progress has been made in the discovery of the mechanisms underlying cell death in PD (Tieu, 2011). Importantly, studies using this neurotoxic model have led to propose the environmental toxicity, as a potential cause in sporadic PD, and the mitochondrial dysfunction and the consequent oxidative stress as a possible pathogenic mechanism of the disease (Le Couteur *et al*., 1999; Tieu, 2011). In addition, several studies have used

this widely known model, for testing therapeutic approaches, trying to delay or even prevent the degeneration of dopaminergic neurons (Le Couteur, 1999; Matthews *et al*., 1999).

3.1.1. MPTP mechanism of action

MPTP is a highly lipophilic molecule that after systemic administration, easily crosses the blood-brain barrier (BBB), entering the brain (Emborg, 2004). Once inside the brain, the pro-toxin MPTP is metabolized to 1-methyl-4-phenyl-2,3-dihydropyridium ($MPDP^+$), by the enzyme monoamine oxidase B (MAO-B), within non-dopaminergic neurons, like glial cells (Dauer and Przedborski, 2003). After that, $MPDP^+$ is converted to the active toxic metabolite, 1-methyl-4-phenylpyridinium (MPP^+), possibly by spontaneous oxidation (Dauer and Przedborski, 2003). Most importantly, it was demonstrated in several studies that MAO-B inhibition prevented both clinical and neuropathological evidence associated to MPTP and therefore, this enzyme is considered obligatory for the MPTP-induced neurotoxicity (Chiba *et al*., 1984; Langston *et al*., 1984).

Since MPP^+ is a polar compound, it cannot freely exit from glial cells and enter the dopaminergic neurons (Smeyne and Jackson-Lewis, 2005). Therefore, as described by Javitch and co-workers (1985) MPP^+ is taken up into dopaminergic neurons, selectively, through its high affinity for the plasma membrane DAT. It is also essential to underline that the relevance of DAT in the MPTP neurotoxic mechanism, is proved in the studies of Javitch and colleagues (1985) and Bezard and collaborators (1999), where the blocking of DAT with antagonists or the ablation of DAT expression, respectively, results in the prevention of the neurotoxicity induced by MPTP. Accordingly, Donovan and co-workers (1999) demonstrated in their study, involving increased DAT levels in the brain of transgenic mice, that these animals are more susceptible to MPTP toxicity. Together, these studies reveal that the presence of DAT is obligatory for the neurotoxicity induced by MPTP. The schematic representation of the MPTP metabolism is shown in Figure I.5 – A**.**

Once inside the dopaminergic neurons (Figure I.5 – B), MPP^+ can follow three different pathways: i) it can be sequestered into synaptic vesicles, by the action of the proton-dependent vesicular monoamine transporter (VMAT); ii) it can enter into the mitochondria, through the inner membrane, by a mechanism actively driven by the membrane electrical gradient, where it interferes with mitochondrial respiration, by blocking complex I (NADH dehydrogenase or NADH ubiquinone oxireductase) of the ETC, firstly described by Nicklas and colleagues (1985); and iii) it can remain in the cytosol and interact with different cytosolic enzymes (Dauer and Przedborski, 2003). Importantly, the blockage of mitochondrial complex I, by MPP^+, results in increased ROS production, leading to oxidative stress (lipid peroxidation, protein peroxidation and DNA damage), impairment of ATP production, elevated intracellular calcium levels, decreased oxygen consumption and disruption of ion homeostasis

(Watanabe *et al*., 2005). Together, all these situations, recapitulate the deleterious events observed in PD patients' brains, and culminate in neuronal death (Watanabe *et al*., 2005).

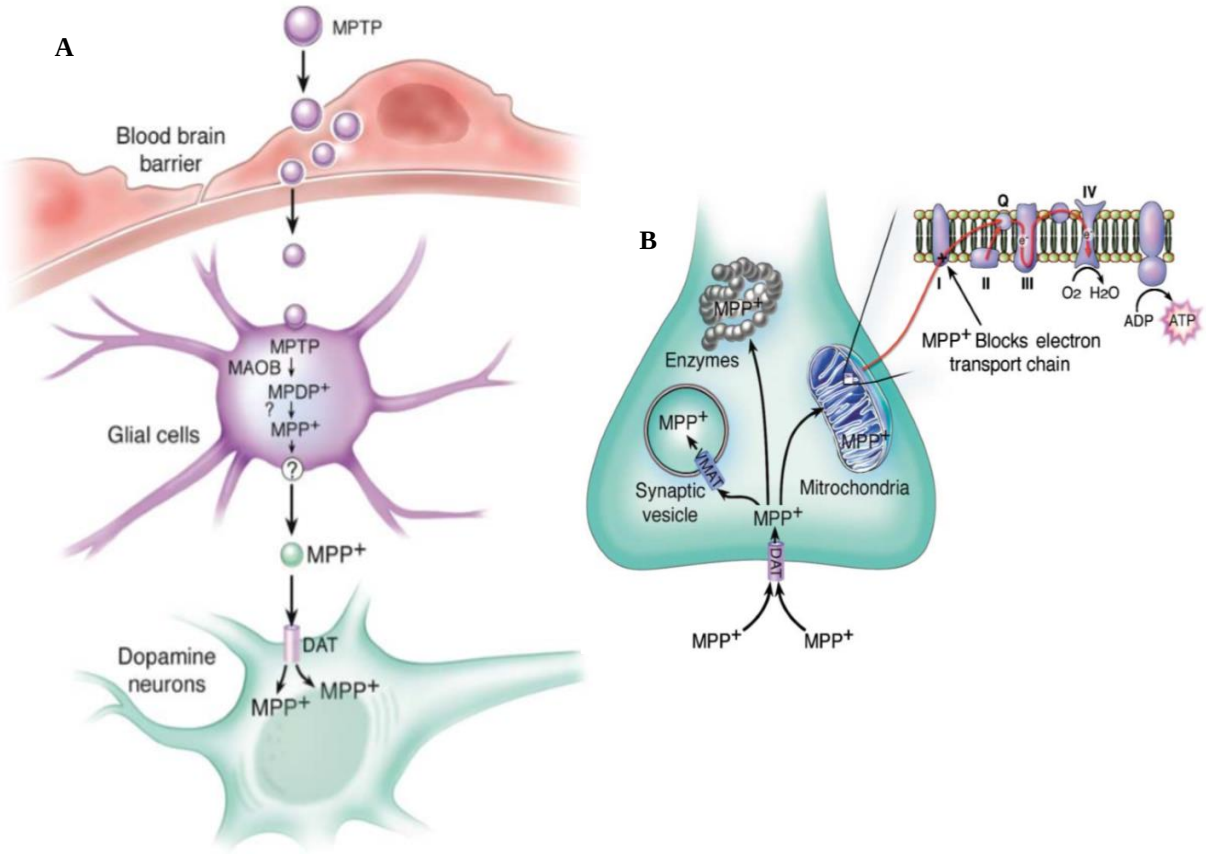


Figure I.5 – Schematic illustration of MPTP metabolism and intracellular pathways. **A –** *MPTP metabolism*. MPTP crosses the blood-brain barrier where is first metabolized to $MPDP^+$ by glial MAO-B, and then converted to its active metabolite MPP^+, probably by spontaneous oxidation. Thereafter, MPP^+ is released into the extracellular space and taken up into dopaminergic neurons through dopamine transporters. **B** – *Intracellular pathways of* MPP^+. Once inside the dopaminergic neurons, MPP^+ can move through several cellular compartments: it can concentrate within the mitochondria, where it inhibits complex I of the mitochondrial electron transport chain; it can interact with cytosolic enzymes; and it can be sequestered into synaptic vesicles by VMAT. DAT – Dopamine transporter; MAO-B – Monoamine oxidase B; $MPDP^+$ – 1-methyl-4-phenyl-2,3-dihydropyridinium; MPP^+ – 1-methyl-4-phenylpyridinium; MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; VMAT – Vesicular monoamine transporter. *In Dauer and Przedborski (2003).*

3.2. Animal models of PD

To be considered a good PD animal model some requisites have to be fulfilled, such as: i) have reproducible nigral damage; ii) the neurodegeneration of dopaminergic cells must be steady over time, without unprompted recuperation and iii) should provide an opportunity for the implementation of a neuroprotective strategy (Emborg, 2004). Besides that, an ideal animal model should resemble the clinical and pathological features of the disease, including therefore, the loss of neurons in SNpc and

the formation of LB, as well as, α-synuclein aggregation and the clinical symptoms that appear during the progress of the disease (Potashkin *et* al., 2010). Once the requirements are achieved, they become an extreme valuable tool, in the therapeutic field, allowing to predict the capacity of a particular substance to protect dopaminergic neurons, against severe damage, as well as uncover potential problems associated with the therapeutic use of the substance in question (Emborg, 2004).

Each animal model, currently available, presents specific advantages and disadvantages, for instance, rodents are the most widely used models to study the underlying mechanisms of PD, by comparison to larger animals such as, cats, dogs and non-human primates, due to the fact that rats and mice are extensively accessible, genetically manageable, they reproduce easily and in large scale, their cost are quite affordable, and they do not need large spaces nor complex feeding conditions (Emborg, 2004; Potashkin *et al*., 2010). It is also important to emphasize that despite the existence of considerable studies using cats, dogs and non-human primates in PD, these models bring countless ethical issues and elevated costs and therefore, their utility has been limitated (Potashkin *et al*., 2010).

3.2.1. The neurotoxin MPTP in rodent models

Susceptibility to the neurotoxin MPTP varies, not only across species, but also across animal strains, as reviewed by Betarbet and collaborators (2002). For example, it is currently known that rodents are more resistant to MPTP than humans and primates, and among rodents, mice exhibit more susceptibility to MPTP toxicity, when compared to rats (Betarbet *et al*., 2002). It is believed that the cause of this resistance, verified in rats, is due to their lower intracerebral levels of MAO-B and for this reason, mice are the most widely used animal models of PD (Emborg, 2004). In addition, it has been shown that only specific strains of mice are sensitive to MPTP, and that mice gender, age and body weight affect MPTP sensibility and reproducibility of the characteristic damage (Emborg, 2004). Therefore, female mice, mice under 8 weeks and mice smaller than 25g are more resistant to MPTP and their lesions are more variable, when compared to male mice, mice older than 8 weeks and heavier than 25g (Emborg, 2004). Accordingly, Przedborski and co-workers (2001) described that an optimal reproducibility of MPTP-lesioning is obtained in C57BL/6 male mice.

MPTP can be administered by a number of different ways, including oral, intracerebral, systemically or intracarotid artery injections (Emborg, 2004). The most common way is done by systemic administration, which in turn, can be done by subcutaneous, intraperitoneal, intravenous or intramuscular injections (Betarbet *et al*., 2002). Lastly, MPTP schemes of administration can also follow different routes. In fact, MPTP is usually administered to mice in three different schemes: acute, sub-acute or chronic administration (Przedborski and Vila, 2001; Emborg, 2004). The acute scheme consists of four intraperitoneal injections of MPTP, with 2 h intervals between the injections, on the same day, as described by Vila and collaborators (2000). In turn, the sub-acute scheme consists of a single injection

of MPTP on the same day (Saporito *et al*., 2000). Both acute and sub-acute schemes cause about 70-80% of dopaminergic cell loss in SNpc, by a mechanism involving essentially oxidative stress, which is associated with non-apoptotic morphology of cell death (Jackson-Lewis *et al*., 1995; Castro-Caldas *et al*., 2009). The chronic scheme consists of one intraperitoneal injection of MPTP per day, for 5 consecutive days (Vila *et al*., 2000). Equally important, this scheme is characterized by 30-50% of dopaminergic cell loss in SNpc, by a mechanism involving essentially apoptosis (Tatton and Kish, 1997). Crucial information regarding the MPTP schemes of administration, in mice, and the key features of the MPTP mouse model are described, respectively in Table I.2 and Table I.3.

Depending on the laboratories and the studies in question, different doses and regimens of MPTP administration can be used.

Table I.2 – Schemes of MPTP administration in mice. *Source: Jackson-Lewis et al., 1995; Tatton and Kish, 1997; Saporito et al., 2000; Vila et al., 2000; Przedborski and Vila, 2001*

	Acute	**Sub-acute**	**Chronic**
Dosage	20 mg/Kg (4 x at 2 h interval)	40mg/Kg (single injection)	30 mg/Kg/day (1 x 5 days)
Extent of cell death	70 – 80%	70 – 80%	30 – 50 %
Mechanism	Oxidative stress	Oxidative stress	Activation of genetic programs
Morphology	Non-apoptotic	Non-apoptotic	Apoptotic

Table I.3 – Key features of the MPTP mouse model in PD. *Adapted from Betarbet et al. (2002).*

Clinical Features	**Histopathology**	**Pathogenic Relevance**	**Applications**	**Disadvantages**
Akinesia	α-synuclein aggregation	Environmental toxin	Screen pharmacological and genetic therapies designed to protect dopaminergic neurons	Inclusion bodies are rare
Rigidity	Degeneration of dopaminergic neurons in SNpc	Oxidative Stress		
Tremor		Inhibition of mitochondrial complex I		

3.3. Cellular models of PD

In vitro cellular models became an important tool in the field of neurological diseases, for the study of molecular mechanisms at a cellular level (Dayem *et al*., 2014).

Between the available *in vitro* cellular models, the most widely used are the proliferative cell lines, primary cells and induced pluripotent stem cells (Krishna *et al*., 2014). The proliferative cell lines, such as human neuroblastoma cells, show some advantages, when compared to the other cellular models displayed. For instance, they are cost effective and easy to use and, since they are tumor derived cells, they constantly divide and are able to provide a large quantity of cells needed for different and numerous

assays, without showing great variability, in a short period of time and in a much less laborious way (Constantinescu *et al*., 2007). Moreover, these cell lines are the most widely used cells in studies related to neurotoxicity, oxidative stress and neurological diseases models, such as PD (Dayem *et* al., 2014; Krishna *et al*., 2014). However, results obtained with these cells should be carefully analyzed, since these models also show some disadvantages, such as genotype, phenotype, natural function and responsiveness to stimuli alterations, causing heterogeneity in cultures at a single point in time and also, possible contaminations with other cell lines and mycoplasma, as described by Kaur and Dufour (2012).

For example, the human dopaminergic neuroblastoma cell line, SH-SY5Y, described by Biedler and co-workers (1978), is a sub-clone of the parent cell line SK-N-SH (derived from a metastatic neuroblastoma in 1970), and is considered one of the most widely used proliferative cell line for modeling certain aspects of neurodegeneration and neurotoxicity in PD (Krishna *et al*., 2014). Notably, SH-SY5Y cells, in an undifferentiated state, have biochemical properties of human dopaminergic neurons (tyrosine hydroxylase, dopamine-β-hydroxylase and dopamine transporter expression), and after differentiation into a functional mature neuron, these cells express a larger number of neuronal markers, such as neurofilament proteins and also, muscarinic, opioid, dopamine and acetylcholine receptors (Constantinescu *et al*., 2007; Xie *et al*., 2010). Essentially, and despite the fact that undifferentiated SH-SY5Y cells possess the phenotype of immature neurons, it was demonstrated by Cheung and colleagues (2009) that the undifferentiated state is more susceptible to neurotoxins. Therefore, the authors concluded that undifferentiated SH-SY5Y cells might be a better cellular model for the study of the neurotoxicity of PD.

4. Tauroursodeoxycholic acid: antioxidant and neuroprotective properties

PD is a severe neurodegenerative disease that despite the efforts in research, has no effective therapies to slow or prevent the neurodegeneration. Currently, the most effective treatment of PD (levodopa) is symptomatic and targets the deficit of DA in striatum (Smith *et al*., 2012). Despite the fact that this therapy provides symptomatic relief, it becomes more inefficient with the progress of the disease, due to the onset of motor complications, such as involuntary movements (Jankovic and Aguilar, 2008). Therefore, the constant search for novel therapeutic strategies is of utmost importance. In addition, therapeutic approaches to slow or even prevent the neurodegeneration should target oxidative stress, and this can be achieved by using pharmacological agents that possess either antioxidant or free radical scavenging properties. In this context, we propose to study the efficacy of the endogenous bile acid, tauroursodeoxycholic acid (TUDCA), in limiting ROS-mediated damage, in two different experimental models of PD, due to its antioxidant and neuroprotective features that will be further discussed.

Bile acids are hydrophilic molecules synthesized in the liver and secreted into the intestine where they play crucial roles, such as lipid solubilization (Amaral *et al*., 2009). Notably, some bile acids are considered cytotoxic molecules, while others, due to chemical structure alterations, may be considered cytoprotective (Amaral *et al*., 2009). For instance, Rodrigues and collaborators (1998*a*) demonstrated that ursodeoxycholic acid (UDCA) is capable of modulating the apoptotic threshold in several cell types. Moreover, this bile acid is an FDA approved drug for the treatment of liver diseases, such as primary biliary cirrhosis (Yanguas-Casás *et al*., 2014), and has been recently used, by Min and colleagues (2012), in clinical trials for the treatment of amyotrophic lateral sclerosis. In addition, after oral administration of UDCA, this bile acid can be conjugated, in the liver, with glycine or taurine, originating respectively, glycoursodeoxycholic acid (GUDCA) or TUDCA (Rodrigues *et al*., 2002).

TUDCA is an endogenous bile acid normally produced at very low levels in humans that easily crosses the BBB, with no associated toxicity (Keene *et al*., 2002; Rodrigues *et al*., 2002; Castro-Caldas *et al*., 2012*b*). Interestingly, it was demonstrated that TUDCA has the ability to prevent ROS production, showing the antioxidant role of this bile acid, as well as, attenuate mitochondrial toxicity and prevent apoptosis (Rodrigues *et al*., 1998*b*; Keane *et al*., 2002). Apart from this, Keene and co-workers (2002) also demonstrated that TUDCA prevents striatal degeneration, and ameliorate locomotor and cognitive deficits, in the *in vivo* 3-nitropropionic acid rat model, of Huntington's disease. Moreover, Rodrigues and colleagues (2002, 2003) revealed that in the presence of TUDCA, lesion volumes in rat models of ischemic and hemorrhagic stroke were reduced. Crucially, it was also proved, by our group, that TUDCA prevents MPTP-induced dopaminergic cell death, in a mouse model of PD (Castro-Caldas *et al*., 2012*b*). All of these studies allowed to demonstrate the neuroprotective and antioxidant features of TUDCA.

Taken together the mechanisms underlying the pathophysiology of PD and the beneficial effects of TUDCA demonstrated in several studies, it is plausible to think of the potential therapeutic properties of this bile acid in the treatment of PD.

5. Aims

The main goal of this thesis is to investigate the mechanisms involved in TUDCA neuroprotection, against MPTP/MPP^+ neurotoxicity, and if part of its neuroprotective effects are modulated through Nrf2 activation. Therefore, we want to demonstrate that Nrf2 is a promising target for TUDCA to limit ROS-mediated damage in PD. To accomplish the aims of this thesis, two different experimental models of the disease were used.

The specific aims are:

- **Characterize the effect of TUDCA on the Nrf2 pathway, in the presence of MPTP/MPP^+.** In this point, we will assess if TUDCA has the ability to up-regulate the master regulator of cellular redox status, Nrf2, and if its nuclear translocation is increased in the presence of this bile acid.

- **Characterize the effect of TUDCA on Nrf2 downstream target enzymes, as well as on Nrf2 stabilizer, DJ-1, in the presence of MPTP/MPP^+.** We will investigate if the expression levels and biological activity of the different cytoprotective enzymes, whose expression is dependent on Nrf2, are improved in the presence of TUDCA. We will also assess if the expression levels of the redox-sensitive protein, DJ-1, are increased in the presence of this bile acid.

- **Evaluate the effect of TUDCA on cellular redox status, in the presence of MPTP/MPP^+.** Finally, we will evaluate if TUDCA is able to attenuate the levels of oxidative stress indicators, such as intracellular ROS production and lipid peroxidation.

The final purpose of this thesis is to provide insights about the molecular mechanisms involved in TUDCA neuroprotection, which potentially could lead to interesting therapeutic approaches in PD.

II. Materials and Methods

1. Materials

1.1. Supplements and chemicals

Minimum Essential Medium (MEM), Nutrient Mixture (Ham's F-12), fetal bovine serum (FBS), Penicillin/Streptomycin, L-glutamine, non-essential amino acids (NEAA) and TriplE Express were purchased from GIBCO ® (Life Technologies, Inc., Grand Islands, USA); TUDCA, MPTP, MPP^+, 2',7'-dichlorofluorescein diacetate (DCF-DA) probe, Bovine Serum Albumin (BSA) (fraction V), Complete Mini Protease Inhibitors Cocktail, Triton X-100, Hoechst 33258 dye, and Mowiol mounting media, for fluorescence microscopy, were acquired from Sigma Aldrich (St Louis, MO, USA); ECL Western blotting detection reagent was purchased from GE Healthcare (Buckinghamshire, UK); SuperSignal® West Femto Maximum Sensitivity Substrate was obtained from Thermo Scientific (Rockford, USA); Gpx activity kit was acquired from Enzo® Life Sciences (New York City, USA); Click-iT® Lipid Peroxidation Detection with Linoleamide Alkyne (LAA) was acquired from Invitrogen™/Molecular Probes (Eugene, OR, USA); Izol-RNA lysis reagent was purchased from 5 PRIME (Hamburg, Germany); random primers for reverse-transcribed complementary DNA (cDNA) were obtained from Promega (Sunnyvale, CA, USA); SuperScript II reverse-transcriptase kit was acquired from Invitrogen (Grand Island, NY, USA); SensiFAST™ SYBR® Hi-ROX kit was purchased from Bioline (London, UK); Bio-Rad's Protein Assay Reagent was obtained from Bio-Rad Laboratories (Hercules, CA, USA); Polyvinyl difluoride (PVDF) membrane was from Millipore (Bedford, MA, USA). Other chemicals and reagents of the highest analytical grade were purchased from local commercial sources.

1.2. Antibodies

Table II.1 – Primary antibodies used for Western blot and immunocytochemistry

Primary Antibody (antigen)	Host	Brand	Dilution
β-actin	Mouse	Santa Cruz (CA, USA)	1:40000
DJ-1	Rabbit	Millipore (MA, USA)	1:1000
Gpx1	Rabbit	AbCam (Cambridge, UK)	1:1000
HO-1	Rabbit	Enzo Life Sciences (NYC, USA)	1:1000
Nrf2 (for Western blot analysis)	Mouse	R & D (MN, USA)	1:1000
Nrf2 (for immunocytochemistry)	Rabbit	AbCam (Cambridge, UK)	1:50
SOD2	Rabbit	Santa Cruz (CA, USA)	1:500

Table II.2 – Secondary antibodies used for Western blot and immunocytochemistry

Secondary Antibody	Host	Brand	Dilution
Alexa Fluor® 488 anti-rabbit	Goat	Invitrogen Corporation™ (OR, USA)	1:200
Horseradish peroxidase conjugated anti-mouse	Goat	Invitrogen Corporation™ (OR, USA)	1:5000
Horseradish peroxidase conjugated anti-rabbit	Goat	Invitrogen Corporation™ (OR, USA)	1:5000

2. Methods

2.1. Animal treatments

All animal experiments were carried out in accordance with the institutional, Portuguese and European guidelines (*Diário da República, 2ª série Nº 121* of 27 June 2011; and 2010/63/EU European Council Directive), and methods were approved by the Direcção Geral de Alimentação e Veterinária (DGAV, reference 021943) and the Ethical Committee for Animal Experimentation of the Faculty of Pharmacy, University of Lisbon.

Twelve-week-old C57BL/6 male mice were purchased from Harlan and were housed under standardized conditions, on a 12 h light/dark cycle with free access to a standard diet and water *ad libitum*.

TUDCA and MPTP were dissolved in saline and were both administered intra-peritoneally (i.p). TUDCA was daily injected (1 injection/day), for three consecutive days, at a dose of 50 mg/Kg body weight, and MPTP was administered at a single dose of 40 mg/Kg body weight, as previously described by Castro-Caldas and colleagues (2012*b*).

Mice were divided in 5 groups: i) control mice that received saline (Control group); ii) mice that received only TUDCA injections for 3 consecutive days, and were sacrificed 6 h after the last TUDCA injection (TUDCA group); iii) mice treated only with MPTP that were sacrificed 3 h (MPTP, 3h group)

or 6 h (MPTP, 6h group) after MPTP administration; iv) mice that received daily injection of TUDCA beginning on day 1, followed by i.p administration of MPTP on day 3, and were sacrificed 3 h (T+M, 3h group) or 6 h (T+M, 6h group) after MPTP administration and v) mice treated with MPTP followed by TUDCA injection, 3 h (M+T, 3h group) or 6 h (M+T, 6h group) after neurotoxin administration, and were sacrificed 6 h after the last TUDCA injection, on day 3.

The time course studies were carried out in four independent experiments (*n*=4) with groups of three mice per time point. Previous studies showed that evaluated parameters in control animals did not change through the time course, therefore, to avoid increasing the number of animals needed in this study, control animals were sacrificed together with TUDCA-treated animals. The schematic representation of TUDCA and MPTP treatment is illustrated in Figure II.1.

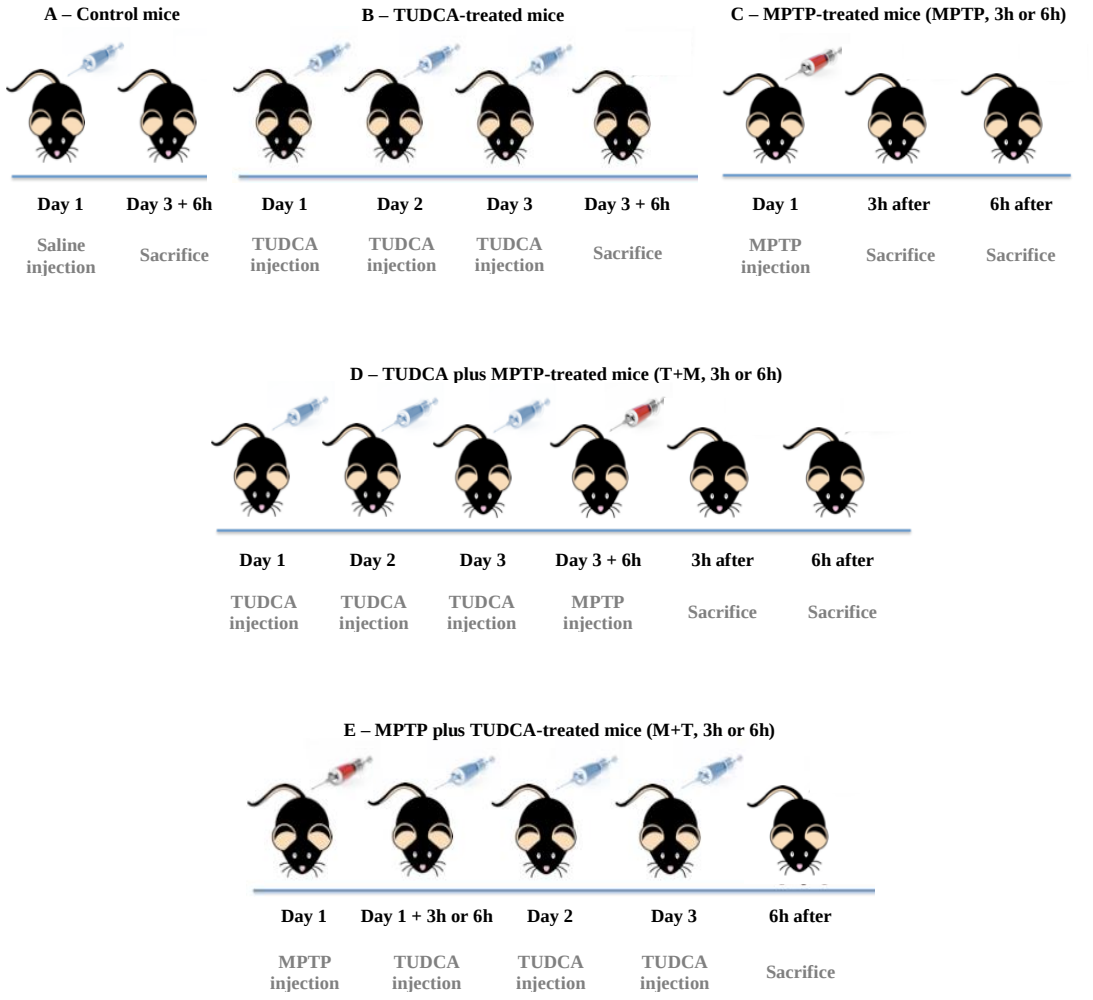


Figure II.1 – **Experimental scheme of C57BL/6 male mice treatment course.** C57BL/6 male mice were i.p injected with TUDCA (50mg/Kg body weight), for three consecutive days. MPTP was administered i.p at a single dose of 40 mg/Kg body weight. **A –** Control mice received saline. **B –** Mice were injected with TUDCA for three consecutive days, and were sacrificed 6 h after the last TUDCA injection. **C –** Mice were treated with a single dose of MPTP, for 3 or 6 h. **D –** MPTP injection was administered 6 h after the last TUDCA injection, on day 3, and mice were sacrificed 3 or 6 h after MPTP administration. **E –** TUDCA administration, for three consecutive days, occurred 3 or 6 h after MPTP injection on day 1, and mice were sacrificed 6 h after the last TUDCA injection, on day 3. i.p – intra-peritoneally; MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; TUDCA – Tauroursodeoxycholic acid.

2.2. Culture conditions and cell treatment

In vitro studies were carried out using the human neuroblastoma cell line, SH-SY5Y, obtained from American Type Culture Collection. This cell line has biochemical properties of human dopaminergic neurons and therefore, it has been widely used as a cell model of PD (Constantinescu *et al*., 2007; Dayem *et al*., 2014).

Cells were maintained in T75 flasks in MEM-Ham's F-12 (1:1) medium supplemented with 15% FBS, 1% non-essential amino acids, 100 µg/mL streptomycin and 100 U/mL penicillin, at 37ºC in a humidified atmosphere of 5% CO_2, in HERAcell 150 incubators (Thermo Scientific, Waltham, MA, USA). When cells reached about 80% confluence, they were detached from the T-flasks using 1.5 mL of TryplE express, for 5 min at 37ºC and were then counted, with a hemocytometer, and diluted in MEM-Ham's F-12, according to their initial concentration and specific assay.

Depending on the experiment to be performed, cells were seeded in 96-well culture plates, at a concentration of $2x10^4$ cells per well, in 35x10 mm culture plates, at a concentration of $5x10^5$ cells/mL or in 60x15 mm culture plates, at a concentration of $1x10^6$ cells/mL. After seeding, and prior to TUDCA or MPP^+ treatment, cells were left to stabilize for 24 h. After that, the medium was changed and the cells were immediately treated with 100 µM of TUDCA for 12 h. After treatment with TUDCA, cells were incubated with 1mM of MPP^+ for another 10 h (T+M, 10h), for Nrf2 detection by immunocytochemistry, or for 24 h (T+M, 24h), for lipid peroxidation detection, measurement of intracellular ROS production and detection of HO-1 expression, by quantitative real time polymerase chain reaction (qRT-PCR). In parallel, cells were treated with 1 mM of MPP^+ alone, for 10 (MPP^+, 10h) or 24 h (MPP^+, 24h). Controls were always included, and consisted of treating the cells with vehicle (control) or with TUDCA.

Each assay, from at least three independent experiments, was performed in duplicate for each condition. The schematic representation of time course treatment is illustrated in Figure II.2.

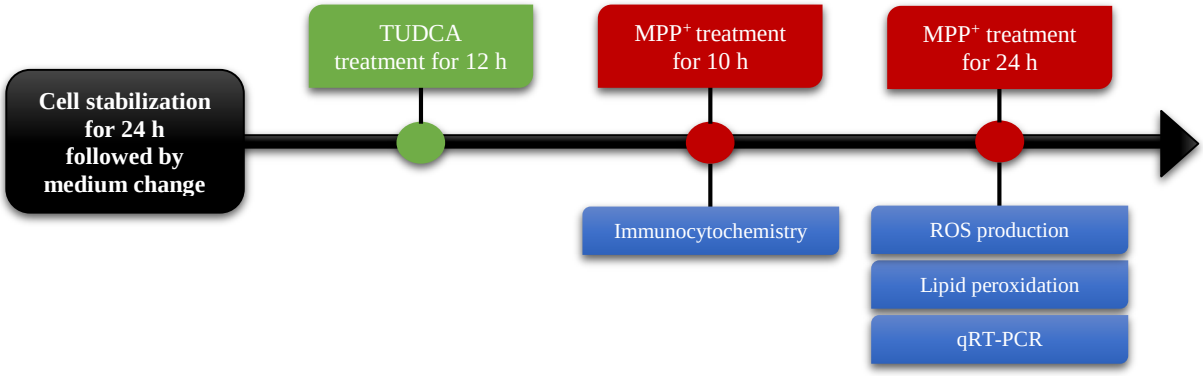


Figure II.2 – Simplified scheme of SH-SY5Y cells treatment course. After a 24 h period of stabilization, the culture medium was changed and SH-SY5Y cells were treated with 100 µM of TUDCA, prior to MPP^+ treatment, for a period time of 12 h. These cells were further incubated with 1 mM of MPP^+, during 10 or 24 h, depending on the specific assay, alone or in the presence of TUDCA. Controls were always included and consisted of treating the cells with vehicle or TUDCA alone. MPP^+ – 1-methyl-4-phenylpyridinium; TUDCA – Tauroursodeoxycholic acid

2.3. Western blot analysis

Mice were decapitated, after being anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and brains were removed and placed in ice-cold freshly made phosphate buffered saline (PBS). The entire midbrain region, containing the SNpc, and the whole striatum were dissected as previously described (Castro-Caldas *et al.*, 2009). Dissected mice midbrains (containing the SNpc) and striata were homogenized in ice-cold PBS, centrifuged at 3000 rpm, for 10 min, at 4ºC. Pellets were then homogenized in lysis buffer 1x [20 mM Tris-HCL (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µg/mL leupeptin] plus Complete Mini Protease Inhibitors Cocktail (200 mM Na_3VO_4, 1M NaF). After sonication, in the Ultrasonic Processor UP100H (Hielscher-Ultrasound Technology, Teltow, Germany), six times for 5 sec each, on ice, samples were centrifuged 13000 rpm for 15 min, at 4ºC, and the supernatant was collected and frozen at -80ºC. Total Protein concentration was determined by the Bradford method (Bradford, 1976), using Bio-Rad's Protein Assay Reagent. Tissue extracts were added (2:1) to denaturing buffer [0.25 M Tris-HCl, 4% sodium dodecyl sulfate (SDS), 4% glycerol, 0.004 % bromophenol blue, 1% β-mercaptoethanol, pH 6.8] and boiled for 5 min. Samples were then resolved on a 12,5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), in running buffer (25 mM Tris Base, 190 mM glycine, 0.1% SDS, pH 8.3) with fixed amperage of 35 mA per gel, for about 3 h. After running the gel, proteins were electrotransferred to an activated PVDF membrane (1 min in ethanol, 2 min in H_2O, 5 min in transfer buffer), in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol), with a fixed amperage of 500 mA during 2 h. The membranes were then blocked with 5% (w/v) non-fat dry milk in TBS-T (25 mM Tris Base, 137 mM NaCl, 2.7 mM KCl, 0.05% Tween 20, pH 7.5), for at least, 1 h at room temperature and further incubated with the specific primary antibodies: rabbit anti-DJ-1 (1:1000, 5% non-fat dry milk), rabbit anti-HO-1 (1:1000, 3% BSA), rabbit anti-Gpx1 (1:1000, 3% BSA), mouse anti-Nrf2 (1:1000, 5% BSA) or rabbit anti-SOD2 (1:500, 5% non-fat dry milk) overnight, at 4ºC, with shaking, followed by incubation with horseradish peroxidase-conjugated anti-mouse (1:5000, 3% non-fat dry milk) or anti-rabbit (1:5000, 3% non-fat dry milk) secondary antibodies, for at least, 1 h at room temperature. After washing membranes with TBS-T, the chemiluminescent immunocomplexes were detected by using ECL or Femto reagents. The relative intensities of protein bands were analyzed using the Image Lab ™ analysis software, after scanning with ChemiDoc™, both from Bio-Rad Laboratories (Hercules, CA, USA). After the evaluation of the different proteins, membranes were stripped, with stripping solution [1.5% glycine, 40% glacial acetic acid, 1% SDS, 10% Tween 20] for 10 min, and rinsed several times in TBS-T. Stripped membranes were then blocked as described and incubated with mouse anti-β-actin (1:40000, 5% non-fat dry milk) primary antibody, followed by incubation with horseradish peroxidase-conjugated anti-mouse secondary antibody. β-actin expression analyzed in stripped membranes was used as a loading control.

2.4. Measurement of Gpx activity

Gpx activity was measured in striatum and midbrain tissue homogenates, using the commercial kit Glutathione Peroxidase Activity, according to the manufacturer's instructions. The reaction measures the conversion of NADPH to NAD^+, during the reduction of the oxidized GSH, which is accompanied by a decrease in absorbance at 340 nm. Importantly, the rate of this decrease is directly proportional to Gpx activity in the sample.

Briefly, the multidetection microplate reader FLUOStar Omega (BMG LABTECH, Ortenberg, Germany) was set to measure absorbance at 340 nm every 30 sec, for 15 min, with orbital shake, at a constant temperature of 25ºC. Each well from 96-well plate contains 1x Assay Buffer, 10x Reaction Mix (Glutathione Reductase, reconstituted GSH + NADPH and 1x Assay Buffer), freshly prepared, and striatum or midbrain samples to be tested. The reactions were initiated after quick addition of cumene hydroperoxide to each well. Positive and negative controls were always included, according to the manufacturer's instructions.

The absorbance at 340 nm, measured every 30 seconds, was used to calculate Gpx activity, according to the manufacturer's instructions, which was then normalized to protein concentration. Each assay from, at least, three independent experiments, was performed in duplicate.

2.5. Total RNA isolation and qRT-PCR analysis

Total RNA, from mice striatum samples or SH-SY5Y cells, was extracted using Izol-RNA lysis reagent, according to the manufacturer's instructions. RNA integrity and concentration were evaluated by agarose gel electrophoresis and spectrophotometry at UV light, using a Nanodrop 1000 (Thermo Scientific, Rockford, USA). Briefly, 1.5 µg of RNA from each sample was submitted to reverse transcription, using the SuperScript II reverse-transcriptase kit, with random primers, using a 732-1200 thermocycler (VWR, Lisbon, Portugal). For qRT-PCR analysis, each reaction was performed in a total volume of 10 µL, including 5 µL SensiFAST™ SYBR® Hi-ROX kit, 0.4 µL of primer forward, 0.4 µL of primer reverse, 2.2 µL H_2O and 2 µL of the previously reverse-transcribed cDNA, using an ABI 7300 sequence detection system (Applied Biosystems, Foster, CA, USA). The cycling program was set as follows: denature at 50ºC for 2 min and at 95ºC, for another 2 min, followed by the 40 cycles of 45ºC for 5 sec and 62ºC for 30 sec. Primer sequences used are shown in Table II.3. The melting curve analysis showed the specificity of the amplifications. Threshold cycle, which inversely correlates with the target mRNA level, was measured as the cycle number at which the reporter fluorescent emission appears above the background threshold (data not shown). To ensure that equal amounts of cDNA were added to the PCR, β-actin (for mRNA analysis of SH-SY5Y cells) or hypoxanthine-guanine phosphoribosyl transferase (HPRT; for mRNA analysis of mice striatum samples) housekeeping genes were also

amplified. Data analysis is based on the ΔCt method with normalization of the raw data to housekeeping genes, as described in the manufacturer's manual. All PCR reactions were performed in duplicate.

Table II.3 – Sequences of primers used for qRT-PCR analysis

Gene	Species	Forward	Reverse
B-actin	Human	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATCCA
HO-1	Human	CGGGCCAGCAACAAAGTG	AGTGTAAGGACCCATCGGAGAA
DJ-1	Mouse	AGCCGGGATCAAAGTCACTG	GGTCCCTGCGTTTTTGCATC
Gpx1	Mouse	AGTCCACCGTGTATGCCTTCT	GAGACGCGACATTCTCAATGA
HO-1	Mouse	CACGCATATACCCGCTACCT	AAGGCGGTCTTAGCCTCTTC
HPRT	Mouse	GGTGAAAAGGACCTCTCGAAGTG	ATAGTCAAGGGCATATCCAACAACA
SOD2	Mouse	CAGACCTGCCTTACGACTATGG	CTCGGTGGCGTTGAGATTGTT

2.6. Measurement of intracellular ROS production

Intracellular ROS production was measured with the cell-permeable and non-fluorescent probe DCF-DA that measures hydroxyl, peroxyl and other ROS activity within the cell. This probe, after diffusion into the cell, is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into DCF, a highly fluorescent compound.

Measurement of ROS was performed in mice striatum and midbrain tissue extracts and also in SH-SY5Y cells. Briefly, 50 µg of midbrain and striatum tissue extracts were incubated with 10 µM of DCF-DA probe, diluted in PBS, at 37ºC for 1 h, in the dark. Alternatively, SH-5YSY cells, treated as previously described, were washed with PBS and immediately incubated with 10 µM of DCF-DA probe, diluted in MEM-Ham's F-12 medium, without FBS, at 37ºC for 45 min, in the dark.

The fluorescence intensity of DCF was measured in the microplate reader GloMax® Multi Detection System Promega (Sunnyvale, CA, USA), at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Blanks, consisting of PBS alone, and negative controls, consisting of MEM-Ham's F-12 medium without FBS and DCF-DA probe, were always included. Each assay from, at least, four different experiments, was performed in duplicate and the results were normalized to total protein concentration, using the Bradford method (Bradford, 1976).

2.7. Detection of lipid peroxidation

Lipid peroxidation was evaluated in SH-SY5Y cells, using a commercial kit that detects lipid-peroxidation-derived protein modifications, in fixed cells. In short, this kit uses the LAA reagent, an alkyne-modified linoleic acid, which is incorporated into cellular membranes, being converted to

reactive aldehydes, upon lipid peroxidation. These products can be subsequently detected using the reaction cocktail, according to the manufacturer's instructions.

Briefly, 50 µM of Click-iT® LAA, diluted in MEM-Ham's F-12 medium, was added to the cells. After that, cells were immediately treated with vehicle, MPP^+ or TUDCA. Twenty four hours after MPP^+ addition, cells were washed with PBS and fixed with 4% (w/v) paraformaldehyde (PFA), for 15 min at room temperature, followed by permeabilization with 0.5% Triton® X-100, for another 10 min at room temperature. Cells were then blocked with 1% BSA for 30 min, followed by LAA detection using the reaction cocktail (1x Click-iT® reaction buffer, $CuSO_4$, Alexa Fluor® 488, 1x Click-iT® buffer additive) incubated for 30 min at room temperature, protected from light.

The fluorescence of Alexa Fluor® 488 was measured in the microplate reader GloMax®, at an excitation wavelength of 495 nm and an emission wavelength of 519 nm. Blanks (PBS alone) and positive controls (according to the manufacturer's instructions) were always included. Each assay from, at least, five different experiments, was performed in duplicate and the results were normalized with total protein content in each well, using the Bradford method (Bradford, 1976).

2.8. Immunocytochemistry

The detection of Nrf2 intracellular localization was performed by immunocytochemistry, in SH-SY5Y cells that were treated with MPP^+ for 10 h, in the presence or absence of TUDCA, as previously described.

After treatment course, SH-SY5Y cells were washed with PBS and then fixed, with freshly prepared 4% (w/v) PFA. Cells were then blocked with blocking solution (10% FBS, 0.05% Tween-20 in PBS), for 1 h at room temperature. After that, cells were incubated overnight, at 4ºC, with a rabbit anti-Nrf2 primary antibody (1:50 in blocking solution), in a humidified chamber. The secondary antibody used was goat anti-rabbit Alexa Fluor® 488 (1:200 in blocking solution) and the incubation was performed for 2 h at room temperature. After staining cell nuclei with Hoechst 33258 dye (5µg/mL), the cells were mounted in glass slides with Mowiol anti-fading mounting medium.

Green (for Nrf2) and blue (for nuclei) fluorescence and UV images of, at least, fifteen random microscopic fields, were acquired per sample, under 400x magnification, using a fluorescence microscope (model AxioScope.A1) with integrated camera AxioCam HR (Carl Zeiss, Inc. – North America). The results were expressed as the percentage of green merged nuclei per total number of cells, using the ImageJ software analysis (National Institutes of Health, USA).

2.9. Statistical analysis

All results are expressed as mean ± SEM values. Data were analyzed by one-way ANOVA and differences between groups were determined by post hoc Bonferroni's test, using GraphPad Prism 5.0 (San Diego, CA, USA). Means were considered statistically significant at a *p* value bellow 0.05.

III. Results

1. Evaluation of the antioxidant role of TUDCA in the MPTP mouse model of PD

1.1. TUDCA up-regulates the expression levels of Nrf2, as well as its downstream targets, in C57BL/6 male mice striatum and midbrain

As previously described in section *I. Introduction*, in order to maintain a proper physiological redox balance, the transcription factor Nrf2, upon ROS exposure, is responsible for activating the expression of several cytoprotective enzymes. Based on different studies presented earlier in this thesis, it was demonstrated that the dysregulation of the Nrf2 pathway is deeply implicated in the pathogenesis of PD, therefore, its activation is considered a promising strategy to limit ROS-mediated damage in this disease (Burton *et al*., 2006; Jackel *et al*., 2007; Chen *et al*., 2009). Thus, to evaluate how the presence of TUDCA may protect from ROS-mediated damage, following MPTP/MPP^+ exposure, we decided to investigate its ability to modulate the Nrf2 signaling pathway.

The expression levels of Nrf2 were evaluated by Western blot assay using a specific antibody. In striatum samples, this protein levels, upon exposure to MPTP alone, were increased in both time points evaluated, being more obvious 6 h after the toxic insult (MPTP, 6h), although the values did not reach statistical significance (Figure III.1 – A). In contrast, results from midbrain revealed that MPTP alone did not induce an increase in the expression of Nrf2 (Figure III.1 – B). However, when mice were treated with TUDCA prior to MPTP administration, the expression levels of Nrf2 increased, in both striatum and midbrain samples. In these conditions, Nrf2 levels were more evidently increased in mice striatum, when MPTP was administered for 3 h (T+M, 3h). Importantly, this increment was found to be significantly different from control ($p<0.01$), as well as from the corresponding MPTP-treated mice ($p<0.05$). In midbrain samples, it was possible to observe a significant increase in Nrf2 levels, when MPTP was administered for 6 h (T+M, 6h), being considered statistically different from the corresponding MPTP-treated mice ($p<0.05$). Interestingly, when TUDCA was administered after MPTP, Nrf2 levels were also increased in mice striatum and midbrain, and this rise was more obvious

in mice that were exposed to MPTP for 3 h (M+T, 3h), when compared to those that were exposed for a longer period (M+T, 6h). In these conditions, the increments observed were found to be significantly different from control, in mice striatum (p<0.05), as well as from MPTP-treated mice, in both striatum (p<0.05) and midbrain (p<0.01). In addition, the rise observed, in midbrain samples, in M+T, 6h mice group revealed also statistical significance, when compared to the corresponding MPTP-treated mice (p<0.05).

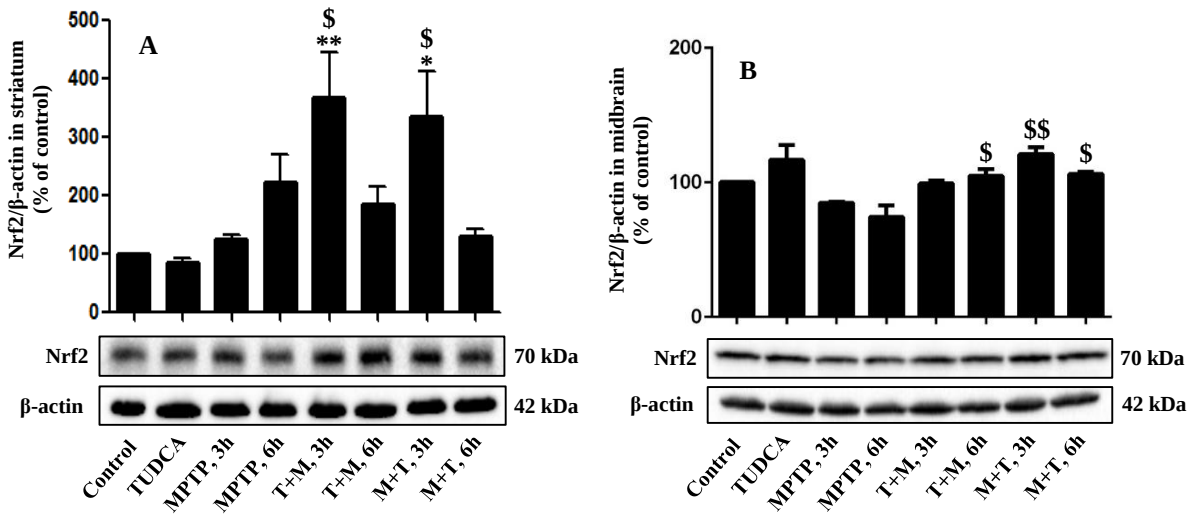


Figure III.1 – **TUDCA increases Nrf2 expression in mice striatum and midbrain.** C57BL/6 mice were treated with MPTP and/or TUDCA as indicated in Methods. Tissue extracts from mice striatum (A) and midbrain (B) were analyzed by Western blot with an anti-Nrf2 antibody. Anti β-actin antibody was used as loading control. The intensity of the bands was quantified using image analysis software (Image Lab). Data are expressed as the mean values ± SEM, indicated as percentage of the respective controls. Results from striatum and midbrain are representative of four or two independent experiments, respectively. Control – Mice received saline; TUDCA – Mice received TUDCA, for three consecutive days; MPTP – Mice received single-dose administration of MPTP, for 3 (MPTP, 3h) or 6 h (MPTP, 6h); T+M – Mice received TUDCA injections, for three consecutive days, followed by MPTP administration, for 3 (T+M, 3h) or 6 h (T+M, 6h); M+T – Mice received single-dose administration of MPTP, for 3 or 6 h, followed by TUDCA injection for three consecutive days (M+T, 3h or M+T, 6h). *p<0.05 vs. Control; **p<0.01 vs. Control; \$ p<0.05 vs. Mice treated with MPTP in absence of TUDCA; \$\$ p<0.01 vs. Mice treated with MPTP in absence of TUDCA.

Since the induction of Nrf2-activated genes has been recently identified as an important cellular defense mechanism against oxidative stress, next we investigated if the expression levels of these genes are up-regulated *in vivo*, upon TUDCA and/or MPTP treatment. Regarding the evaluation of antioxidant enzymes expression, namely Gpx1, HO-1 and SOD2, it was possible to notice that single-dose administration of MPTP induced an apparent increase in the expression of all these cytoprotective enzymes analyzed, in both striatum and midbrain (Figure III.2). Interestingly, when mice were pre-treated with TUDCA, the expression levels of these enzymes were up-regulated in striatum and midbrain samples. Specifically, in these conditions, Gpx1 expression levels were significantly increased, when compared to control, in mice striatum, at both time points evaluated (p<0.05 at 3 h and p<0.01 a 6 h)

(Figure III.2 – A). In contrast, pre-treatment with TUDCA did not have a significant impact in Gpx1 expression levels (Figure III.2 – B). In turn, HO-1 expression was considered statistically different from control ($p<0.01$), as well as from the corresponding MPTP-treated animals ($p<0.05$), in T+M, 3h mice group (Figure III.2 – C). As opposed, the rise observed in mice midbrain, in TUDCA plus MPTP-treated animals, was not considered statistically different (Figure III.2 – D). Finally, SOD2 levels were also increased in mice striatum, when mice were treated with TUDCA and MPTP, reaching statistical relevance in T+M, 3h mice (Figure III.2 – E). This observed increment was statistically different from control ($p<0.01$), as well as from the corresponding MPTP-treated mice ($p<0.05$). On the other hand, in midbrain samples, there were no significant differences in SOD2 expression, in any of the evaluated conditions (Figure III.3 – F). Notably, when mice were treated with MPTP prior to TUDCA administration, the expression levels of the different enzymes increased, in both striatum and midbrain, with an exception of SOD2 expression that were only increased in striatum samples. Despite the increment observed in mice striatum, in all of the cytoprotective enzymes, only Gpx1 expression levels were statistically different from control, in both time points evaluated ($p<0.01$ at 3 h and $p<0.05$ at 6 h).

The results so far presented revealed that TUDCA, when administered before MPTP, increases significantly Nrf2 expression levels, in both striatum and midbrain, and also the expression of the different cytoprotective enzymes, in mice striatum. Curiously, the expression peak observed in these cytoprotective enzymes, when the animals were treated with TUDCA followed by 3 h treatment with MPTP, in mice striatum, was coincident with the previous observed expression peak of Nrf2, further confirming its activation by TUDCA. Interestingly, it was demonstrated that this bile acid is also effective when administered after the neurotoxic insult.

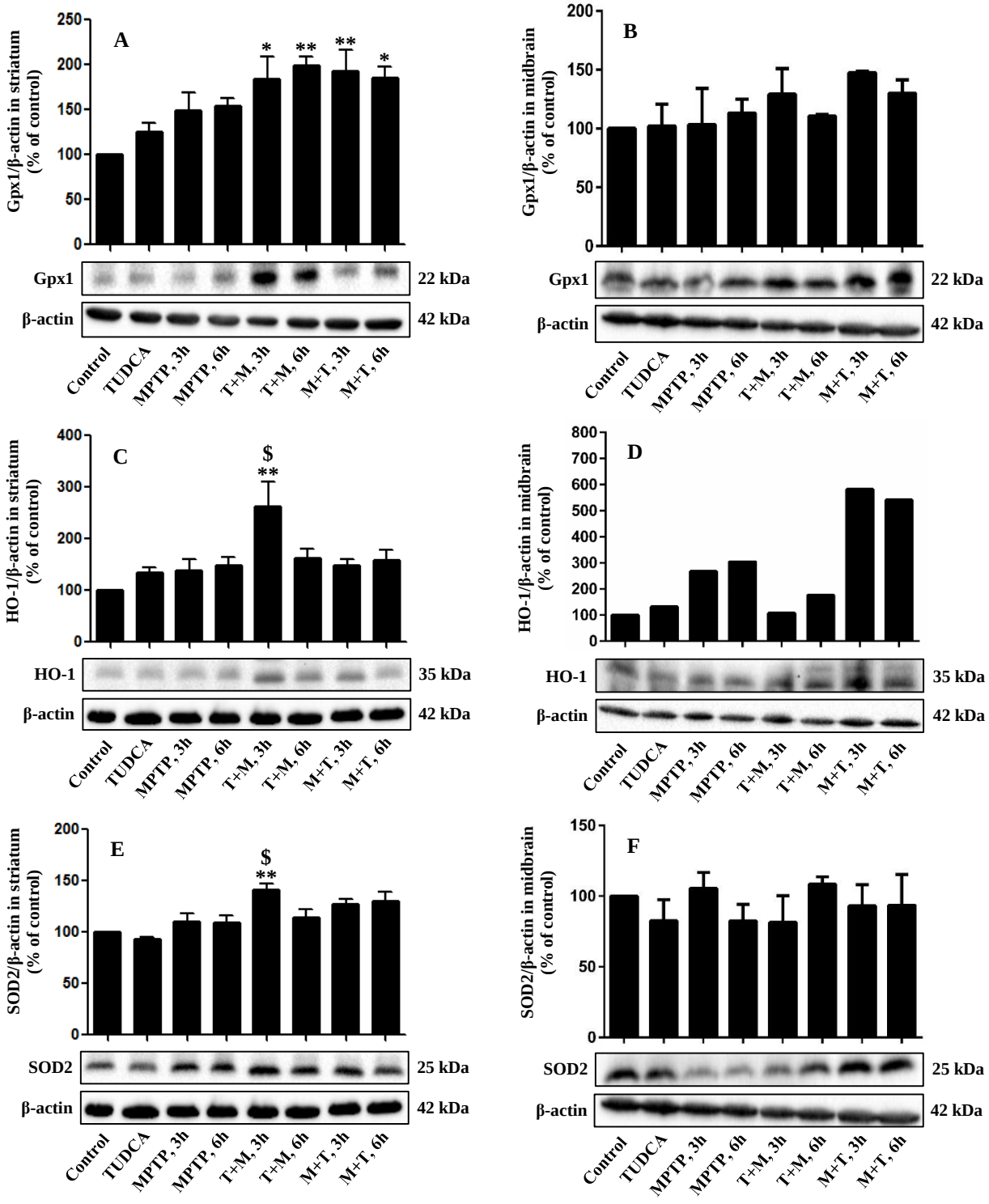


Figure III.2 – TUDCA increases the expression of Nrf2 downstream target enzymes in mice striatum and midbrain. C57BL/6 mice were treated with MPTP and/or TUDCA as indicated in Methods. Tissue extracts from mice striatum (A, C and E) and midbrain (B, D and F) were subjected to 12.5% SDS-PAGE, and the corresponding blots were probed with antibodies against Gpx1 (A and B), HO-1 (C and D), SOD2 (E and F) or β-actin (as loading control). Results from striatum and midbrain are representative of four and two (for HO-1 detection) or three (for Gpx1 and SOD2 detection) independent experiments, respectively. The intensity of the bands was quantified using computerized image analysis (Image Lab). Data are expressed as the mean values ± SEM indicated as percentage of the respective controls. Control – Mice received saline; TUDCA – Mice received TUDCA, for three consecutive days; MPTP – Mice received single-dose administration of MPTP, for 3 (MPTP, 3h) or 6 h (MPTP, 6h); T+M – Mice received TUDCA injections, for three consecutive days, followed

by MPTP administration, for 3 (T+M, 3h) or 6 h (T+M, 6h); M+T – Mice received single-dose administration of MPTP, for 3 or 6 h, followed by TUDCA injection for three consecutive days (M+T, 3h or M+T, 6h). *p<0.05 vs. Control; **p<0.01 vs. Control; $ p<0.05 vs. Mice treated with MPTP in absence of TUDCA.

1.2. DJ-1 expression levels are modulated by TUDCA in C57BL/6 male mice striatum and midbrain

Taking into consideration our previous results that showed an increase in Nrf2 and its downstream targets expression levels, in the presence of TUDCA, we thought that it would be interesting to investigate if there were any changes in the expression levels of the redox-sensitive and Nrf2 stabilizer protein, DJ-1, under TUDCA and/or MPTP administration. Therefore, DJ-1 expression levels were evaluated by Western blot analysis, using a specific antibody against this protein. Our results demonstrated that DJ-1 expression levels were increased, when mice were exposed to MPTP, in mice striatum and midbrain, although the values did not reach statistical significance (Figure III.3). An increase in the expression of DJ-1 was also induced in TUDCA plus MPTP-treated mice, especially when MPTP was administered for 3 h, in both striatum and midbrain. These increments were more evident in striatum samples (Figure III.3 – A), when compared to midbrain (Figure III.3 – B**)**, being statistically significant from control (p<0.01 at 3 h or p<0.05 at 6 h), as well as from the corresponding MPTP-treated mice (p<0.05 at 3 h). In turn, post-treatment with TUDCA, also raised DJ-1 protein levels, in both striatum and midbrain samples, at the different time points evaluated. However, and despite the notorious tendency, these increments were not considered statistically significant. Curiously, TUDCA alone was also capable of inducing DJ-1 expression, in both striatum and midbrain samples.

In conclusion, these results show that TUDCA is clearly capable of modulating DJ-1 protein levels, in both striatum and midbrain of C57BL/6 male mice. Notably, the significant increment of DJ-1 observed in T+M, 3h mice striatum was coincident with the increment previously observed in the expression levels of Nrf2 and its downstream targets.

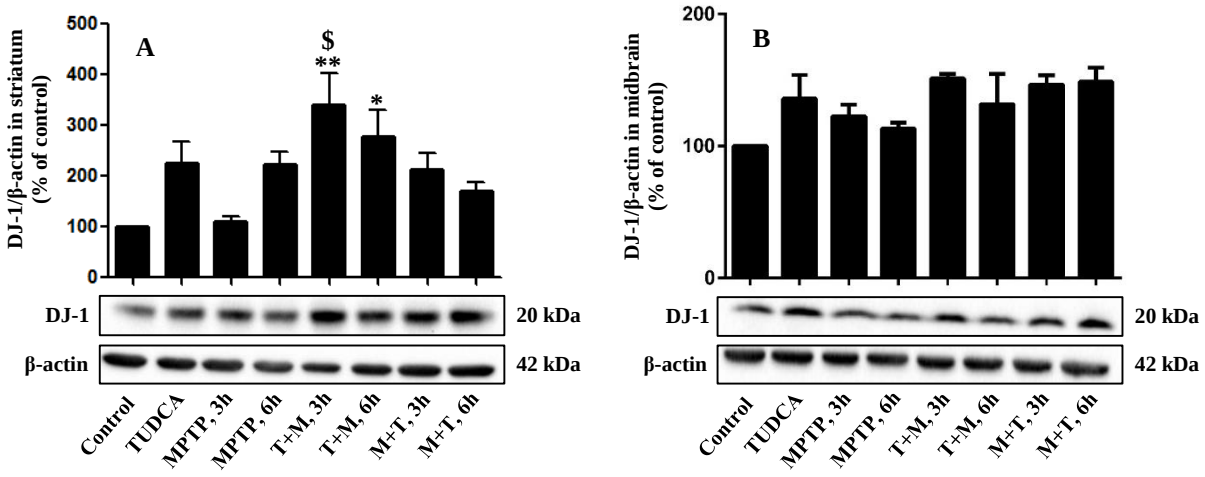


Figure III.3 – DJ-1 expression is modulated by TUDCA in mice striatum and midbrain. C57BL/6 mice were treated with TUDCA and/or MPTP as indicated in Methods. The corresponding blots from mice striatum (A) and midbrain (B) tissue extracts were probed with anti-DJ-1 or anti-βactin (as loading control) antibodies. The intensity of the bands was quantified using image analysis software (Image Lab). Data are expressed as the mean values ± SEM from four independent experiments, in both striatum and midbrain, indicated as percentage of the respective controls. Control – Mice received saline; TUDCA – Mice received TUDCA, for three consecutive days; MPTP – Mice received single-dose administration of MPTP, for 3 (MPTP, 3h) or 6 h (MPTP, 6h); T+M – Mice received TUDCA injections, for three consecutive days, followed by MPTP administration, for 3 (T+M, 3h) or 6 h (T+M, 6h); M+T – Mice received single-dose administration of MPTP, for 3 or 6 h, followed by TUDCA injection for three consecutive days (M+T, 3h or M+T, 6h). *p<0.05 vs. Control; **p<0.01 vs. Control; $ p<0.05 vs. Mice treated with MPTP in absence of TUDCA.

1.3. TUDCA enhances Gpx activity in C57BL/6 male mice striatum and midbrain

To investigate whether the observed increase in protein levels of the cytoprotective enzyme Gpx is coincident with an increase in its biological activity, we decided to evaluate the *in vivo* activity of this enzyme, with a commercial kit, in both striatum and midbrain samples, from mice treated with MPTP, in the presence or absence of TUDCA. This kit measures the conversion of NADPH to NAD^+ that is accompanied by a decrease in absorbance at 340 nm, which is directly proportional to Gpx activity in the sample. Importantly, the absorbance measured at 340 nm, was used to calculate the enzymatic activity (Units/mL) using the following equation:

$$\textit{Gpx activity} = \frac{\Delta A_{340}/\text{min}}{0.00379\ \mu M^{-1}} \times \frac{0.2\ \text{mL}}{\text{Vol. of sample mL}} \times \text{Sample dilution}$$

Results from Figure III. 4 – A show that Gpx activity is enhanced, in striatum samples, by comparison to control mice, in the presence of TUDCA alone, when the animals were treated with MPTP for 3 h, as well as in both time points evaluated of TUDCA plus MPTP and MPTP plus TUDCA-treated mice. Interestingly, these increments were significantly different from control (p<0.05), in animals treated with TUDCA after MPTP administration (M+T, 3h mice group). Remarkably, these results are in agreement with those previously observed by Western blot analysis, regarding Gpx1 expression

levels, in mice striatum. In midbrain samples (Figure III.4 – B), neither TUDCA alone nor the single dose administration of MPTP enhanced Gpx activity. However, when mice were treated with the combination of TUDCA plus MPTP, or *vice versa*, Gpx activity levels increased, being statistically different from the corresponding MPTP-treated mice (p<0.01), when the animals were treated with MPTP for 3 h, followed by TUDCA administration for three consecutive days. Once again, these results are in agreement with those previously observed, by Western blot analysis, in midbrain samples.

Together, these observations suggest that TUDCA is capable of enhancing Gpx biological activity, further supporting our previous results.

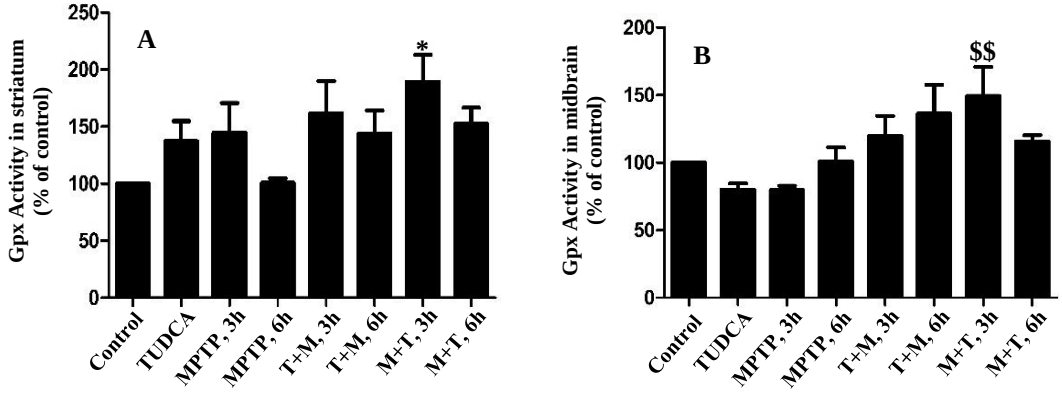


Figure III.4 – **TUDCA increases Gpx activity in mice striatum and midbrain.** C57BL/6 mice were treated with TUDCA and/or MPTP as indicated in Methods. Gpx activity was measured, in the microplate reader FLUOstar Omega, at 340 nm, in tissue homogenates from mice striatum (A) and midbrain (B). Data are expressed as the mean values ± SEM from at least, three independent experiments, performed in duplicate. All the results were normalized with total protein content and are presented as a percentage of control. Control – Mice received saline; TUDCA – Mice received TUDCA, for three consecutive days; MPTP – Mice received single-dose administration of MPTP, for 3 (MPTP, 3h) or 6 h (MPTP, 6h); T+M – Mice received TUDCA injections, for three consecutive days, followed by MPTP administration, for 3 (T+M, 3h) or 6 h (T+M, 6h); M+T – Mice received single-dose administration of MPTP, for 3 or 6 h, followed by TUDCA injection for three consecutive days (M+T, 3h or M+T, 6h). *p<0.05 vs. Control; $$ p<0.01 vs. Mice treated with MPTP in absence of TUDCA.

1.4. TUDCA regulates mRNA levels of antioxidant enzymes in C57BL/6 male mice striatum

The results presented so far demonstrated that TUDCA modulates protein levels of Nrf2 and its downstream target enzymes, as well as the expression of DJ-1, in mice striatum and midbrain. To further investigate the ability of TUDCA to up-regulate the different antioxidant enzymes, total RNA was extracted from striatum samples and Gpx1, HO-1 and SOD2 mRNA levels were quantified by qRT-PCR.

The preliminary results obtained demonstrated that in TUDCA plus MPTP-treated mice or vice versa, mRNA levels of Gpx1, HO-1 and SOD2 increased, when compared to control mice (Figure III.5), being in agreement with the previous results obtained, regarding the evaluation of protein expression.

These preliminary observations indicate that TUDCA increases the expression of the different cytoprotective enzymes, possibly by increasing their mRNA levels, further supporting the role of TUDCA as an inducer of the Nrf2 pathway. However, to further confirm this we would need to increase the number of independent experiments, which was not possible in the course of this thesis, but it will be done in a near future.

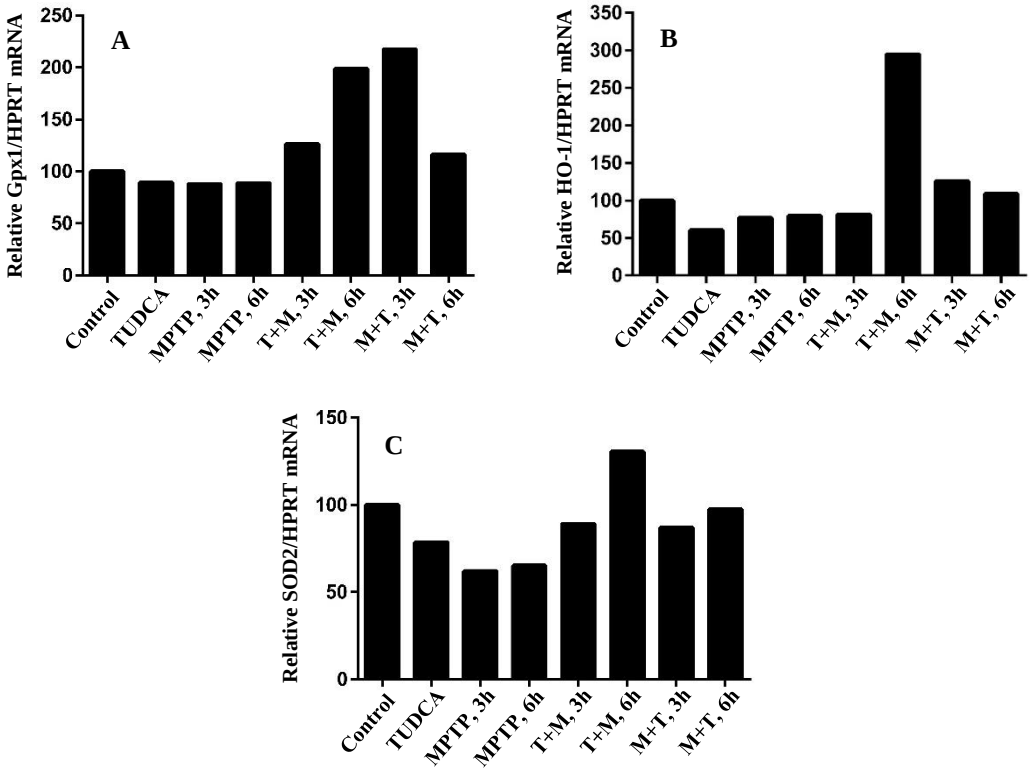


Figure III.5 – TUDCA modulates mRNA levels of the different cytoprotective enzymes in mice striatum. C57BL/6 mice were treated with MPTP and/or TUDCA as indicated in Methods. Total RNAs extracted from mice striata were analyzed by quantitative real-time PCR using specific primers. The relative amounts of Gpx1, HO-1 and SOD2 products were calculated using the ΔΔCt method, normalizing for the expression of the housekeeping gene HPRT. Results from mice striatum are only preliminary and representative of one independent experiment. Control – Mice received saline; TUDCA – Mice received TUDCA, for three consecutive days; MPTP – Mice received single-dose administration of MPTP, for 3 (MPTP, 3h) or 6 h (MPTP, 6h); T+M – Mice received TUDCA injections, for three consecutive days, followed by MPTP administration, for 3 (T+M, 3h) or 6 h (T+M, 6h); M+T – Mice received single-dose administration of MPTP, for 3 or 6 h, followed by TUDCA injection for three consecutive days (M+T, 3h or M+T, 6h).

1.5. Role of TUDCA on ROS generation in C57BL/6 male mice striatum and midbrain

MPTP toxicity, as mentioned before in this thesis, is associated with increased levels of ROS generation, which in turn activates an efficient free radical scavenging system (like the antioxidant enzymes), in an effort to protect cells against ROS-mediated damage. In this context, and since we verified an increase in the expression and activity of these enzymes, and to further explore the

antioxidant role of TUDCA, in mice striatum and midbrain, we investigated the *in vivo* ability of this bile acid to modulate ROS production, in the presence of MPTP, using DCF-DA probe.

As illustrated in Figure III.6, intracellular ROS levels were not significantly altered in any of the conditions tested, neither in striatum nor in midbrain. Importantly, in striatum samples (Figure III.6 – A), and despite the inexistence of statistical relevance, it was possible to notice a slight decrease in ROS production in T+M, 3h mice that is coincident with the previous observed increase, in the expression levels of the different cytoprotective enzymes. In midbrain (Figure III.6 – B), although there are no statistical differences between groups, TUDCA seems to downregulate ROS levels in the presence of MPTP, either injected before or after the neurotoxin. These TUDCA-dependent slight decreases in ROS levels also occurred in coincident time points when the expression levels of the cytoprotective enzymes were increased.

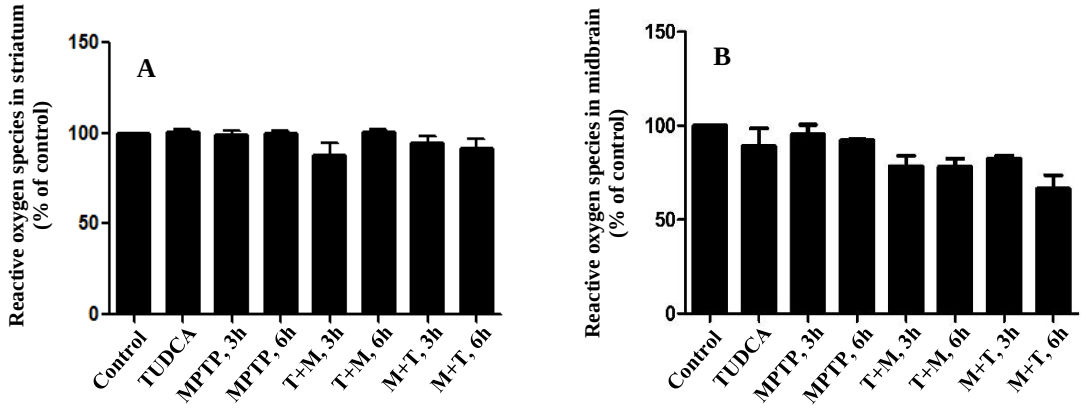


Figure III.6 – Role of TUDCA on ROS generation in mice striatum and midbrain. C57BL/6 mice were treated with TUDCA and/or MPTP as indicated in Methods. ROS production in mice striatum (A) and midbrain (B) was determined as previously described in Methods using DCF-DA probe. Fluorescence intensity of DCF was measured in the microplate reader (Glomax) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm, and the results, performed in duplicate, are presented as a percentage of control. Data are expressed as the mean values ± SEM from four independent experiments. Control – Mice received saline; TUDCA – Mice received TUDCA, for three consecutive days; MPTP – Mice received single-dose administration of MPTP, for 3 (MPTP, 3h) or 6 h (MPTP, 6h); T+M – Mice received TUDCA injections, for three consecutive days, followed by MPTP administration, for 3 (T+M, 3h) or 6 h (T+M, 6h); M+T – Mice received single-dose administration of MPTP, for 3 or 6 h, followed by TUDCA injection for three consecutive days (M+T, 3h or M+T, 6h).

2. Evaluation of the antioxidant role of TUDCA in the MPP^+ cell model of PD

2.1. TUDCA prevents MPP^+-induced ROS formation in SH-SY5Y cells

As demonstrated above, we investigated if TUDCA was capable of modulating the intracellular oxidative environment, by measuring the intracellular ROS levels, in C57BL/6 mice striatum and midbrain, but the results were inconclusive. Therefore, to overcome the limitation of using more animals, and since in the Lab we have previously confirmed that TUDCA protects SH-SY5Y cells

against MPP^+-induced cell death (data published in I. Fonseca Master thesis, 2015), we decided to explore if TUDCA was capable of modulating ROS formation, in these cells, using DCF-DA probe.

The results presented in Figure III.7 demonstrate that MPP^+ treatment resulted in a significant increase in ROS formation, when compared to control (p<0.05), in SH-SY5Y cells. In contrast, when these cells were pre-treated with TUDCA, the ability of MPP^+ to generate ROS was abrogated, since the fluorescence observed was similar to control values. Importantly, this decrease in ROS levels, elicited by TUDCA, was considered statistically different from MPP^+-treated cells (p<0.01).

These results suggest that TUDCA is capable of modulating the intracellular oxidative environment in SH-SY5Y cells, further confirming the antioxidant role of TUDCA, *in vitro*.

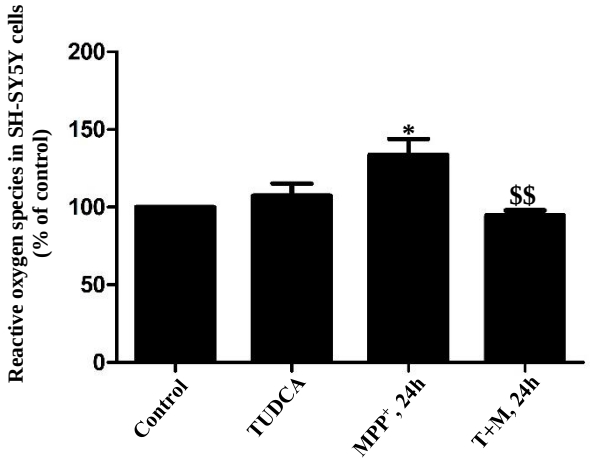


Figure III.7 – TUDCA prevents MPP^+-induced ROS formation in SH-SY5Y cells. SH-SY5Y cells were cultured and treated with TUDCA and/or MPP^+ as previously described in Methods. ROS generation in these cells was determined by a fluorimetric assay where the fluorescence intensity of DCF was measured in a microplate reader (Glomax), at an excitation wavelength of 485 nm and an emission wavelength of 528 nm, and the results, performed in duplicate, are presented as a percentage of control. Data are expressed as the mean values ± SEM from five independent experiments. The results were normalized with total protein content. Control – Cells were treated with vehicle; TUDCA – Cells were treated only with TUDCA, for 12 h; MPP^+, 10h – Cells were treated with MPP^+, for 10 h; T+M, 10h – Cells were pre-treated with TUDCA for 12 h, following MPP^+ administration for another 10 h. *p<0.05 vs. Control; $$ p<0.01 vs. Cells treated with MPP^+ in absence of TUDCA.

2.2. MPP^+-dependent lipid peroxidation is attenuated by TUDCA in SH-SY5Y cells

To strengthen the antioxidant role of TUDCA, we decided to elucidate how the presence of this bile acid affects another oxidative stress indicator. Therefore, we detected lipid peroxidation *in vitro*, with a commercial kit, upon TUDCA and/or MPP^+ administration.

Results shown in Figure III.8 demonstrate that MPP^+ treatment triggered a significant increase of lipid peroxidation, by comparison with control values (p<0.05), in SH-SY5Y cells. Notably, when these

cells were pre-treated with TUDCA, MPP^+-induced lipid peroxidation was significantly attenuated, when compared to MPP^+-treated cells ($p<0.05$).

Together, these results strengthen the antioxidant role of TUDCA, since this bile acid has the ability to modulate two major oxidative stress indicators, namely ROS formation and lipid peroxidation, in SH-SY5Y cells.

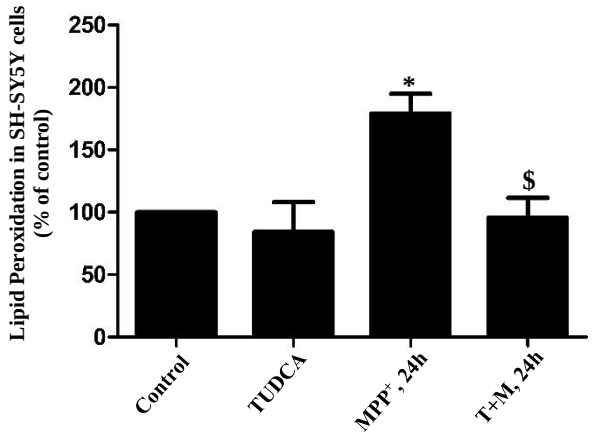


Figure III.8 – TUDCA attenuates lipid peroxidation in SH-SY5Y cells. SH-SY5Y cells were cultured and treated with TUDCA and/or MPP^+ as previously described in Methods. Lipid peroxidation was measured in a microplate reader (Glomax), at an excitation wavelength of 495 nm and an emission wavelength of 519 nm, and the results, performed in duplicate, are presented as a percentage of control. Data are expressed as the mean values ± SEM from five independent experiments. All the results were normalized with total protein content. Control – Cells were treated with vehicle; TUDCA – Cells were only treated with TUDCA; MPP^+, 10h – Cells were treated with MPP^+, for 10 h; T+M, 10h – Cells were pre-treated with TUDCA for 12 h, following MPP^+ administration for another 10 h. *$p<0.05$ vs. Control; $ $p<0.05$ vs. Cells treated with MPP^+ in absence of TUDCA.

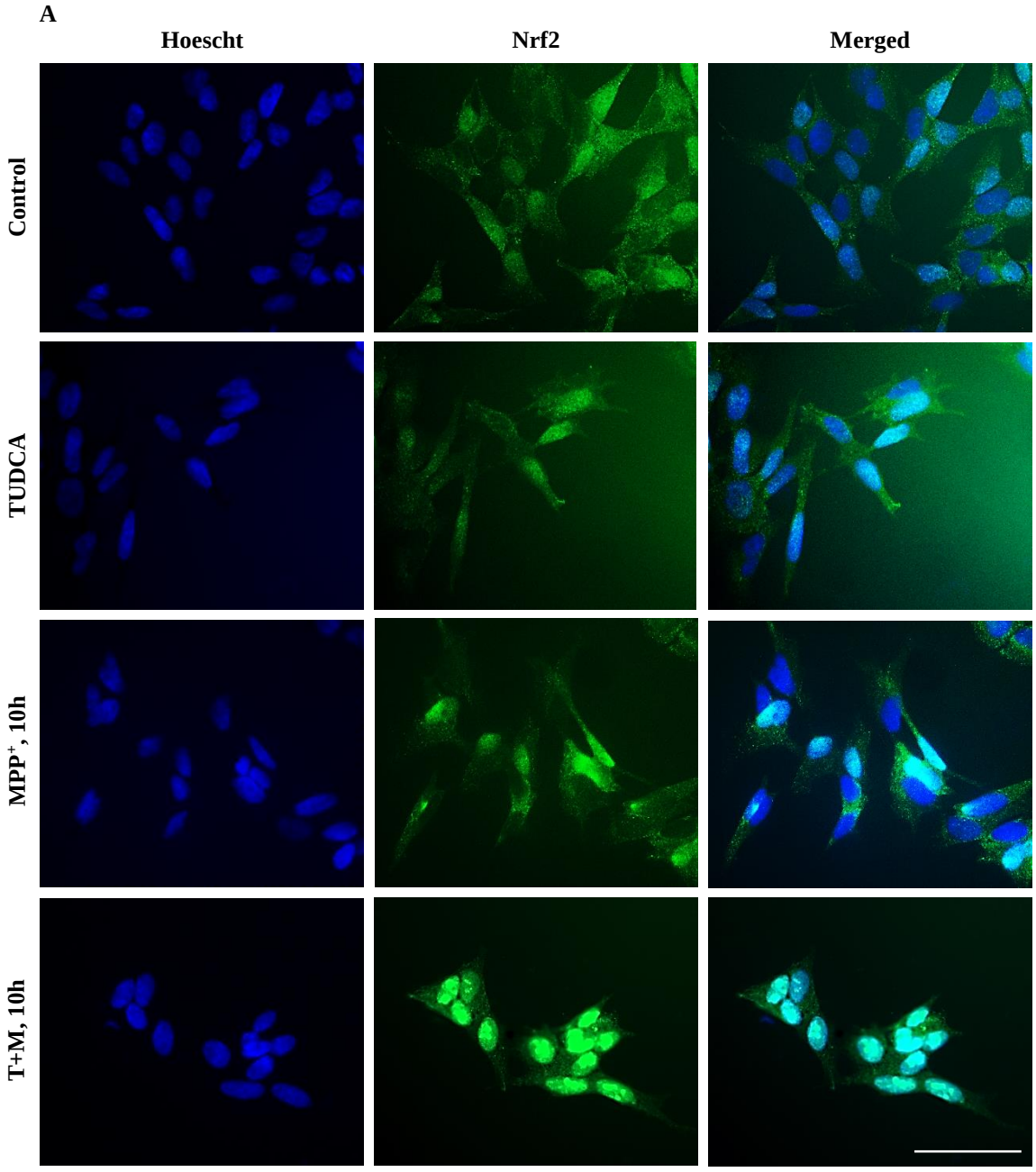
2.3. TUDCA increases Nrf2 nuclear translocation in SH-SY5Y cells

Previous results reported in this work demonstrated that TUDCA induces Nrf2 expression. Therefore, the following question was whether TUDCA was capable of inducing Nrf2 activation, by evaluating its nuclear translocation. Firstly, we attempted to evaluate Nrf2 translocation, *in situ*, in brain sections of C57BL/6 mice striatum, by immunohistochemistry. However, these results were found to be difficult to obtain and inconclusive. Consequently, and taking into consideration our previous results showing that TUDCA prevents MPP^+-induced ROS formation and lipid peroxidation, in SH-SY5Y cells, we proceeded to evaluate Nrf2 translocation in this cell line.

Graphic results expressed in Figure III.9 indicate the percentage of green merged nuclei per total number of cells, in each field. When cells were treated with MPP^+ for 10 h, an increase in Nrf2 nuclear translocation was observed, although the values did not reach statistical significance. Interestingly, pre-treatment with TUDCA for 12 h, significantly increased Nrf2 nuclear translocation, when compared to

control (p<0.001), as well as MPP^+-treated cells (p<0.05). Notably, in the absence of MPP^+, TUDCA was also capable of inducing Nrf2 translocation.

These results presented above show that TUDCA induces Nrf2 activation, by increasing its nuclear translocation, in SH-SY5Y cells, further re-enforcing our previous results obtained in this cell model of PD.



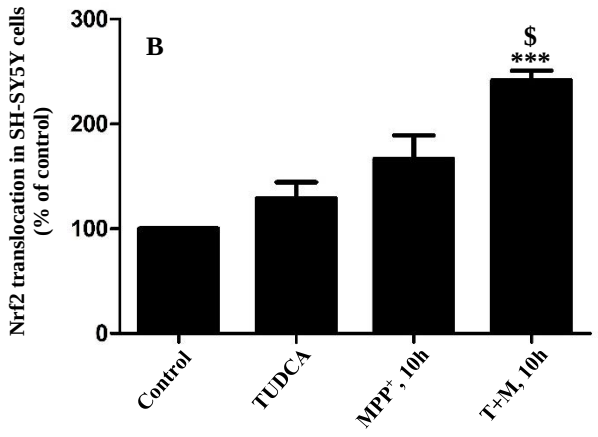


Figure III.9 – **Nrf2 translocation to the nucleus is increased by TUDCA in SH-SY5Y cells.** SH-SY5Y cells were cultured and treated with TUDCA and/or MPP^+ as previously described in Methods. After fixation with paraformaldehyde, cells were stained with an antibody against Nrf2, followed by a fluorescent-labeled secondary antibody (in green), and counterstained with Hoechst for the nuclei (in blue). Green and blue fluorescence was detected using a fluorescence microscope (model AxioScope.A1) coupled with AxioCam HR (Zeiss). **A –** Representative results, from one experiment, of random microscopic fields are shown. Scale bar represents 50 µm. **B –** Data are expressed as the mean values ± SEM from three independent experiments. The results are presented as the percentage of control. Control – Cells were treated with vehicle; TUDCA – Cells were treated only with TUDCA; MPP^+, 10h – Cells were treated with MPP^+, for 10 h; T+M, 10h – Cells were pre-treated with TUDCA for 12 h, following MPP^+ administration for another 10 h. ***p<0.001 vs. Control; $ p<0.05 vs. Cells treated with MPP^+ in absence of TUDCA.

2.4. TUDCA increases HO-1 mRNA levels in SH-SY5Y cells

Based on the previous results, we decided to investigate whether Nrf2 nuclear translocation, elicited by TUDCA, was coincident with an increase at the transcriptional level of the antioxidant enzyme HO-1, in SH-SY5Y cells.

As demonstrated in Figure III.10, in the presence of MPP^+, HO-1 mRNA levels were significantly increased, in SH-SY5Y cells, when compared to control (p<0.01). Remarkably, when these cells were pre-treated with TUDCA, a further increase in HO-1 mRNA levels was observed. Notably, this increment was considered significantly different from control values (p<0.001). Interestingly, when TUDCA was administered in the absence of MPP^+, the mRNA levels of this protein also increased notoriously, being statistically significant when compared to control levels (p<0.001).

Importantly, these results regarding HO-1 mRNA levels are in accordance with Nrf2 nuclear translocation and reflect the induction of this transcription factor, by TUDCA, in SH-SY5Y cells.

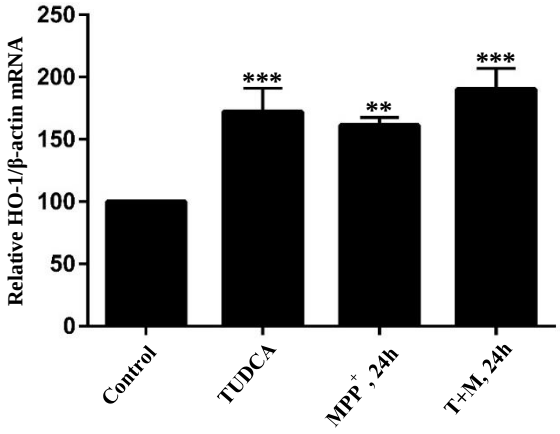


Figure III.10 – HO-1 mRNA levels are increased by TUDCA in SH-SY5Y cells. Cells were cultured and treated with TUDCA and/or MPP^+ as previously described in Methods. Total RNA extracted from SH-SY5Y cells was analyzed by quantitative real-time PCR using specific primers. The relative amount of HO-1 products were calculated using the ΔΔCt method, normalizing for the expression of the housekeeping gene β-actin. Results are representative of three independent experiments. Control – Cells were treated with vehicle; TUDCA – Cells were treated only with TUDCA; MPP^+, 10h – Cells were treated with MPP^+, for 10 h; T+M, 10h – Cells were pre-treated with TUDCA for 12 h, following MPP^+ administration for another 10 h. $^{**}p<0.05$ vs. Control; $^{***}p<0.001$ vs. Control.

Overall, the results obtained suggest that TUDCA exerts an antioxidant role against ROS-mediated damage, following MPTP/MPP^+ exposure, by activating the master regulator of cellular redox status, Nrf2, and consequently increasing the expression of the different cytoprotective enzymes.

IV. Discussion

Mitochondrial dysfunction and oxidative stress play a critical role in the pathogenesis of PD. In this way, therapeutic compounds that target mitochondria are considered promising therapies to counteract oxidative stress. Unfortunately, the use of exogenous antioxidants, as a possible therapeutic strategy, failed due to two situations: i) since these exogenous antioxidants do not cross efficiently the BBB, high amounts are needed to achieve considerable protective effects; and ii) these antioxidants develop toxic effects at higher doses, limiting their administration (Moosmann and Behl, 2002). In this regard, the need for alternative therapeutic strategies to counteract ROS-mediated damage and restore the redox cellular balance is of utmost importance. Thus, a promising arising target against oxidative stress is the transcription factor Nrf2, which is responsible for inducing the expression of several endogenous cytoprotective enzymes. Importantly, in a pathological situation, ROS production overwhelms the cytoprotective effects of the Nrf2-controlled genes, allowing ROS-mediated damage to occur and further neurodegeneration (de Vries *et al*., 2008). Consequently, the use of potential Nrf2 inducers may be a possible therapeutic strategy to be implemented in PD. Therefore, in this thesis, we investigated if TUDCA, an endogenous bile acid with antioxidant and neuroprotective properties, could be considered a promising agent to limit ROS-mediated damage, through Nrf2 activation, in two experimental models of PD.

Our results regarding the effect of TUDCA in the Nrf2 pathway, in C57BL/6 male mice, revealed that pre-treatment with TUDCA positively modulates Nrf2 protein levels, in both striatum and midbrain. Notably, we found that when TUDCA is administered after the toxic stimulus, it is also capable of modulating Nrf2 expression. This is a very interesting result suggesting that the neuroprotective properties of TUDCA are also effective after the toxic insult.

As previously mentioned, Nrf2 activation, under oxidative stress situations, results in the transcription of different cytoprotective enzymes (Bryan *et al*., 2013). In accordance, our results demonstrated that in the presence of MPTP, protein levels of the cytoprotective enzymes Gpx1, HO-1 and SOD2 were increased in both striatum and midbrain, revealing possibly a compensatory response,

by the cell, against MPTP-induced oxidative stress. Similar results were obtained in previous studies, suggesting that Nrf2 activation may be part of a general adaptive response by the cells against oxidative stress (Tripanichkul *et al*., 2007; Chen *et al*., 2009 and Wang *et al*., 2014). Interestingly, our results in mice striatum showed that TUDCA treatment further enhanced the MPTP-triggered expression of Nrf2 downstream targets. These findings are in agreement with several reports that demonstrated that Nrf2 activation can be further induced by antioxidant agents, increasing the expression of different antioxidant enzymes, allowing cell protection against the deleterious effects of ROS (Na *et al*., 2008; Zou *et al*., 2014). Unfortunately, preliminary results from midbrain samples were not so clear. Due to technical problems, that we could not solve in the course of this thesis, we only performed two (for HO-1 detection) or three (for Gpx1 and SOD2 detection) independent experiments, whereas for striatum samples we were able to perform four independent experiments. Therefore, to evaluate completely the effect of TUDCA in mice midbrain, further experiments are needed, and are planned for a near future. In agreement with our previous results regarding the evaluation of Gpx1, HO-1 and SOD2 protein levels, qRT-PCR analysis also revealed that TUDCA has a positive effect in the mRNA levels of these cytoprotective enzymes. Together, these results confirm the ability of TUDCA to activate Nrf2, in an experimental model of PD, with consequences on the expression of its downstream target enzymes, by increasing their protein, as well as mRNA levels. Remarkably, we also showed that TUDCA up-regulates Gpx activity in mice striatum and midbrain. This particularly important result shows that TUDCA not only induces the expression of antioxidant enzymes, but may also increase their biological activity, supporting once more the positive effect of TUDCA in Nrf2-controlled genes.

In addition, to strengthen the role of TUDCA in the activation of Nrf2 signaling pathway, we tried to detect Nrf2 translocation *in situ*, in the presence of TUDCA, in brain sections from C57BL/6 male mice, by immunohistochemistry. However, and despite our efforts, these results were not at all conclusive. This difficulty in obtaining these results may be due to the lack of specific antibodies for immunohistochemistry in mouse tissues, as stated by experts on the Nrf2 pathway studies (Zhang, 2006; Cuadrado *et al*., 2009; Zhang, 2010; Cuadrado *et al.*, 2014). Moreover, Nrf2 translocation is a transient effect, and possibly undetected in brain slices from the selected time points evaluated. Consequently, we did not further pursue with this assay. Due to these experimental issues, that we expect to overcome in a near future by analyzing Nrf2 protein levels, in the presence of TUDCA, in cytoplasmic and nuclear extracts from mice striatum and midbrain, we decided to evaluate the effect of TUDCA in Nrf2 translocation, *in vitro*, using SH-SY5Y human cells. According to our initial expectations, these results revealed that pre-treatment with TUDCA culminates in an obvious and significant increase in Nrf2 nuclear translocation in these cells.

Considering the fact that TUDCA increased Nrf2 nuclear translocation in SH-SY5Y cells, we decided to confirm if this nuclear translocation resulted in a positive modulation of HO-1 mRNA levels in this cell line and, as we expected, in the presence of TUDCA mRNA levels of HO-1 were increased.

These results obtained in neuroblastoma cells are in agreement and complement the results obtained in mice brains.

Summarizing, we have demonstrated in mice treated with MPTP that TUDCA administration increases Nrf2 expression levels, culminating in an increase in both protein and mRNA levels of the different antioxidant enzymes, as well as in an increase in the biological activity of the cytoprotective enzyme Gpx. Moreover, in MPP^+ treated cells, TUDCA induces Nrf2 nuclear translocation, with the consequent increase in HO-1 mRNA levels. Together, these findings strongly suggest that TUDCA up-regulates the different cytoprotective enzymes, through modulation of the Nrf2 signaling pathway, opening a possibility for the beneficial effects of this bile acid in human disease.

The multifunctional protein, DJ-1, upon exposure to oxidative stress, is responsible for the activation of several different antioxidant responses (Canet-Avilés *et al*., 2004). As previously mentioned in section *I. Introduction*, several studies demonstrated that the antioxidant response of DJ-1 may result from its modulation of the Nrf2 signaling pathway. Interestingly, our results demonstrated that upon exposure to MPTP, protein levels of DJ-1 increased, in both striatum and midbrain, contrary to our initial expectations, since several studies demonstrated that MPTP insult reduces the expression of this beneficial protein (Zhou *et al*., 2011; Khasnavis and Pahan, 2014). However, these results are in agreement with ours, demonstrating an up-regulation of Nrf2 by MPTP, as a possible protective strategy in order to fight oxidative stress. These differences between our results and the previously described could be due to experimental variabilities such as, the age of the mice that were used, as well as dose and exposure time to MPTP. Importantly, and in line with our previous findings, pre-treatment and post-treatment with TUDCA enhanced DJ-1 expression, in both striatum and midbrain samples. Since DJ-1 is responsible for Nrf2 stabilization and further nuclear translocation, these results may suggest that TUDCA modulation of the Nrf2 signaling pathway involves the activation of this redox-sensitive protein. Further studies should be performed to investigate this possibility.

Oxidative stress plays a crucial role in both PD and MPTP-mediated Parkinsonism (Carvalho *et al*., 2013; Dias *et al*., 2013). In fact, it has been well documented that MPTP administration results in severe oxidative stress, caused by an excessive increase in ROS production (Sriram *et al*., 1997; Castro-Caldas *et al*., 2012*b*). Therefore, to further explore the antioxidant role of TUDCA on cellular redox status, we analyzed its ability to modulate ROS formation and lipid peroxidation, in the presence of MPTP and/or MPP^+. Our results, in striatum and midbrain samples, demonstrated that in the presence of MPTP, the intracellular ROS levels were practically unaltered, when compared to controls, contradicting our initial expectations and the previous studies mentioned. In mice striatum, it was also possible to observe that neither pre-treatment nor post-treatment with TUDCA caused any significant changes in the intracellular ROS levels, in the presence of MPTP. However, it was possible to notice a slight decrease in ROS production at the time point we have previously observed the expression peak of the different cytoprotective enzymes, i.e., when the animals were treated with TUDCA for three consecutive days, followed by MPTP administration for 3 h. In mice midbrain, it was possible to observe

that in the presence of TUDCA, ROS levels were always slightly decreased. Although not significant, these results suggest that TUDCA, in the presence of MPTP, is capable of modulating ROS formation. Relevantly, these unexpected results, mainly regarding the lack of ROS induction by MPTP, and consequently an unobserved significant protective role of TUDCA, may result from the time points and conditions evaluated in these experiments. Probably, we failed to detect the peak of ROS production, and significant results could emerge if we had used mice exposed to MPTP for shorter periods. Unfortunately, these experiments could not be performed in the time course of this thesis. Since these results were inconclusive regarding the role of TUDCA in the modulation of ROS levels, and to avoid the use of more animals, we decided to explore the role of TUDCA on cellular redox status, *in vitro*. Our results using SH-SY5Y cell cultures revealed that pre-treatment with TUDCA attenuated significantly the MPP^+-triggered ROS formation. These results suggest that TUDCA is capable of modulating ROS production in this cell model of PD, confirming its antioxidant role. Notably, our results also revealed that TUDCA significantly prevented MPP^+-induced lipid peroxidation, in the same cell cultures. This set of results regarding the effect of TUDCA on cellular redox status, together with the previous results *in vitro,* regarding Nrf2 activation and HO-1 expression, demonstrate that TUDCA has effective protective roles against MPP^+ toxicity, by modulating the intracellular oxidative environment, further supporting its antioxidant role in this model of PD.

Ideally neuroprotective agents should be administered before the first symptoms of the disease appear, in other words, when there are still cells to be protected (Emborg, 2004). In this way, our results revealed that when TUDCA is administered before MPTP, this endogenous bile acid is capable of performing its neuroprotective and antioxidant roles, modulating the Nrf2 signaling pathway, as well as the intracellular oxidative environment. Curiously, it was also observed that TUDCA administration after MPTP was also effective. This is an interesting result demonstrating that neuroprotective interventions, with TUDCA, early after the toxic stimulus could be also effective. This is particularly relevant in PD, since its clinical diagnosis only occurs when the degenerative process was already triggered and about 60-80% of DA neurons have already died. Additionally, the ability of TUDCA to exert neuroprotective effects after the toxic insult is also mostly important for long term progressive diseases like PD, to prevent further degeneration of the remaining neurons.

In short, all the results here presented demonstrate that TUDCA modulates the cellular redox status by activating the Nrf2 signaling pathway and consequently, increasing the expression and/or activity of the cytoprotective enzymes, further attenuating the deleterious events caused by MPTP/MPP^+. Therefore, this lead us to conclude that TUDCA is a promising agent to limit ROS-mediated damage in PD, through Nrf2 activation, further supporting its clinical application. However, to further support our conclusions, additional experiments are needed, using Nrf2 knockdown or knockout strategies in cells treated with TUDCA and/or MPP^+, followed by measurement of oxidative stress indicators.

Finally, the benefits of TUDCA, as a neuroprotective and antioxidant agent that efficiently crosses the BBB with no associated toxicity, together with the promising results obtained in prior studies (from

our and other groups) and the work presented in this thesis, it is plausible to think that TUDCA is in fact, a potential therapeutic agent to be implemented in the treatment of Parkinson's disease.

Figure IV.1 illustrates a simplified scheme of the possible involvement of the Nrf2 signaling pathway in TUDCA prevention of ROS-mediated damage.

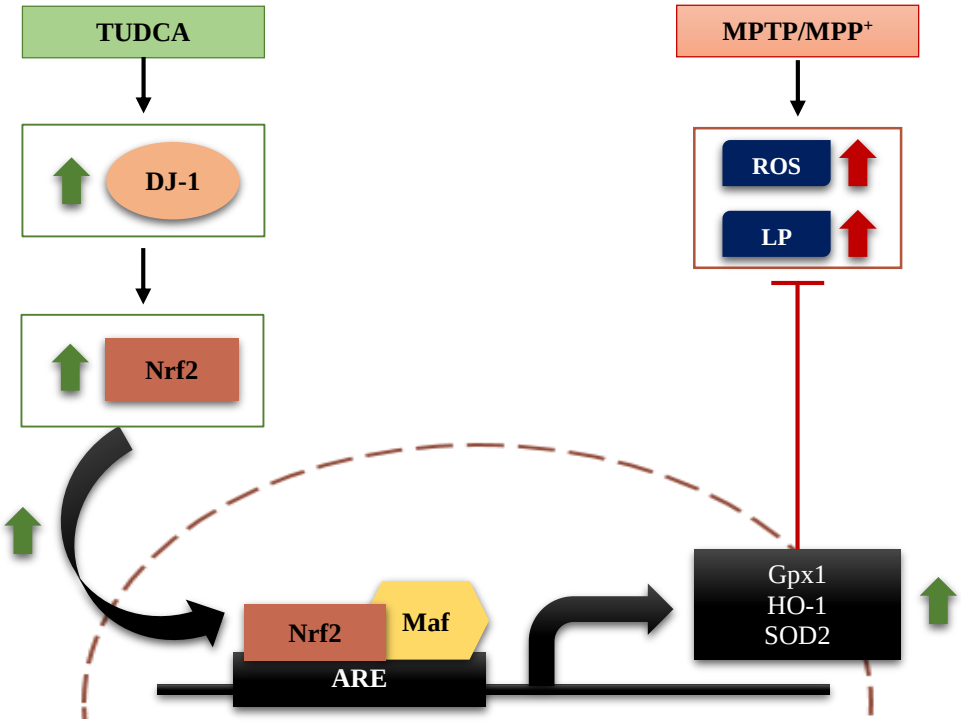


Figure IV.1 – Illustrative representation involving the Nrf2 signaling pathway in the neuroprotective and antioxidant role of TUDCA. TUDCA increases DJ-1 expression, possibly culminating in an increase in Nrf2 stabilization and consequent nuclear translocation, which leads to an increase in the expression of Nrf2-controlled genes (Gpx1, HO-1 and SOD2), as well as in Gpx activity. This rise in the antioxidant defense mechanism, elicited by TUDCA, attenuates lipid peroxidation and also ROS formation induced by the neurotoxin MPTP/MPP^+.

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