

Universidade de Lisboa

Faculdade de Farmácia



Neuroprotective effects of TUDCA in Parkinson's disease: Dissecting the anti-oxidant and anti-inflammatory effects of this bile acid in the mouse cerebral cortex

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Dissertação elaborada para obtenção do Grau de Mestre em Ciências Biofarmacêuticas

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Part of the results discussed in this thesis were presented in the following meetings:

M.O. Mendes, A.I. Rosa, M. J. Nunes, A. Carvalho, S. Duarte-Silva, A. Silva-Fernandes, E. Rodrigues, P. Maciel, C.M.P. Rodrigues, M.J. Gama, M. Castro-Caldas. Neuroprotective effects of Tauroursodeoxycholic acid in MPTP model of Parkinson's disease: A study in the mouse cortex. MitoPorto - Advances in mitochondrial research, 26th May, 2017, Universidade do Porto. [Poster communication];

M.O. Mendes, A.I. Rosa, M. J. Nunes, A. Carvalho, S. Duarte-Silva, A. Silva-Fernandes, E. Rodrigues, P. Maciel, C.M.P. Rodrigues, M.J. Gama, M. Castro-Caldas. Extended neuroprotective effect of Tauroursodeoxycholic acid to the mouse cerebral cortex in a MPTP model of Parkinson's disease. 9th iMed.UL post-graduate students meeting, 13th-14th July, 2017, Faculty of Pharmacy, Universidade de Lisboa. [Poster communication];

This work was supported by National funds (Fundação para a Ciência e Tecnologia – FCT, Portugal) with the project PTDC/NEUNMC/0248/2012 and with the project PEst-OE/SAU/UI4013/2011.



Abstract

Parkinson's disease (PD) is a progressive neurological disorder, mainly characterized by the progressive loss of dopaminergic neurons in the substantia nigra *pars compacta* (SNpc) and by the presence of intracellular inclusions, known as Lewy bodies. Although the main cause of this disorder remains elusive, neurodegeneration has been associated with different mechanisms of cell damage including oxidative stress, mitochondrial dysfunction and neuroinflammation. Despite SNpc being considered the primary affected region in PD, the neuropathological features may not solely be confined to the nigro-striatal axis. It is conceivable that other brain regions are also affected, namely the cerebral cortex, being the neurologic insult to this brain region still not completely unraveled.

Tauroursodeoxycholic acid (TUDCA) is an endogenous bile acid that has been shown to have antioxidant properties and to exhibit a neuroprotective effect in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mice model of PD. Moreover, TUDCA anti-inflammatory properties have been reported in glial cells, making it a prominent therapeutic agent for PD treatment and relevant the pursuit of its mechanism of action in this disease.

In this thesis we used C57BL/6 mice injected with MPTP in a sub-acute paradigm aiming to investigate if the neurotoxic effects of MPTP could be extended to the cerebral cortex. Moreover, we intended to dissect the anti-oxidant and neuroprotective effects of TUDCA in this brain region. In addition, we proposed to investigate the anti-inflammatory mechanisms triggered by this bile acid, in the cerebral cortex and in microglia cells, namely its ability to modulate Annexin-1 (ANXA1) expression and secretion.

Our results revealed that in mice cortex TUDCA: i) increases the expression of nuclear factor erythroid 2 related factor 2 antioxidant downstream targets, namely Glutathione peroxidase 1 and Heme oxygenase 1 enzymes, upon MPTP exposure; ii) up-regulates parkin expression and AMP-activated protein kinase activation, and prevents MPTP-induced ATP depletion; and iii) decreases inflammatory makers of astrogliosis and microgliosis, and up-regulates the expression of the anti-inflammatory protein ANXA1. Additionally, results from cellular models corroborate TUDCA modulation of ANXA1 synthesis and secretion and point to the possibility of this protein being a direct target of this bile acid.

Together, these results reinforce the potential benefits of using TUDCA as a therapeutic strategy in the treatment of PD.

Keywords: ANXA1, Mitochondrial Dysfunction, MPTP, Neuroinflammation, TUDCA;

Resumo

A doença de Parkinson (DP) é uma doença neurodegenerativa progressiva, caracterizada principalmente pela perda gradual de neurónios dopaminérgicos na *substantia nigra pars compacta* (SNpc) e pela presença de inclusões intracelulares denominados corpos de Lewy. Apesar da principal causa desta doença permanecer desconhecida, o processo de neurodegenerescência tem sido associado a diferentes mecanismos de dano celular tais como *stress* oxidativo, disfunção mitocondrial e neuroinflamação. Apesar da SNpc ser considerada a principal região afectada na DP, as características neuropatológicas podem não estar exclusivamente confinadas ao eixo nigro-estriado. É concebível que outras regiões cerebrais possam estar também afectadas, nomeadamente o córtex cerebral, sendo que o insulto neurológico nesta região cerebral ainda não está completamente desvendado.

O ácido tauroursodesoxicólico (TUDCA) é um ácido biliar endógeno que tem mostrado ter propriedades antioxidantes e exibir um efeito neuroprotector num modelo da DP em murganhos tratados com 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP). Para além disso, as propriedades anti-inflamatórias do TUDCA também foram descritas em células da glia tornando-o num agente terapêutico importante para o tratamento da DP, e tornando relevante a investigação sobre o seu mecanismo de acção nesta doença.

Nesta tese usámos murganhos C57BL/6 injectados com MPTP, num regime experimental sub-agudo, com o objectivo de investigar se os efeitos neurotóxicos do MPTP se estendem ao córtex cerebral. Para além disso, dissecámos os efeitos antioxidantes e neuropotectores do TUDCA nesta região cerebral. Procurámos ainda investigar o mecanismo anti-inflamatório despoletado por este ácido biliar, no córtex cerebral e em células da micróglia, nomeadamente a capacidade do TUDCA modular a expressão e secreção da Anexina-1 (ANXA1).

Os nossos resultados, no córtex cerebral de murganhos, revelaram que o TUDCA: i) aumenta a expressão dos alvos antioxidantes do *nuclear factor erythroid 2 related factor 2*, nomeadamente as enzimas Glutathione Peroxidase 1 e Heme-oxigenase 1, após exposição ao MPTP; ii) regula a expressão proteica da *parkin*, induz a activação da *AMP-activated protein kinase*, e previne a depleção de ATP induzida pelo MPTP; e iii) diminui os marcadores inflamatórios característicos de astrogliose e microgliose, e regula a expressão da proteína anti-inflamatória ANXA1. Além disso, os resultados dos modelos celulares corroboraram a regulação da expressão e secreção da ANXA1 pelo TUDCA, sugerindo a possibilidade desta proteína ser um alvo directo deste ácido biliar.

Em conjunto, os resultados apresentados neste trabalho contribuem para reforçar os potenciais benefícios do uso do TUDCA como estratégia terapêutica no tratamento da DP.

Palavras-chave: ANXA1, Disfunção mitocondrial, MPTP, Neuroinflamação, TUDCA;

Agradecimentos

As minhas primeiras palavras de agradecimento são dirigidas à Professora Doutora Cecília Rodrigues pela disponibilidade e orientação ao longo do Mestrado em Ciências Biofarmacêuticas, em especial pela ajuda que me deu na procura do tema desta tese e por me ter recebido no seu grupo “Cellular Function and Therapeutic Targeting”.

Um enorme agradecimento à Professora Doutora Margarida Castro-Caldas por me ter proporcionado esta oportunidade e pela ajuda e orientação constantes. Obrigada por todo o rigor, exigência, sugestões e conhecimento transmitidos. Agradeço ainda a disponibilidade constante para me esclarecer dúvidas e aconselhar (mesmo quando não podia estar presencialmente), toda a paciência que teve comigo e todas as palavras de encorajamento e motivação (até quando os resultados não eram assim tão promissores). Aprendi e cresci bastante neste ano e grande parte deve-o a si, espero não a ter desapontado.

À Professora Doutora Maria João Gama agradeço a disponibilidade em co-orientar esta tese, as valiosas sugestões que me deu e todas as dúvidas que se propôs a responder. Agradeço ainda a preocupação e palavras de incentivo transmitidas ao longo deste ano, principalmente na recta final.

À Professora Doutora Elsa Rodrigues agradeço o rigor e boas práticas que exigiu desde o primeiro dia no laboratório, essenciais para o meu crescimento e sem as quais já não consigo trabalhar. Agradeço ainda a boa disposição constante, a prontidão para esclarecer as minhas dúvidas e todas as vezes que no final de um longo dia validava o meu trabalho dizendo que “todo o meu esforço não era em vão e iria certamente dar frutos”.

À Alexandra deixo o meu obrigada, apesar de não teres estado comigo “no meio” da tese, estiveste presente no início e no fim, e a tua orientação e ajuda foi fundamental para que este trabalho começasse com o pé direito.

À Andreia, a “Doutorada de Serviço”, quero agradecer a simpatia, a ajuda e o encorajamento que me deste. As tuas histórias de experiências trágicas e finais felizes serviram para me dar um pouco de alento quando as coisas não corriam tão bem e a tua boa disposição e relativização dos problemas ajudou-me a descontrair um pouco mais. Obrigada.

A todos os elementos do CellFun quero expressar a minha gratidão, não só pela maneira como me integraram mas também por toda a ajuda que me deram e dúvidas que esclareceram ao longo deste ano. Não é fácil ser a pessoa nova no grupo, muito menos uma aluna de mestrado no

meio de alunos de doutoramento e professores. Dito isto, acredito que também seja complicado lidar com este vai-e-vem de alunos verdinhos e propensos a fazer asneiras. Contudo o companheirismo, a preocupação e a disponibilidade em ajudar são evidentes neste grupo de pessoas fantásticas que nunca me fizeram sentir excluída nem sentir que as minhas questões não eram válidas, por isso muito obrigada Maria, Pedro, André, Simão, Diane, Sofia, Tânia, Marta, Hugo, Sara, Vanda, Susana, Rui, Joana e Dionísio (especialmente por todas as células que me passaste e o tempo que perdeste comigo a desvendar o feitio temperamental das mesmas).

À Carolina, a minha companheira de cave e portuense favorita (sinceramente não conheço muitas pessoas do norte) queria agradecer a companhia, os risos, os desabafos e o desespero conjunto, aquela cave seria muito mais depressiva se não a partilhasse contigo. Orgulho-me em dizer que no final deste ano já estávamos uma “máquina bem oleada” a trabalhar em conjunto, e a única coisa que me arrependo foi não começarmos a ouvir rádio no laboratório mais cedo.

À Marta tenho a agradecer a minha sanidade mental! Obrigada pelos cafés, almoços e jantares, por me aturares 8 a 12 horas por dia, por me fazeres companhia até às 10h da noite no CPM só para eu não ficar sozinha, pelas conversas, gargalhadas e muito mais. Este ano teria sido muito diferente se não o tivesse partilhado contigo, e ainda bem que assim foi.

Queria agradecer a minha família, sem a qual todo o meu percurso académico não seria possível. Um obrigada especial à minha Mãe por tudo o que atura e por ser o meu porto de abrigo, não há palavras para descrever o quão grata eu estou de me teres “calhado na rifa”. Ao meu Pai e ao meu Irmão, que também estão neste barco e tem que levar com as minhas diversas tempestades, mas no final estão sempre lá para me apoiar e encorajar. À minha querida Avó, que apesar de não perceber porque é que eu passo tanto tempo ao computador, está sempre aqui para me mimar, como só as avós sabem. Não podia deixar de agradecer à minha Tia Lurdes por todo o apoio e concelhos que me dá, e ao resto da minha família que estando longe ou perto sei que com eles posso sempre contar.

Por fim, e como não podia deixar de ser, quero agradecer aos meus amigos que me perdoam as longas ausências e estão sempre dispostos a receber-me de volta de braços abertos. Posso não mostrar e ser uma amiga desnaturada, mas sem vocês este meu ser “anti-social” seria muito infeliz, por isso OBRIGADA do fundo do coração pela vossa amizade, paciência e compreensão!
P.S: Já estou de volta ao mundo real!

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Abbreviations

6-OHDA	6-hydroxydopamine
ADP	Adenosine diphosphate
AKT	Protein kinase B
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANXA1	Annexin-1
ARE	Antioxidant response element
ARG1	Arginase 1 enzyme
ATP	Adenosine triphosphate
BAX	Bcl-2-associated X
BBB	Brain blood barrier
BDNF	Brain-derive neurotrophic factor
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
COX-2	Cyclooxygenase 2
CNS	Central nervous system
CRE	cAMP responsive element
CREB	cAMP responsive element binding protein
DA	Dopamine
DAMPs	Danger associated molecular patterns
DAT	Dopamine transporter
DCF	2',7'-dichlorofluorescein
DCF-DA	2',7'-dichlorofluorescein diacetate
DNA	Deoxyribonucleic acid
ESR	Electron Spin Resonance

ETC	Electron transport chain
FADH₂	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GFAP	Glial fibrillary acidic protein
GPBAR1	G protein-coupled bile acid receptor 1
GPxs	Glutathione peroxidases
HBSS	Han's balanced salt solution
HBSS-2	HBSS without Ca ²⁺ and Mg ²⁺
HOs	Heme oxygenases
Iba-1	Ionized calcium binding adaptor molecule-1
IL-1β/4/6/10/12/13	Interleukins
iNOS	Inducible nitric oxide synthase
i.p	Intraperitoneally
Keap1	Kelch-like ECH-associated protein 1
LB	Lewy bodies
LPS	Lipopolysaccharide
LRRK2	Leucine-rich repeat kinase 2
MAO-A	Monoamine oxidase A
MAO-B	Monoamine oxidase B
MAF	Musculoaponeurotic fibrosarcoma
MAP2	Microtubule-associated protein 2
MHC	Major Histocompatibility Complex
MPP	Mitochondrial processing peptidase
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
NADH	Nicotinamide adenine dinucleotide
NF-E2	Nuclear factor erythroid 2
NF_κB	Nuclear factor-kappa B
NO	Nitric Oxide
NRF-1	Nuclear regulatory factors
Nrf2	Nuclear factor erythroid 2 (NF-E2) - related factor 2
OCT-3	Organic cation transporter 3
OMM	Outer mitochondrial membrane
PAMPs	Pathogen-associated molecular patterns
PARIS	parkin-interacting substrate
PARL	Presenilin-associated rhomboid-like protein
PD	Parkinson's disease
PGC-1α	Peroxisome proliferator-activated receptor γ coactivator - 1 α
PINK1	Phosphatase and tensin homolog (PTEN)-induced putative kinase 1
PKA	Protein kinase A
PRRs	Pattern recognition receptors
PVDF	Polyvinyl difluoride
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulphate-polyacrilamide gel electrophoresis
SNpc	Substantia nigra <i>pars compacta</i>
TGF-β	Transforming growth factor β
TGR5	Takeda G protein-coupled receptor 5
TIM	Translocase of the inner membrane

TLR	Toll-like receptors
TNF-α	Tumor necrosis factor- α
TOM	Translocase of the outer membrane
TUDCA	Tauroursodeoxycholic acid
TUNEL	TdT-mediated dUTP nick end labeling
UDCA	Ursodeoxycholic acid
ULK1	UNC-51-like kinase 1
VMAT2	Vesicular monoamine transporter-2
VTA	Ventral tegmental area

I. Introduction

1. Parkinson's disease

This year marks the 200th anniversary of James Parkinson publication on “An Essay on the Shaking Palsy” where he first described a condition that would one day bear its name (Parkinson, 2002). Parkinson's disease (PD) is the second most common neurodegenerative disorder, following Alzheimer's disease. This progressive motor disorder is pathologically characterized not only by the loss of dopaminergic neurons in the Substantia nigra *pars compacta* (SNpc) and the subsequent depletion of the neurotransmitter dopamine (DA) in striatum, but also by the presence of intracellular inclusions composed by aggregates of α -synuclein and other proteins, known as Lewy bodies (LB) (Dauer & Przedborski, 2003; Goedert *et al.*, 2013; Jellinger, 1999).

PD affects approximately 0,3% of the entire world population (Pringsheim *et al.*, 2014). It is believed that incidence of this disorder increases sharply with age, making the prevalence of PD about 3%, in a population over 65 years of age (Poewe *et al.*, 2017). Therefore an early-onset, occurrence below 50 years of age, is rare and usually associated with genetic factors (Klein & Westenberger, 2012). PD prevalence is also reported to vary with gender and geographic distribution, being twice more common in men than in women, probably due to the protective effect of female sex hormones, gender associated genetic mechanisms or differences in exposure to environmental risk factors (Poewe *et al.*, 2017).

Initial motor manifestations characteristics of PD are strongly linked to the main pathological marker of the disorder, progressive loss of dopaminergic neurons in SNpc. The nigrostriatal pathway is comprised of dopaminergic neurons rich in neuromelanin and their projections to the putamen (Mhyre *et al.*, 2012). Neurodegeneration in this area leads to a

SNpc depigmentation, a reduction of the striatal DA and a consequent difficulty in regulating movement activation and cognitive functions (Song & Kim, 2016). In early stages of the disease, neuron loss is confined to the ventrolateral substantia nigra leaving other midbrain areas exempt, though it becomes more widespread by the end-stage of the disease, corroborating that neurodegeneration and LB formation are not only restricted to dopaminergic neurons but also affect noradrenergic, serotonergic and cholinergic systems, as well as the cerebral cortex, olfactory bulb, and autonomic nervous system in PD (Dauer & Przedborski, 2003; Poewe *et al.*, 2017).

Even though the initial diagnosis of PD rests upon clinical observation of prominent motor features, symptoms can be divided in two categories: motor symptoms, including rest tremor, rigidity, bradykinesia (slowness of movement) and postural instability; and non-motor symptoms, including depression and cognitive decline (Rodriguez-Oroz *et al.*, 2009). Additionally, other important non-motor and motor symptoms, as well as their manifestation over time are recount in Figure I.1. However, by the time clinical manifestations of PD are noticeable 50% to 70% of dopaminergic neurons have already been lost, making it more difficult to apply neuroprotective therapies in an early state of the disease (George *et al.*, 2009).

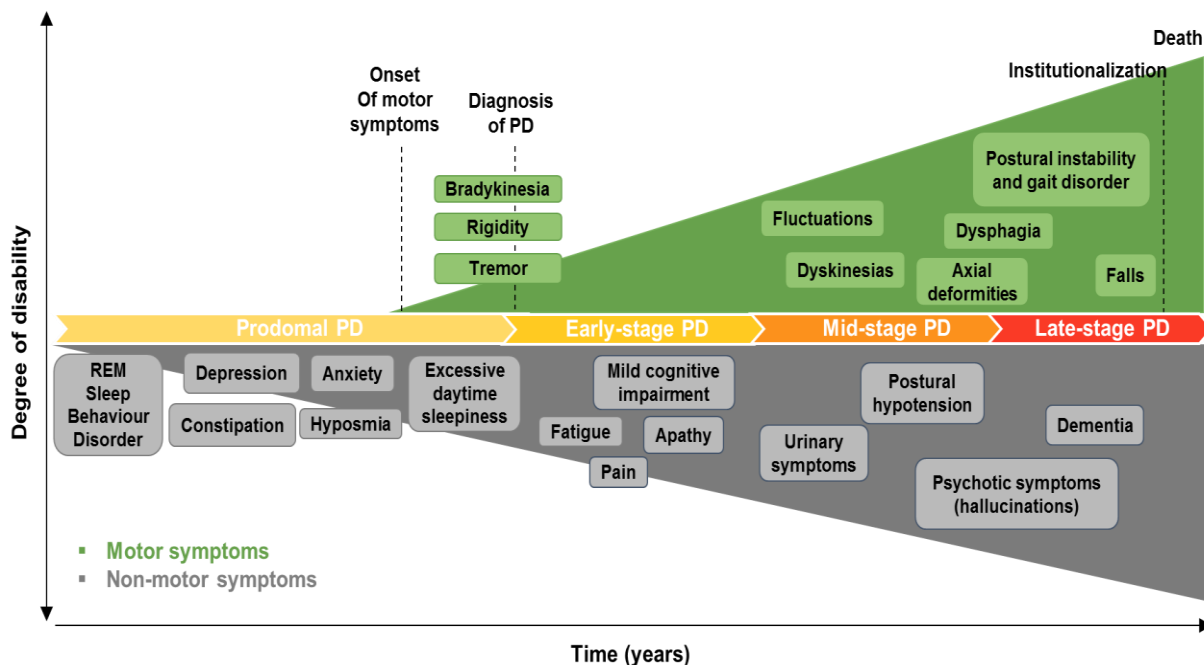


Figure I.1 – Clinical Parkinson symptoms associated with over time disease progression. Parkinson's disease diagnosis occurs with the onset of motor symptoms (early-stage Parkinson disease), but can remain undiagnosed and with manifestation of specific non-motor symptoms for years (prodomal Parkinson disease). Over disease progression, non-motor symptoms become more pronounced and widespread and motor symptoms increase severity. REM – Rapid eye movement; PD – Parkinson's Disease; Adapted from *Poewe et al.* (2017).

So far, PD existing therapies such as replacement of DA by L-Dopa or deep brain stimulation can only relieve disease symptoms but cannot stop disease progression. Therefore, furthering our understanding of PD pathogenesis it is crucial to the development of successful therapeutic strategies (Orth & Tabrizi, 2003).

Despite the extensive research, mechanisms underlying neuropathology of PD are still largely unknown. However it is believed that multiple genetic as well as environmental factors play critical roles in the development of PD. Heritable forms of this disease (Familial PD) only account for 5–10% of incidence, which means that most cases are a sporadic form of the disorder, and likely represent the interplay between genetic and environmental factors (Lill, 2016; Warner & Schapira, 2003). Mutations in genes indisputably related to heritable monogenic PD are responsible for either autosomal dominant or recessive forms of the disease. Loss of function mutations in *α-synuclein* and *Leucine-rich repeat kinase 2* (LRRK2) genes cause underlie dominant PD forms, whereas *parkin*, *Phosphatase and tensin homolog (PTEN)-induced putative kinase 1* (PINK1) and *DJ-1* lead to recessive forms of the disease (Gasser, 2015; Klein & Westenberger, 2012).

Familial PD although rare, has had an enormous contribute in unraveling molecular pathways involved in dopaminergic cell loss, including *α-synuclein* proteostasis, mitochondrial function, oxidative stress, calcium homeostasis, axonal transport and neuroinflammation, expanding our understanding and making possible the discover of new therapeutic targets, illustrated in Figure I.2 (Poewe *et al.*, 2017).

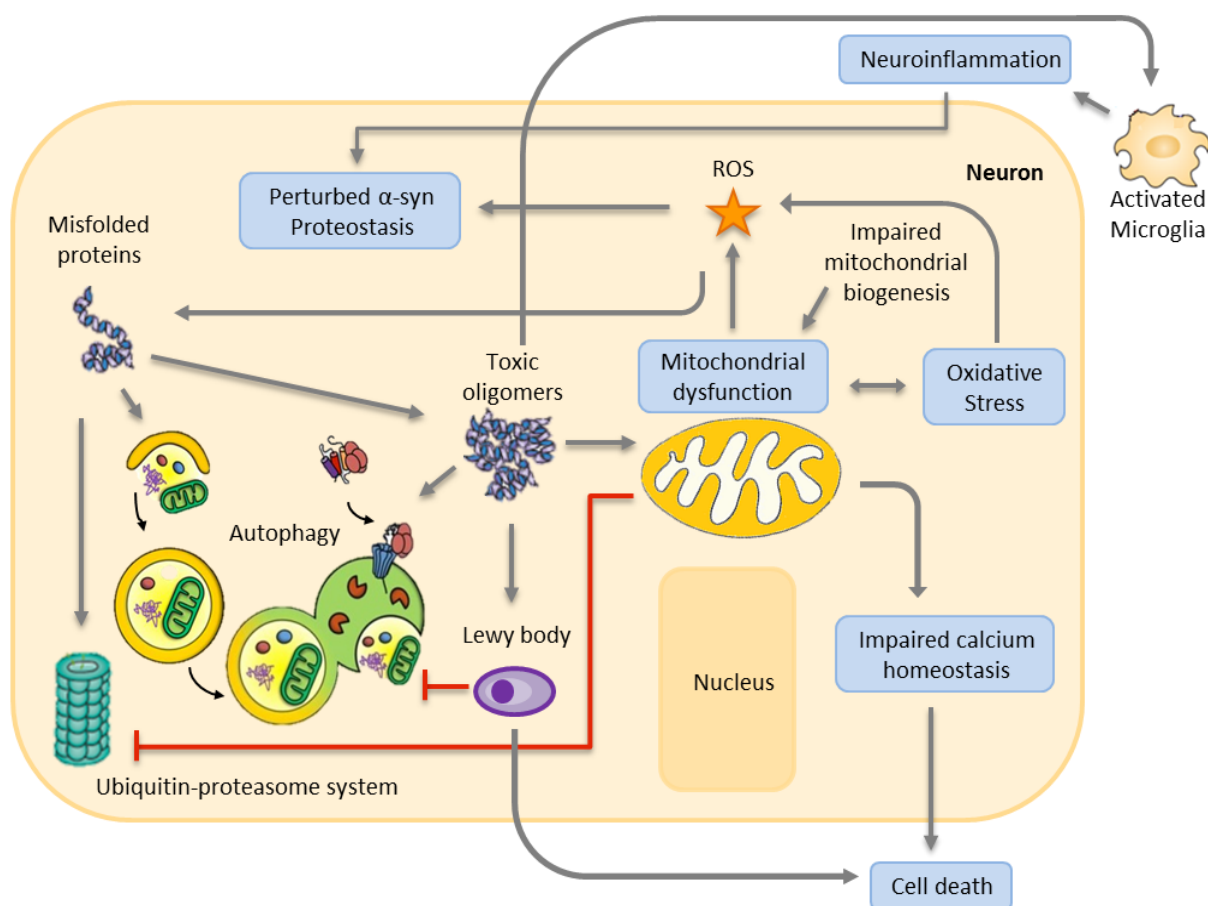


Figure I.2 – Molecular mechanisms involved in Parkinson’s disease. Schematic representation of the molecular pathways implicated in PD pathogenesis and the interplay between them. Adapted from Poewe *et al.* (2017).

2. Experimental Models of PD

Regardless of the progress that has been made to understand the underlying mechanisms that leads to the development of PD and the molecular pathways involved in the progression of this disease, there is still a lot to unravel. Nonetheless, the important advances made in 200 years of PD research are greatly due to experimental models of the disease. These models can be divided in two categories, genetic and neurotoxic models used in both *in vivo* and *in vitro* experiments (Blesa & Przedborski, 2014). Although experimental models are not perfect and cannot precisely reproduce all features of PD, they are essential to broaden our knowledge about the disease in hopes of discovering new therapeutic targets and strategies for combating it.

2.1 Neurotoxin based models of PD

Ideally, models of PD should portrait both pathological and clinical features of the disease. This means that to be considered a fitted model of PD, non-dopaminergic and dopaminergic systems should be involved, as well as non-motor and motor symptoms (Tieu, 2011). Even though none of the current models display all those attributes, the neurotoxin based models remain preferable to genetic ones. Despite giving us great insight into the molecular mechanisms of PD, present genetic models are incapable of reproducing a considerable degeneration of dopaminergic neurons in contrast with some neurotoxic models that generate selective neuronal death (Bove *et al.*, 2005; Chesselet *et al.*, 2008; Dawson *et al.*, 2010).

Within the group of different neurotoxic compounds responsible for inducing degeneration of dopaminergic neurons such as rotenone, paraquat, methamphetamines, 6-hydroxydopamine (6-OHDA), the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) serves as the most widely used and better characterized neurotoxic experimental model of PD (Blesa & Przedborski, 2014; Duty & Jenner, 2011; Orth & Tabrizi, 2003).

MPTP was first discovered around 1982 in California, when a group of young drug addicts started to develop severe symptoms similar to the ones in PD, after intravenously administrating a street preparation of 1-methyl-4-phenyl-4-propionpiperidine (MPPP), a meperidine analog. Thereafter, it was revealed that this new “synthetic heroin” was not only comprised of MPPP but was in fact a mixture of this compound and a synthesis by-product, MPTP. Further investigation in to patients’ condition etiology and progression, unveil MPTP as the culprit behind their clinical state and responsible for nigral cell death, accessed after patients’ *postmortem* evaluation (Langston *et al.*, 1983; Langston *et al.*, 1999).

2.1.1 MPTP mechanism of action

After the discovery of MPTP parkinsonogenic effects it was necessary to understand its underlying molecular mechanisms of action and particularly the high targeted dopaminergic neurons. Although MPTP by itself is not toxic, as a highly lipophilic compound it can easily cross the brain blood barrier (BBB) after being systemically administered (Langston, 2017). Once in the brain, MPTP is oxidize into its toxic metabolite, 1-methyl-4-phenylpyridinium (MPP⁺) by the monoamine oxidase B (MAO-B) enzyme, within astrocytes and serotonergic neurons (Chiba *et al.*, 1984; Langston *et al.*, 1984; Ransom *et al.*, 1987; Shen *et al.*, 1985).

Additional studies narrowed down further by proving that solely MAO-B, not even monoamine oxidase A (MAO-A), was responsible for this biotransformation, but also that MAO-B inhibition would prevent MPTP-induced loss of dopaminergic neurons in SNpc (Heikkila *et al.*, 1984).

MPP⁺ is then release by astrocytes and serotonergic neurons into the extracellular space via the organic cation transporter 3 (OCT-3) (Cui *et al.*, 2009). Since it is a polar molecule and cannot easily pass through the plasmatic membrane, the questions were how and why this toxic metabolite selectively targeted dopaminergic neurons. Both Shen and colleagues (1985) and Javitch and collaborators (1985) drew attention to the fact that MPP⁺ has high affinity for the membrane protein responsible for DA uptake, Dopamine Active Transporter (DAT) thus explaining how MPP⁺ is taken up and concentrated into dopaminergic neurons (Javitch *et al.*, 1985; Shen *et al.*, 1985). Considering that in the ventral tegmental area (VTA) dopaminergic neurons membranes are enriched in DAT, MPP⁺ uptake by those membrane transporters does not clarify the selectivity damage induced by MPTP in the nigrostriatal pathway. However, differences in uptake affinity or the presence of calbindin in VTA neurons could be the reason for a lower susceptibility to MPP⁺ when compared with SNpc neurons (Blanchard *et al.*, 1994; Lavoie & Parent, 1991).

Once taken up by dopaminergic neurons, MPP⁺ can follow three different pathways; i) it can bind to the vesicular monoamine transporter-2 (VMAT2) and be stored in synaptosomal vesicles. Vesicular sequestration of MPP⁺ seems to represent an important protective mechanism against neurodegeneration, being that a reduction in VMAT2 activity or a lower DAT/VMAT2 ratio can enhance MPP⁺ toxicity (Miller *et al.*, 1999; Speciale *et al.*, 1998); ii) it can remain in the cytosol and interact with cytosolic enzymes (Dauer & Przedborski, 2003; Klaidman *et al.*, 1993); and/or iii) it can be concentrated in the mitochondria against a concentration gradient via an uptake mechanism energized by transmembrane potential (Ramsay *et al.*, 1986). Once in the mitochondria, MPP⁺ impairs oxidative phosphorylation by blocking the Complex I of the mitochondrial electron transport chain (ETC) (Nicklas *et al.*, 1985). Consequently, this inhibition leads to a decrease in ATP synthesis and an increase in reactive oxygen and nitrogen species production harmful to the cells (Chan *et al.*, 1991). MPTP mode of action is depicted in Figure I.3.

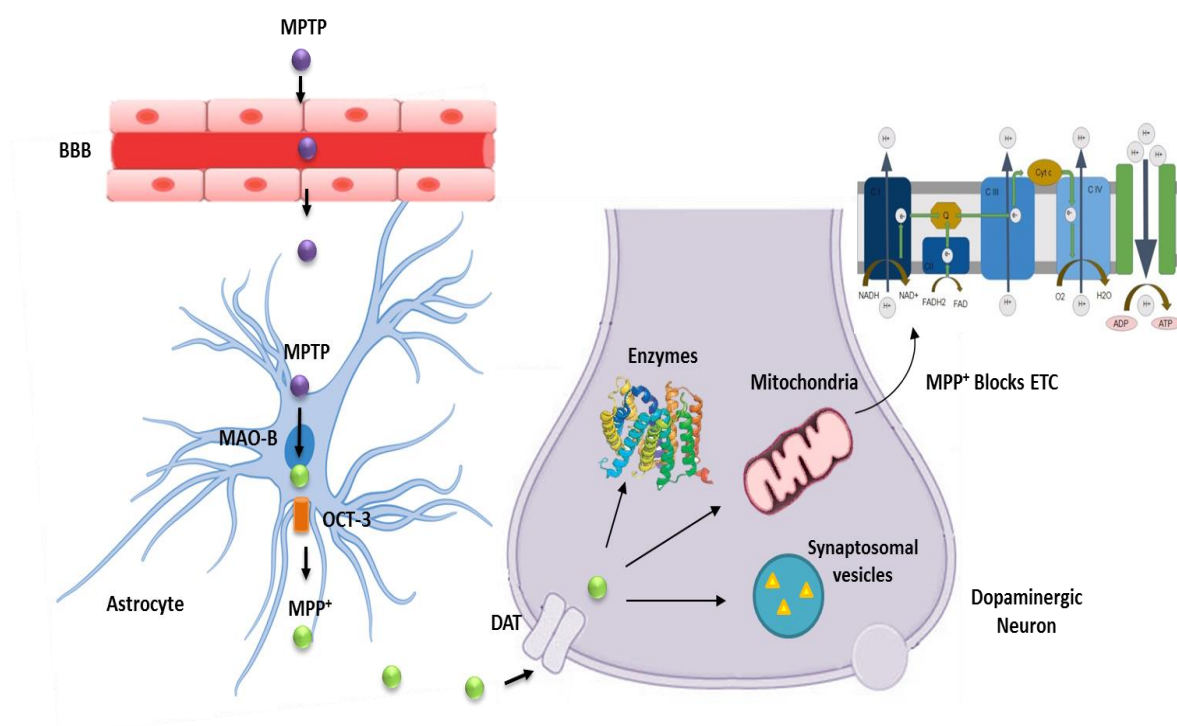


Figure I.3– Schematic representation of MPTP metabolism and MPP⁺ intracellular pathways. MPTP can easily cross the blood-brain barrier (BBB). Once in the brain the neurotoxic is converted to MPP⁺ by Monoamine oxidase B (MAO-B) within astrocytes and serotonergic (not shown), being then released to the extracellular space via the organic cation transporter 3 (OCT-3). Afterwards, MPP⁺ is taken up by dopamine transporter (DAT) allowing it to be concentrated in dopaminergic neurons. Inside the dopaminergic neurons, MPP⁺ it can follow three different pathways: it can concentrate in the mitochondria and inhibit Complex I of the electron transport chain (ETC); it can interact with cytosolic enzymes; and it can be stored in synaptosomal vesicles. MPTP – 1- methyl-4-phenyl-1,2,3,6-tetrahydropyridium; MPP⁺ – 1- methyl-4-phenylpyridium; Adapted from *Dawer and Przedborski (2003)*.

2.1.2 Animal models of PD

In recent years, animal models of PD have played a key role in understanding the pathogenic process behind neuronal loss, conceiving new pharmacological approaches to therapy and developing novel treatment strategies (Duty & Jenner, 2011). Therefore, an ideal animal model should present both clinical and pathological features of PD, including LB formation, steady degeneration of dopaminergic neurons over time without spontaneous recovery and progressive clinical symptoms. Even though no current model mimics the complex neurodegenerative features of PD in humans, a higher similarity to the disease provides a greater model validity for clinical efficacy (Emborg, 2004). Moreover, *in vivo*

models are essential for evaluating neuroprotective capacity of pharmacologic compounds against insults, treatment related complications and side effects (Emborg, 2004).

Each animal model presents specific advantages and disadvantages, being the choice of species, age and sex determined by different aspects including the neurotoxin administered and the questions proposed to be answered. In comparison with non-human primates, cats and dogs, rodent models present various advantages, they are widely available, have high reproductive rates, require reduced cost of maintenance and living space, and bring up less ethical concerns being extensively used as the preferential model of PD (Emborg, 2004; Potashkin *et al.*, 2010).

MPTP susceptibility and lesion not only varies between species, with rats presenting less sensibility to this toxin than mice, but also is affected by gender, age and body weight making male C57BL/6 mice, the optimal reproducible animal model of PD (Przedborski *et al.*, 2001).

3. Mitochondrial dysfunction and oxidative stress

Mitochondria are unique double membrane organelles that carry their own DNA and whose main function is the generation of energy in the form of ATP by oxidative phosphorylation (Keane *et al.*, 2011). Apart from being the powerhouses of eukaryotic cells, mitochondria also play important roles in regulation of cell death via apoptosis, calcium homeostasis and control of cell division and growth (Bose & Beal, 2016; Keane *et al.*, 2011; Szabadkai & Duchen, 2008; Tait & Green, 2013).

Mitochondrial energy production is accomplished by the transport of electrons through a series of five complexes, located in the mitochondrial inner membrane, known as the ETC (Keane *et al.*, 2011). Electrons from oxidative substrates nicotinamide adenine dinucleotide (NADH) or flavin adenine dinucleotide (FADH₂) are conveyed along ETC complexes until they are reduced to water, at the respiratory Complex IV (Winklhofer & Haass, 2010). In the process, enough energy to create an electrochemical gradient is generated and protons are transferred from the mitochondria matrix to the inner membrane by respiratory Complexes I, III and IV. Lastly, as protons re-enter the mitochondrial matrix, ATP is synthesized by respiratory Complex V, ATP synthase (Chan, 2006), depicted in Figure I.4. Even though the electron transport process during oxidative phosphorylation is highly efficient it still can lead to electron leakage and the consequent production of harmful reactive oxygen species (ROS)

(Chan, 2006). Electrons are particularly leaked by respiratory Complexes I and III, and when interact with oxygen originate superoxide species. Although, under normal physiological conditions the imbalance of ROS is relatively low and rapidly converted into water by mitochondrial antioxidants, in a dysfunction mitochondrial state ROS production could lead to the cell death observed in PD (Keane *et al.*, 2011).

Mitochondrial DNA (mtDNA) is also a key factor in mitochondrial respiratory function (Wallace, 2005). Even though the majority of mitochondrial proteins derive from the nuclear genome and are later imported from the cytosol, within the 37 genes of the circular mtDNA, 13 encode protein subunits of respiratory complexes (Chan, 2006). Since mtDNA is closely located near the ETC and its repair mechanisms are limited, becomes more susceptible to damage from ROS generated during oxidative phosphorylation (Tufekci *et al.*, 2011). This damage could lead to mutations that not only implicate mitochondrial respiratory function but also mtDNA replication, transcription or translation processes, being possibly linked to PD pathogenesis (Keane *et al.*, 2011).

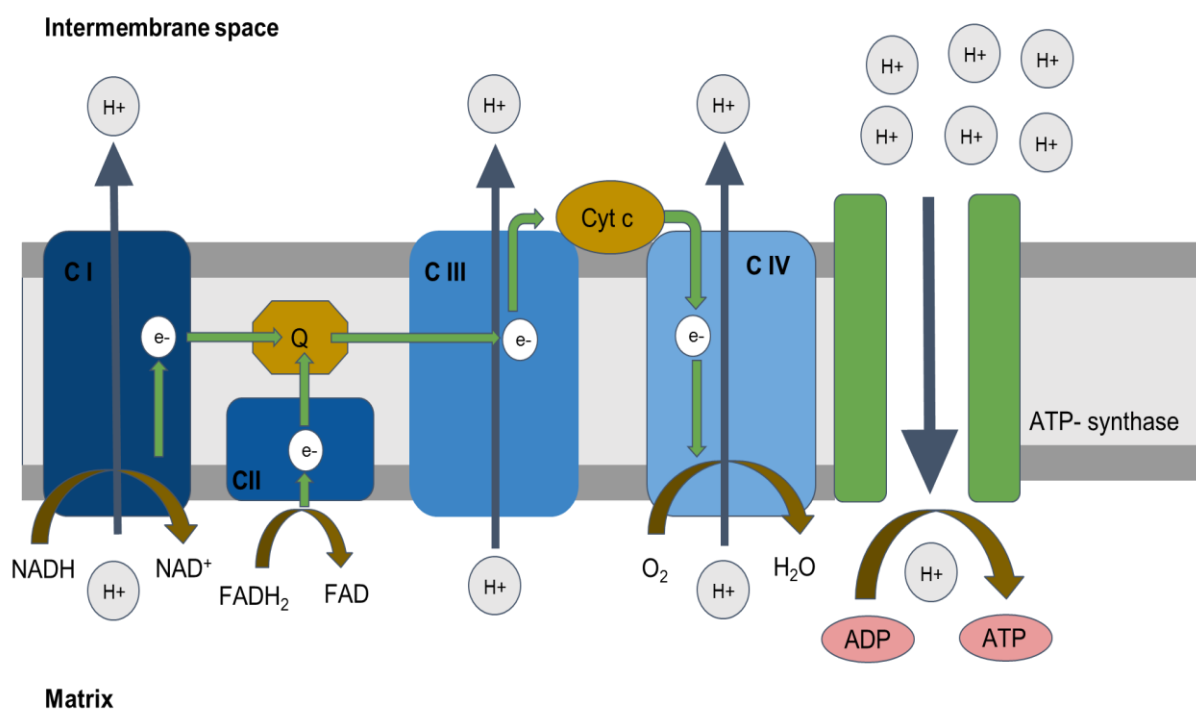


Figure I.4– Schematic illustration of mitochondrial electron transport chain. Respiratory Complexes I and II (CI and CII) transport electrons (e⁻s) generated by the conversion of NADH into NAD⁺ (CI) or FADH₂ into FAD (CII) through ubiquinone (Q), respiratory Complex III (CIII), Cytochrome c (Cyt c) and finally respiratory Complex IV (CIV), where electrons are reduce to water. During electron transfer, protons (H⁺) are pump from the mitochondria matrix to the inner membrane, by CI, CII and CIV, creating an electrochemical gradient that drives formation of ATP from ADP by ATP-synthase (Complex V). Adapted from *In Keane et al. (2011)*.

3.1 Mitochondrial dysfunction in PD

Mitochondria dysfunction was first implicated in PD upon the discovery of MPTP's ability to induce a parkinsonian syndrome in humans, by inhibiting mitochondrial respiratory Complex I (Gautier *et al.*, 2014). This hypothesis was further sustained by reports of Complex I deficiency in SNpc, frontal cortex, peripheral tissues and skeletal muscle, in a *postmortem* analysis of PD patients tissues (Bindoff *et al.*, 1991; Haas *et al.*, 1995; Parker *et al.*, 2008; Schapira *et al.*, 1990).

Complex I impairment causes a decrease in mitochondrial ATP production and an increase in ROS formation. In turn, severe ROS imbalance can lead to protein oxidation, lipid peroxidation, and damage in DNA and mtDNA, thereby affecting components of the respiratory chain and other mitochondrial factors, triggering a vicious cycle between mitochondrial dysfunction and oxidative stress that increases neurodegeneration and PD progression (Exner *et al.*, 2012; Keane *et al.*, 2011).

Even though the exact mechanisms that lead to neurodegeneration in PD remain elusive, studies suggest that together with oxidative stress, defects in mitochondrial dynamics and inflammation contribute to disease pathogenesis (Bose & Beal, 2016). Causes and consequences of mitochondrial dysfunctions are represented in Figure I.5 and Figure I.6.

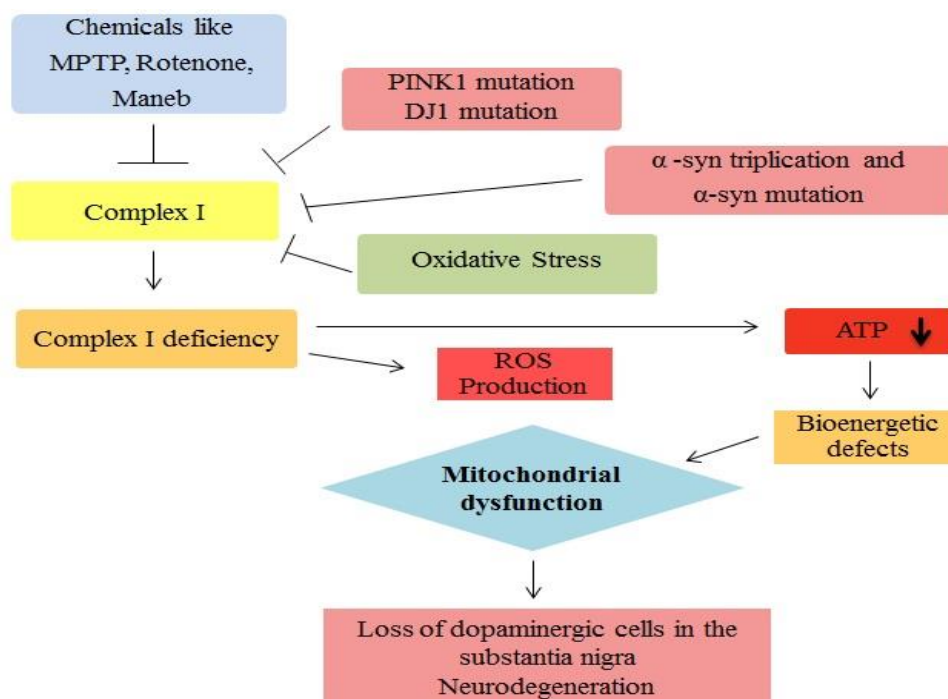


Figure I.5 – Mitochondrial respiration dysfunction in Parkinson’s disease. Exposure to neurotoxins and PD related mutations cause defects in oxidative phosphorylation by inhibition of Complex I. In turn, this inhibition causes ATP depletion and ROS production. Accumulation of ROS leads to oxidative stress and more Complex I deficiency, resulting in bioenergetic defects, mitochondrial dysfunction and neurodegeneration. Adapted from *Bose and Beal, 2016*.

3.1.1 Oxidative stress in PD

Oxidative stress derives from an increase ROS formation coupled with their defective removal, causing a disturbance in the fragile pro-oxidant/antioxidant homeostatic state. Elevated demand in energy production, the neuro biochemical composition rich in high levels of fatty acids and the lack of antioxidant defenses, make the brain the most susceptible organ to ROS generation (Uttara *et al.*, 2009; Winklhofer & Haass, 2010). Moreover, elevated markers of oxidative stress and damage are present in PD patients’ brains, establishing it as crucial feature of PD (Dauer & Przedborski, 2003; Keane *et al.*, 2011; Keeney *et al.*, 2006). Finally DJ-1 mutations, responsible for familial PD, which impair its neuroprotective role of function as ROS scavenger further reinforce oxidative stress’ impact in PD (Ariga *et al.*, 2013; Milani *et al.*, 2013). Hence, better understanding of oxidative stress regulatory pathways, like the nuclear factor erythroid 2 (NF-E2) - related factor 2 (Nrf2) - Kelch-like

ECH-associated protein 1 (Keap1) pathway and its downstream targets is integral to develop novel therapeutic strategies or unveil new pathogenic avenues to PD.

A vital cellular defense mechanism against oxidative stress is the activation of Nrf2 pathway (Nguyen *et al.*, 2009). Nrf2 is a basic leucine zipper transcription factor that regulates gene expression by interacting with the antioxidant response element (ARE) present in the promoter region of many cytoprotective genes (Yu & Kensler, 2005). Under physiological conditions, Nrf2 can be found in the cytosol bound to its inhibitor, Keap1. Keap1 is an oxidative stress sensor that regulates the rate of Nrf2 turnover through ubiquitin signaling and proteosomal degradation, by facilitating the interaction of Cullin-based E3 ligase complex with Nrf2 (Tong *et al.*, 2006). During oxidative stress Keap1 is oxidized and the latch to Nrf2 is weakened, changing the conformation of Nrf2-Keap1 complex and stopping ubiquitination and degradation of the nuclear factor (Stepkowski & Kruszewski, 2011). This permits stabilization of Nrf2 and its consequent translocation to the nucleus. Once it reaches the chromatin, Nrf2 can form a heterodimer with a basic leucine zipper transcription factor, small musculoaponeurotic fibrosarcoma (MAF), bind to ARE sequence and activate the transcription of Nrf2 dependent genes. Thereafter, when basal conditions are restored, Nrf2 returns to the cytoplasm where Keap1 continues to promote its sequestration and degradation (de Vries *et al.*, 2008).

The activation of the Nrf2 pathway induces the transcription of several endogenous antioxidant enzymes including glutathione peroxidases (GPxs) and heme oxygenases (HOs). GPxs are a group of eight enzymes (Gpx1-8) that play an important antioxidant role in catalyzing the reduction of hydrogen peroxide to water. Amongst the four major selenium dependent GPx isozymes in mammalian tissues, the classical GPx1 stands out. This enzyme is present in both neurons and glia cells, where it can be found in the mitochondria, nucleus and the cytosol (Smeyne & Smeyne, 2013). PD patients' brain studies revealed that GPx1 was present in highest levels in microglia, but was expressed in an overall low concentration in brain (Power & Blumbergs, 2009). In addition, mice lacking GPx1 gene showed increased vulnerability towards MPTP exposure than control mice (Klivenyi *et al.*, 2000).

Interestingly, has been reported that overexpression of GPx enzymes decreases accumulation of oxidizing agents and neuronal loss, highlighting its importance as antioxidant defense mechanism (Wang *et al.*, 2003).

HOs are a family of enzymes responsible for the degradation of intracellular heme into free iron, carbon monoxide and biliverdin. Subsequently, free iron is sequestered by ferritin and biliverdin is converted into bilirubin, a powerful ROS scavenger (Hung *et al.*, 2008).

Besides bilirubin, carbon monoxide also has an important anti-apoptotic and antioxidant role, highlighting HOs neuroprotective ability, not only by degrading proteins such as oxidases and peroxidases, but also generating important antioxidants end products (Hung *et al.*, 2008; Zhang *et al.*, 2013).

In mammals, two HOs isoforms were characterized, one whose expression is inducible (HO-1), and another that is constitutively expressed, (HO-2). In contrast with HO-2, whose expression does not vary in response to environmental stress, HO-1 mRNA and protein expression are reported to be increased in stress conditions (Takeda *et al.*, 1996). *Postmortem* analysis of PD patients' brains, shown a greater upregulation of HO-1 in SN in comparison to other brain regions, emphasizing the importance of this antioxidant protein in the stress context of this disease (Schipper *et al.*, 1998; Schipper *et al.*, 2009).

The evidence that Nrf2 expression is altered in neurodegenerative disorders combined with reports that its downstream targets are increased in stress conditions as a tentative endogenous defense mechanism, emphasize its antioxidant and neuroprotective role in PD as well as its importance as a therapeutic strategy for regulating oxidative stress.

3.1.2 Mitophagy in PD

Mitochondrial dynamics and mtDNA maintenance play an integral part in preserving mitochondria health (Bose & Beal, 2016). Consequently, removal of damaged mitochondria through autophagy (mitophagy) is crucial to protect cellular homeostasis and function (Ni *et al.*, 2015). Impairments in this process result in the persistence of dysfunctional mitochondria and a subsequent increase in ROS production, which culminates in cellular stress and death, as illustrated in Figure I.6.

3.1.2.1 PINK1/parkin pathway in mitophagy

PINK1 selective activation of parkin is an important mitophagic pathway, vital for mitochondria quality control (Scarffe *et al.*, 2014). The mitochondrial damage sensing mechanism relies on PINK1 degradation rate. In healthy mitochondria PINK1 is transferred from the translocase of the outer membrane (TOM) complex into the translocase of the inner membrane (TIM) complex where it is first cleaved by the mitochondrial processing peptidase (MPP) and then by presenilin-associated rhomboid-like protein (PARL). PARL proteolysis

generates a 52 kDa N-terminal fragment of PINK1 that is later released into the cytosol. There, cleaved PINK1 is recognized by an N-degron type 2 E3 ubiquitin ligase and marked for proteosomal degradation. Conversely, in damage mitochondria PINK1's import to TIM complex is disrupted, preventing MPP and PARL proteolysis and forcing the accumulation of full-length PINK1, bound to TOM complex, in the outer mitochondrial membrane (OMM). Subsequently, PINK1 precedes with selective parkin recruitment to the mitochondria, by phosphorylating ubiquitin chains linked to OMM. Once in the mitochondria, parkin can be both activated by phospho-ubiquitin and PINK1 itself, promoting a maximal parkin activity. parkin is an E3 ubiquitin ligase that once activated ubiquitynates a number of mitochondrial proteins, triggering the recruitment of autophagy receptors to mitochondria and the general autophagy process (Pickrell & Youle, 2015).

Mutations in certain genes such as *PINK1* and *parkin* cause mitochondrial dysfunction and are known to be associated with familial PD (Bose & Beal, 2016). Studies revealed that in mice lacking parkin mitochondria integrity is reduced, Complexes I and IV have lower activity, respiratory capacity decreases and mice become highly susceptible to neurotoxic compounds like rotenone (Casarejos *et al.*, 2006; Palacino *et al.*, 2004). In turn, models of PINK1 knockdown present reduced rates of mitochondria respiration and as a result, a decrease in ATP synthesis. Moreover, mice lacking PINK1 appear to be more sensitive to the oxidative stress toxic effect and to an increase in mitochondrial dysfunction (Gautier *et al.*, 2008; Liu *et al.*, 2009). PINK1 deficiency also reported to affect brain regions in distinct manners. For example, cortex tissues appear to compensate mitophagy failure with stimulation of alternative catabolic processes, increasing its resistance against oxidative damage (Diedrich *et al.*, 2011). In addition, mutations found in PD patients in both PINK1 and parkin are also accountable for preventive recruitment of parkin to the mitochondria by PINK1 thus disrupting this mitophagic pathway (Pickrell & Youle, 2015).

Together, these results reinforce the role of mitochondrial dysfunction in sporadic PD and the importance of PINK1/parkin pathway as a possible therapeutic target (Pickrell & Youle, 2015).

3.1.2.2 AMPK pathway in PD

AMP-activated protein kinase (AMPK) is a highly conserved energy and nutrient sensor that its activated in cases of ATP depletion and/or glucose starvation (Mihaylova & Shaw, 2011). AMPK is essential in mitochondrial homeostasis regulation, and it is involved in both

processes of mitochondrial biogenesis and clearance, sharing some similarities with parkin (Canto *et al.*, 2010; Jager *et al.*, 2007; Kim *et al.*, 2011; Zong *et al.*, 2002). Upon energy stress conditions, including mitochondria dysfunction, intracellular ATP/AMP ratio decreases leading to AMPK phosphorylation (He & Klionsky, 2009). This allows the direct phosphorylation of UNC-51-like kinase 1 (ULK1) and the consequent promotion of autophagy processes, including mitophagy (Hang *et al.*, 2015). Therefore, it is proposed that AMPK-ULK1 pathway may play a neuroprotective role by rescuing mitophagy defects in parkin deficient cases. Moreover, in a MPTP model of PD, AMPK activation is recognized as a protective strategy against the neurotoxic stimuli (Choi *et al.*, 2010). Collectively, this secondary pathway to mitophagy gains relevance as a compensatory neuroprotective strategy in PD and once more exposes mitochondrial dysfunction role in this disease.

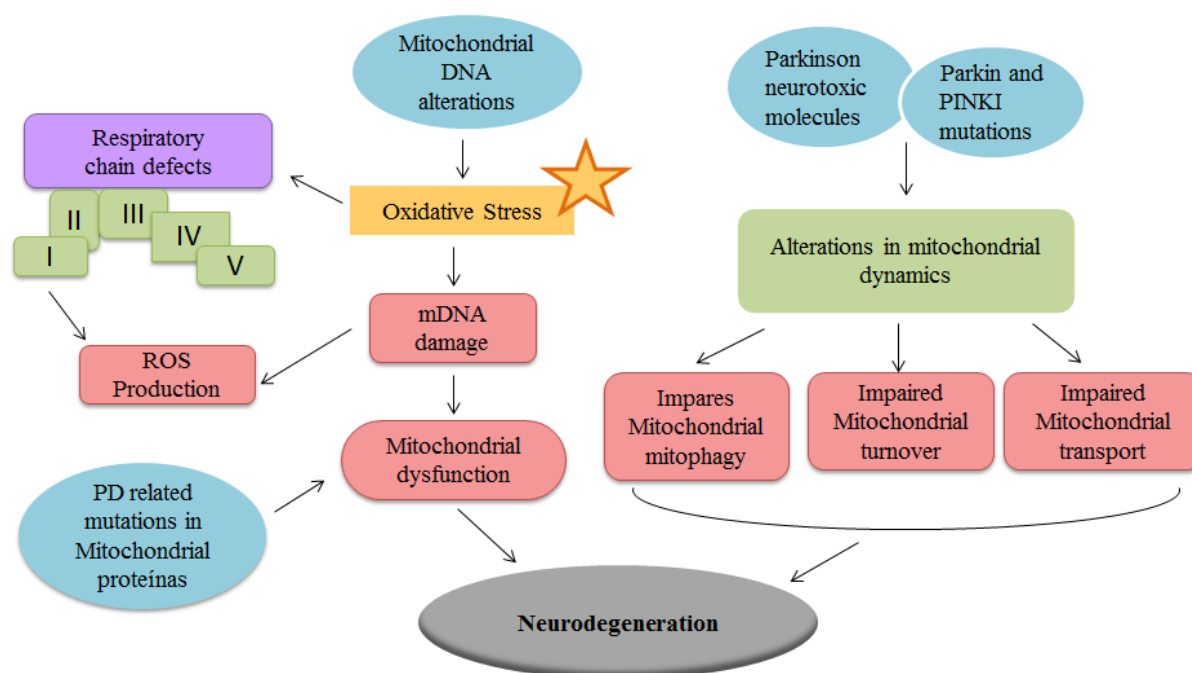


Figure I.6 – Alterations in mitochondrial DNA and mitochondrial dynamics, and Parkinson’s disease.

Alterations in mitochondrial DNA (mtDNA) can increase generation of reactive oxygen species (ROS) and lead to mtDNA damage and ultimately neurodegeneration. Parkinson’s disease related mutations in parkin, PINK1, DJ-1, α -synuclein and LRRK2 can also be the source of mitochondrial dysfunction. In addition, environmental neurotoxins or mutations in parkin and PINK1 lead to alterations in mitochondrial dynamics that result in altered mitochondrial clearance, turnover and transport, and eventually leading to neurodegeneration. Adapted from Bose and Beal, 2016.

4. Glia Activation and Neuroinflammation in PD

The initial neuroinflammation process is a protective mechanism that restores damaged neuronal cells and glial cells in the central nervous system (CNS) (Kempuraj *et al.*, 2016). Nevertheless, a sustain neuroinflammatory response can become detrimental and contribute to the cascade of events that lead to neuronal degeneration, thus playing an important role in the onset and progression of neurodegenerative disorders like PD (Kempuraj *et al.*, 2016).

Neuroinflammation is mostly mediated by neurons, infiltrating leukocytes and glial cells, including microglia and astrocytes (Carson *et al.*, 2006). These innate immune system cells can express pattern recognition receptors (PRRs), sensible to pathogen-associated molecular patterns (PAMPs) and host-derived PRR ligands, which can trigger inflammatory signaling pathways. Host-derived PRR ligands, also known as danger associated molecular patterns (DAMPs), can be found in brain diseases in the form of misfolded or aggregated proteins, mislocalized nucleic acids and damaged cells, serving as direct triggers of inflammation. The sustain exposure to pro-inflammatory mediators diverts immune-competent cells from their beneficial “housekeeping” role and contributes to the development and progression of neurodegenerative diseases (Heneka *et al.*, 2014).

4.1 Microglia activation in PD

Microglia represents a key cellular component of the innate immune system in the CNS. These cells are able to interact with neighboring neurons, blood vessels and astrocytes, and have a number of transporters, channels and receptors in its cell surface that recognize neurotransmitters, cytokines, chemokines and PRRs (Heneka *et al.*, 2014).

Under physiological conditions, microglia actively maintain homeostasis, neuroprotection and neuro repair by releasing growth factors such as brain-derived neurotrophic factor (BDNF) and transforming growth factor β (TGF- β), while retaining a ramified morphology. Under pathological conditions and in response to PAMPs and DAMPs, microglia becomes activated, proliferates and changes its conformation to a macrophage-like morphology (Wang, Tan, *et al.*, 2015). Depending on the pathologic event and their activation status, microglia can acquire one of two phenotypes with polar inflammatory responses, M1 microglia displaying a pro-inflammatory response whereas M2 microglia displays an anti-

inflammatory reaction, making the balance between them essential to maintain homeostasis (Tang & Le, 2016).

Classic microglia activation state, M1 phenotype, is associated with the production of pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and others (IL-6 and IL-12), superoxide, nitric oxide (NO), ROS and proteases (Wang, Liu, *et al.*, 2015). In turn, M2 phenotype comprises both states of alternative activation and acquired deactivation. Alternative activation is dependent on IL-4 or IL-13 treatment, and associated to genes that promote anti-inflammation, tissue repair and extracellular matrix reconstitution. Acquired deactivation is induced by uptake of apoptotic cells and exposure to anti-inflammatory cytokines like IL-10 and TGF- β counteracting the acute inflammation (Tang & Le, 2016). Microglia interaction and active state are illustrated in Figure I.7.

In 1988, the first evidence of neuroinflammation involvement in PD pathogenesis was proven by the presence of reactive microglia in the SNcp region of human brain (McGeer *et al.*, 1988). Further *postmortem* studies revealed the presence of microglia markers, cytokines like TNF- α and IL-1 β , NO and inducible NO synthase (iNOS) in SNcp as well as the presence of TGF- β , TNF, IL-6 and IL-1 β in the striatum of PD patients (Hirsch & Hunot, 2009). Moreover, PD patients serum and cerebrospinal fluid analyzed before death, showed an increased in cytokines (Hirsch & Hunot, 2009). MPTP models of PD were also investigated and showed the presence of reactive microglia in both mice and monkey models (Hurley *et al.*, 2003; Liberatore *et al.*, 1999).

In addition to cytokines and Major Histocompatibility Complex (MHC), the ionized calcium binding adaptor molecule-1 (Iba-1) is also a widely used marker for active microglia in tissues (Walker & Lue, 2015). Iba-1 is found to be expressed in microglia alone, both *in vivo* and *in vitro*, making it a good marker of this monocyte cell type (Ito *et al.*, 1998). Moreover, Doorn and colleagues described an increase in Iba-1 in microglia found in the substantia nigra of PD patients compared with the control (Doorn *et al.*, 2014).

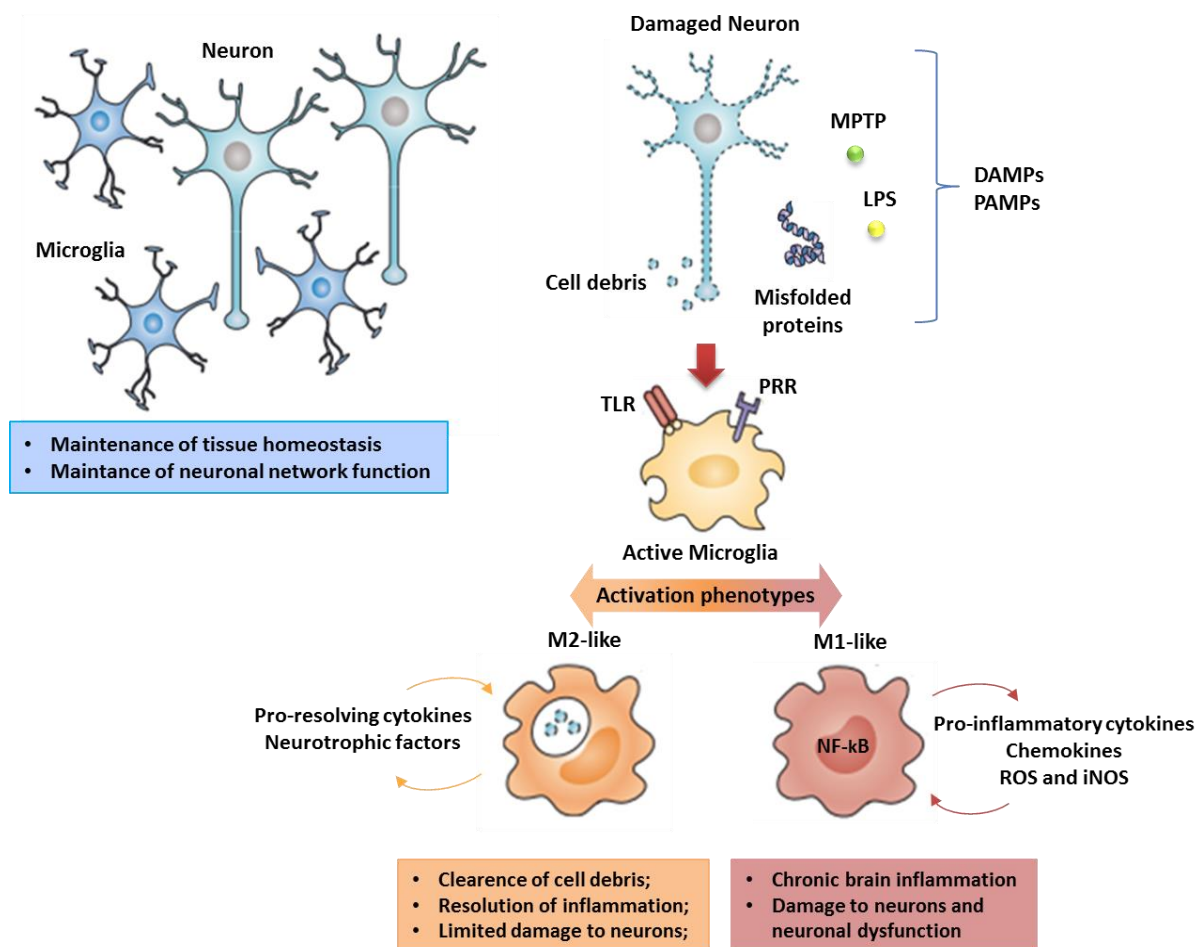


Figure I.7 – Beneficial and detrimental functions of microglia in the brain. In physiological conditions, microglia is responsible for maintaining tissue homeostasis, neuroprotection and neuro repair by secretion of neurotrophic factors, and synaptic remodeling. Danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) can bind to pattern recognition receptors (PRR) like Toll-like receptors (TLR), expressed on the surface of microglia and promote their activation. Depending on the signal microglia may adopt “M1-like” or “M2-like” phenotype, and can either respond by secreting inflammatory mediators or by enhancing the removal of the stimulant. “M1-like” activation of microglia is associated with the expression of inducible nitric oxide synthase (iNOS), production of reactive oxygen species (ROS) and pro-inflammatory mediators by NF-kB. “M2-like” activation of microglia is associated with the increase secretion of neurotrophic factors and the production pro-resolving cytokines. The difference responses determine whether microglial cell activity leads to debris clearance and resolution of the inflammatory response or leads to chronic neuroinflammation. MPTP - 1- methyl-4-phenyl-1,2,3,6-tetrahydropyridium; LPS – Lipopolysaccharide; Adapted from *Heneka et al., (2014)* and *Tang & Le (2016)*.

4.2 Reactive Astrocytes in PD

Astrocytes are greatly abundant in the CNS. These glial cells make numerous contributions to maintain brain homeostasis, by providing metabolites and growth factors to neurons, participating in synaptic formation and plasticity, regulating blood flow and

maintaining the extracellular balance of neurotransmitters, fluid and ions (Sofroniew, 2009). Astrocytes also play a vital role in the inflammatory process. Just like microglia, these immune-competent cells are able to detect PAMPs and DAMPs and respond through the secretion of cytokines and chemokines, thus activating an adaptive immune defense (Colombo & Farina, 2016). Reactive astrogliosis is characterized by upregulation of glial fibrillary acidic protein (GFAP), a main component of astrocyte intermediate filaments, and morphological changes comprised of proliferation and glial scar formation in the most severe cases (Pekny & Pekna, 2014).

The interplay between astrocytes and other immune-competent cells is also important for the inflammatory process. Astrocytes are responsible for the production of chemokines essential for the infiltration of macrophages and lymphocytes in the brain, thus promoting an increased inflammatory response (Barcia, 2013). In turn, astrocytes response can also be amplified by the conjugation of CNS immune stimulus with pro-inflammatory mediators released by active microglia, leading to a potentially prejudicial sustained neuroinflammatory response (Wang, Liu, *et al.*, 2015).

Various studies in PD models as well as in affected brain regions of PD patients have presented characteristics of reactive astrogliosis, namely increase expression of GFAP and morphologic transformations in astrocytes, highlighting the contribution of neuroinflammation in PD progression (Hirsch & Hunot, 2009; Wang, Liu, *et al.*, 2015).

4.3 Anti-Inflammatory mechanisms

Even though the mechanism underlying the progressive neurodegeneration in PD is still elusive, the hypothesis that links neuroinflammation with PD progression is more and more plausible (Kempuraj *et al.*, 2016).

An insult to the CNS triggers an immune response mainly carried out by microglia. Microglia activation leads to production of inflammatory mediators that stimulate the recruitment of other immune competent cells, and together promote clearance of cell debris and secretion of neurotrophic factors. The initial protective inflammatory mechanism can rapidly become detrimental. Inflammatory mediators not only target immune cells but also act on neurons, promoting neurodegeneration. In turn, neuronal death acts as a stimulus for the inflammatory response, creating a vicious cycle between inflammation and neuronal death that contributes to neurodegeneration (Rocha *et al.*, 2015). Therefore, anti-inflammatory

mechanisms are essential for maintaining homeostasis and avoid the damaging effects of chronic inflammation, making them an interesting therapeutic target to be explored in PD.

4.3.1 Annexin-1: an important anti-inflammatory protein

Inflammation process regulation is of the utmost importance. In physiological conditions, inflammation does not progress to a chronic state due to a resolution response comprised of downregulating the production of inflammatory mediators and clearance of leukocytes and cellular debris from the inflammation site (Perretti *et al.*, 2017). This crucial process is carried out by anti-inflammatory and pro-resolving molecules like Annexin-1 (ANXA1), and is essential for restoring tissue structure, function and homeostasis (Sugimoto *et al.*, 2016).

ANXA1 is a phospholipid-calcium-binding protein that intracellularly plays a role in differentiation, proliferation, plasma membrane repair and apoptosis. Additionally, extracellular ANXA1 is also implicated in important anti-inflammatory processes, namely regulation of neutrophil migration and apoptosis, glucocorticoids activation, and modulation of macrophage phagocytosis and reprogramming (Solito *et al.*, 2008; Sugimoto *et al.*, 2016).

ANXA1 is a 37 kDa protein that in its full-length form remains inactive. However in inflammatory conditions this protein can be cleaved by neutrophil elastase and proteinase-3, generating a 33 kDa cleaved isoform and an active peptide derived from ANXA1 N-terminus. Once the inflammation state is resolved ANXA1 regains its full-length form (Sugimoto *et al.*, 2016). Schematic representation of ANXA1 mediated inflammatory processes are illustrated in Figure I.8.

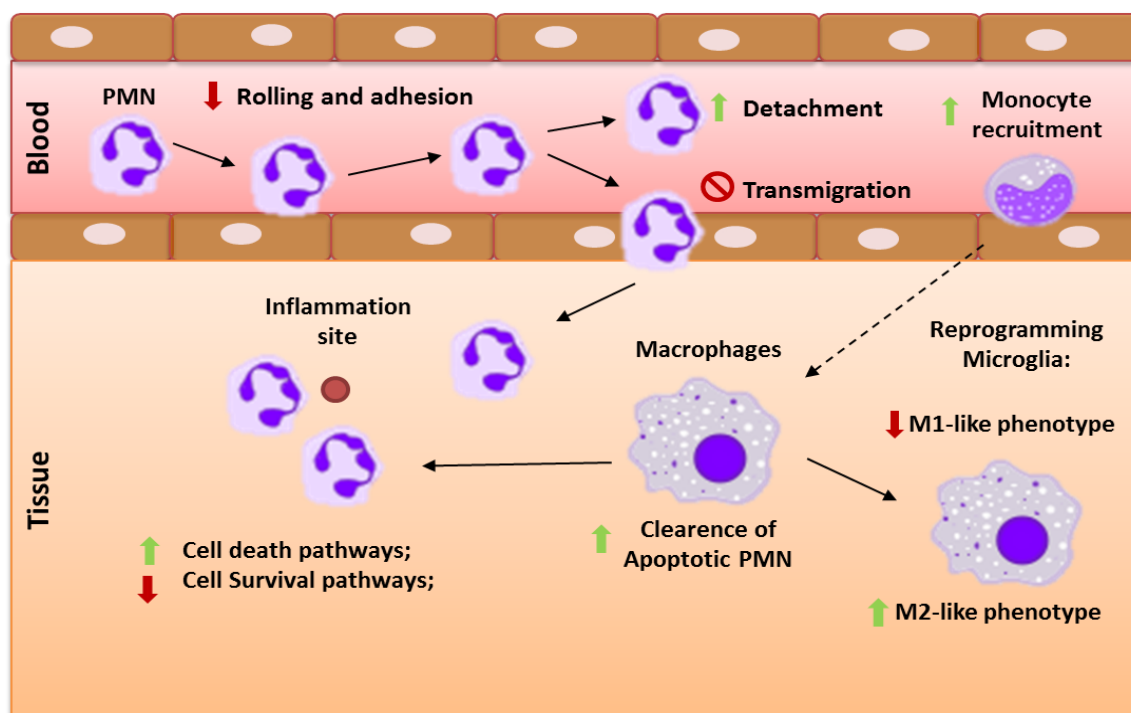


Figure I.8 – Schematic anti-inflammatory and proresolving effects of ANXA1. ANXA1 modulates a range of cellular mechanisms that are activated to resolve the inflammatory response. This anti-inflammatory protein decreases neutrophil (PMN) movement and adhesion to the endothelium, increases detachment of adherent cells and inhibits neutrophil migration to the tissue. ANXA1 induces apoptosis of neutrophils while surmounting the prosurvival signals to these cells. In addition, endogenous and exogenous ANXA1 also promote monocyte recruitment and clearance mediated by macrophages, coupled with the release of anti-inflammatory signals and decrease in proinflammatory cytokines levels. Besides, ANXA1 is also involved in macrophages reprogramming, from a proinflammatory to a proresolving phenotype. Adapted from *Sugimoto et al., (2016)*.

In the brain, ANXA1 is mostly expressed in glial cells, especially microglia, but absent in the majority of neurons (*McArthur et al., 2010*). This anti-inflammatory protein was implicated in PD when Knott and colleagues reported an increased expression of ANXA1 in activated microglia surrounding degenerating dopaminergic neurons, in SNpc of PD patients. A raise in ANXA1 expression was not only detected in PD but also in other neurodegenerative disorders such as Alzheimer's disease and Multiple Sclerosis. This upregulation could present a neuronal rescue mechanism, making it relevant the study of ANXA1 anti-inflammatory mechanism as a therapeutic strategy in PD (*McArthur et al., 2010*; *Solito et al., 2008*).

5. Tauroursodeoxycholic acid

Tauroursodeoxycholic acid (TUDCA) is an endogenous hydrophilic bile acid formed in liver by conjugation of ursodeoxycholic acid (UDCA) with taurine (Gronbeck *et al.*, 2016).

Bile acids are chemical detergents produced in the liver crucial in the absorption and transport of fats and lipid-soluble nutrients (Ackerman & Gerhard, 2016). Some of these molecules are considered cytotoxic while others like UDCA appear to be cytoprotective. Mostly, UDCA protective role derives from its ability to reduce the apoptotic threshold in certain cell types (Amaral *et al.*, 2009). Moreover, UDCA is approved by the U.S. Food and Drug Administration (FDA) for human therapeutic use, being clinically adopted in primary biliary cirrhosis treatment (Yanguas-Casas *et al.*, 2017). Clinical results obtain with UDCA led the way to TUDCA administration, which comparatively had a greater cytoprotective effect (Rodrigues *et al.*, 1995). Additionally, among its therapeutic advantages TUDCA is quickly bioavailable via oral, subcutaneous, or intravenous administration, has no associated toxicity and crosses the BBB (Ackerman & Gerhard, 2016; Rodrigues *et al.*, 2002).

The neuroprotective role of TUDCA has been supported by various experimental studies, being currently used in a promising clinical trial for the treatment of amyotrophic lateral sclerosis (Elia *et al.*, 2016). Rodrigues and colleagues demonstrated that TUDCA modulates apoptosis by suppressing mitochondrial membrane perturbation and consequently inhibiting Bcl-2-associated X (BAX) protein association to the mitochondria (Rodrigues *et al.*, 2003). Those results were proven to reduce neurodegeneration in Alzheimer's and Huntington's disease models as well as to protect against locomotor and cognitive deficits in the 3-nitropropionic rat model of Huntington's disease (Keene *et al.*, 2001; Rodrigues *et al.*, 2000; Sola *et al.*, 2006). In addition, Yanguas-Casás and co-workers recently revealed that TUDCA displayed anti-inflammatory properties in microglia cells by decreasing nuclear factor-kappa B (NF- κ B) activation and/or by increasing cyclic adenosine monophosphate (cAMP) levels that mediate an anti-inflammatory response (Yanguas-Casas *et al.*, 2017). Together, TUDCA's neuroprotective characteristic along with its antioxidant and anti-inflammatory capacity make this bile acid a promising therapeutic compound for neurodegenerative disorders as well as other traumatic brain injuries (Ackerman & Gerhard, 2016; Gronbeck *et al.*, 2016).

5.1 TUDCA in PD

Despite the intensive research in PD field, there are still no effective therapies to delay, stop or revert neurodegeneration and disease progression. Current therapeutic strategies are only capable of relieve PD symptoms and restore some quality of life to patients (Taylor *et al.*, 2013).

Giving the age relevance in PD and the increasing life expectancy of the world population, incidence of this disease has been growing exponentially, making the discovery of new approaches to treatment an urgent matter (Valadas *et al.*, 2015). PD's late diagnose, mainly based on motor symptoms, together with the fact that this disease is believed to be multifactorial and differs from case to case, hinders the strategy development process (Poewe *et al.*, 2017).

Although the cause of PD remains elusive, neurodegeneration has been associated with different mechanisms of cell damage including inflammation, mitochondrial dysfunction, oxidative stress, apoptosis and protein aggregation (Emborg, 2004). As a result, TUDCA's neuroprotective and antioxidant features appeared to represent a potentially successful therapeutic agent in PD treatment.

Our group began to demonstrate that TUDCA prevented MPTP-induced dopaminergic cell loss and ROS production, in a mice model of PD (Castro-Caldas, Carvalho, Rodrigues, Henderson, Wolf, Rodrigues, *et al.*, 2012). Furthermore, TUDCA's antioxidant role was determine by promoting Nrf2 activation in a MPP⁺ cell model as well as by increasing expression of Nrf2 and its downstream antioxidant targets in striatum and midbrain regions of an MPTP mice model (Moreira *et al.*, 2017). Additionally, TUDCA was showed to display a neuroprotective role by modulating PINK1/parkin mitophagic pathway and promoting mitochondrial biogenesis in response to MPP⁺/MPTP models of PD (Rosa *et al.*, 2017).

Together, these results validated the beneficial effects of TUDCA reported by other studies in striatum and midbrain regions of an MPTP mice model of PD. However, little is known about the TUDCA's broad neuroprotective response in other brain regions such as cortex, making it a relevant study to better understand the mechanisms involved in this prominent therapeutic agent for PD treatment.

6. Aims

The main goal of this thesis is to investigate if the neurotoxic effects of MPTP are extended to the cerebral cortex in a mouse model of PD. This study also consists in dissecting the anti-oxidant effects of TUDCA in this brain region, comparing the obtained results with our previous data from MPTP mice models. In addition, we propose to investigate the anti-inflammatory mechanism of this bile acid, in the cortex region and in glia cells, and explore its capacity to modulate an important anti-inflammatory protein, ANXA1.

Our specific aims are:

- **Validate MPTP neurotoxic effects in the cerebral cortex region.** We will start by confirming MPTP toxicity in the cortex region of mice subjected to short and long term evaluation. The expression level of proteins involved in mitochondrial dysfunction as well as main dysfunctional markers such as ROS production and ATP depletion will be assessed.
- **Determine TUDCA anti-oxidant effects in cerebral cortex region.** The broad neuroprotective effect of TUDCA in response to MPTP toxicity will be evaluated. Expression of proteins involved in mitophagy and oxidative stress will be analyzed and compared to the previous results obtained by our group in the striatum and midbrain regions from mice models.
- **Explore TUDCA anti-inflammatory effect in an MPTP mice model.** We will explore TUDCA role in resolving/preventing neuroinflammation and glia activation. Glia activation markers such as Iba-1 and GFAP and the expression of ANXA1 anti-inflammatory protein will be analyzed in the brain cortex region.
- **Explore the direct effect of TUDCA in ANXA1.** Lastly, we will examine TUDCA direct effect on ANXA1 and microglia's activity *in vitro*. We hope to establish a direct connection, highlighting the promising therapeutic role of both TUDCA and ANXA1.

Finally, in this work we intent to provide extended knowledge into TUDCA broad neuroprotective effect and draw attention to its promising therapeutic role in PD treatment.

II. Materials and Methods

1. Materials

1.1 Supplements and chemicals

Bio-Rad's Protein Assay Reagent was acquired from Bio-Rad Laboratories (Hercules, CA, USA); Polyvinyl difluoride (PVDF) membrane was obtained from Millipore (Bedford, MA, USA). ATP-Glo Bioluminometric Cell Viability was purchased from Biotium Inc. (Fremont, CA, USA); ECL Western blotting detection reagent and Western Bright™ ECL-Spray were bought from GE Healthcare (Buckinghamshire, UK) and Advansta Inc. (CA, USA), respectively. SuperSignal® West Femto Maximum Sensitivity Substrate was acquired from Thermo Scientific (Rockford, USA); RPMI Media 1640, fetal bovine serum (FBS) Penicilin/streptomycin and TripleE Express were purchased from GIBCO ® (Life Technologies, Inc., Grand Islands, USA); TUDCA, MPTP, 2',7'-dichlorofluorescein diacetate (DCF-DA) probe, Bovine Serum Albumin (BSA) (fraction V), Hoechst 33258 dye, Triton X-100, and Mowiol mounting media, were obtained from Sigma Aldrich (St Louis, MO, USA); Other chemicals and analytical grade reagents were ordered from local commercial sources.

1.2 Antibodies

Table II.1 – Primary antibodies used for Western Blot analysis

Primary Antibody (<i>antigen</i>)	Host	Brand	Dilution
p-AMPK	Rabbit	Cell Signaling Technology (MA, USA)	1:1000
AMPK	Rabbit	Cell Signaling Technology (MA, USA)	1:1000
ANXA1	Rabbit	Invitrogen (CA, USA)	1:1000
β -III-tubulin	Mouse	Covance (NJ, USA)	1:1000
β -Actin	Mouse	Sigma Aldrich (MO, USA)	1:80000
DJ-1	Rabbit	Millipore (MA, USA)	1:1000
GFAP	Mouse	Millipore (MA, USA)	1:20000
Gpx1	Rabbit	Abcam (Cambridge, UK)	1:1000
HO-1	Rabbit	Enzo Life Sciences (NYC, USA)	1:1000
p-parkin	Rabbit	Abcam (Cambridge, UK)	1:300
Parkin	Mouse	Cell Signaling Technology (MA, USA)	1:1000
PINK-1	Rabbit	Novus Biologicals (CO, USA)	1:1000

Table II.2 – Primary antibodies used in immunohistochemistry

Primary Antibody	Host	Brand	Dilution
GFAP	Rabbit	Sigma Aldrich (MO, USA)	1:200
Iba-1	Rabbit	Wako Pure Chemicals (VA, USA)	1:100

Table II.3 – Secondary antibodies used for Western Blot analysis and immunohistochemistry assay

Secondary Antibody	Host	Brand	Dilution
Anti-rabbit Alexa Fluor® 488	Goat	ThermoFisher Scientific (IL, USA)	1:200
Horseradish peroxidase conjugated anti-mouse	Goat	Bio-Rad Laboratories (CA, USA)	1:5000
Horseradish peroxidase conjugated anti-rabbit	Goat	Bio-Rad Laboratories (CA, USA)	1:5000

2. Methods

2.1 Animal treatments

All animal experiments were carried through in accordance with the institutional, Portuguese and European guidelines (*Diário da República*, 2.^a série N.º121 of 27 June 2011; and 2010/63/EU European Council Directive) and methods were approved by the Direcção Geral de Alimentação e Veterinária, the Órgão Responsável pelo Bem-Estar Animal (ORBEA) of the Faculty of Pharmacy, University of Lisbon and the Animal Ethics Committee of the Life and Health Sciences Research Institute, University of Minho (SECVS 013/2017). Animal facilities and the people directly involved in animal experiments were certified by the Portuguese regulatory entity - Direcção Geral de Alimentação e Veterinária.

Twelve-week-old male C57BL/6 mice were purchased from Harlan (Spain) and Charles River Laboratories and subjected to short and long term MPTP treatments, respectively. Animals were housed in groups of 4 or 5 animals in filter-topped polysulfone cages 267 × 207 × 140 mm (370 cm² floor area) (Tecniplast, Buguggiate, Italy), with corncob bedding (Scobis Due, Mucedola SRL, Settimo Milanese, Italy) in a conventional animal facility. All animals were maintained under standard laboratory conditions: an artificial 12 h light/dark cycle (lights on from 8:00 to 20:00 h), with an ambient temperature of 21±1°C and a relative humidity of 50–60%; the mice were given a standard diet (4RF21, Mucedola SRL, Settimo Milanese, Italy) and water *ad libitum*. Health monitoring was performed according to FELASA guidelines (Guillen, 2012; Nicklas *et al.*, 2002) confirming the Specified Pathogens status of sentinel animals maintained in the same animal room. Humane endpoints for experiments were defined (20% reduction of the body weight, inability to reach food and water, presence of wounds in the body, dehydration), but not needed in practice, as animals did not reach these endpoints in the study period.

TUDCA and MPTP were dissolved in saline and administered intraperitoneally (i.p.). TUDCA was daily injected (1 injection per day), for three consecutive days, at a dose of 50 mg/Kg body weight whereas MPTP was administered at a single dose of 40 mg/Kg body weight, as previously described (Castro-Caldas, Carvalho, Rodrigues, Henderson, Wolf, Rodrigues, *et al.*, 2012).

Mice evaluated for a short period of time were divided in 6 groups: i) mice that received saline (Control group); ii) mice treated with TUDCA for 3 consecutive days, and sacrificed 6 h after the last TUDCA injection (TUDCA group); iii) mice injected with MPTP and

sacrificed 3 h after MPTP administration (MPTP, 3h group); iv) mice that received daily injection of TUDCA during 3 consecutive days, followed by i.p. administration of MPTP on day 3, 6 h after the last TUDCA injection, and sacrificed 3 h after MPTP administration (T+M, 3h group); v) mice injected with MPTP followed by TUDCA injection 3 h after neurotoxin administration, and sacrificed on day 3, 6 after the last TUDCA injection (M+T, 3h group); and vi) mice injected with MPTP and sacrificed at 3 days after MPTP administration (MPTP, 3D group);

Mice evaluated during a long period of time were divided in 4 groups: 1) mice that received saline (Control group); 2) mice injected with MPTP and sacrificed 52 days after MPTP administration (MPTP, 52D group); 3) mice that received daily injection of TUDCA during 3 consecutive days, followed by i.p. administration of MPTP on day 3, 6 h after the last TUDCA injection, and sacrificed 52 days after the first MPTP administration (T+M, 52D group); and 4) mice injected with MPTP followed by a daily injection of TUDCA during 3 consecutive days, starting 3 h after the neurotoxin administration, and sacrificed 52 days after MPTP injection (M+T, 52D group).

Treatments were carried out in at least 3 independent experiments ($n=3$) for short and long term evaluation experiences respectively. Since previous studies showed that evaluated parameters in control animals did not change throughout the time course, control animals were sacrificed together with TUDCA-treated animals in order to avoid increasing the number of animals needed for the experiments. The schematic representation of mice treatments is illustrated in Figure II.1.

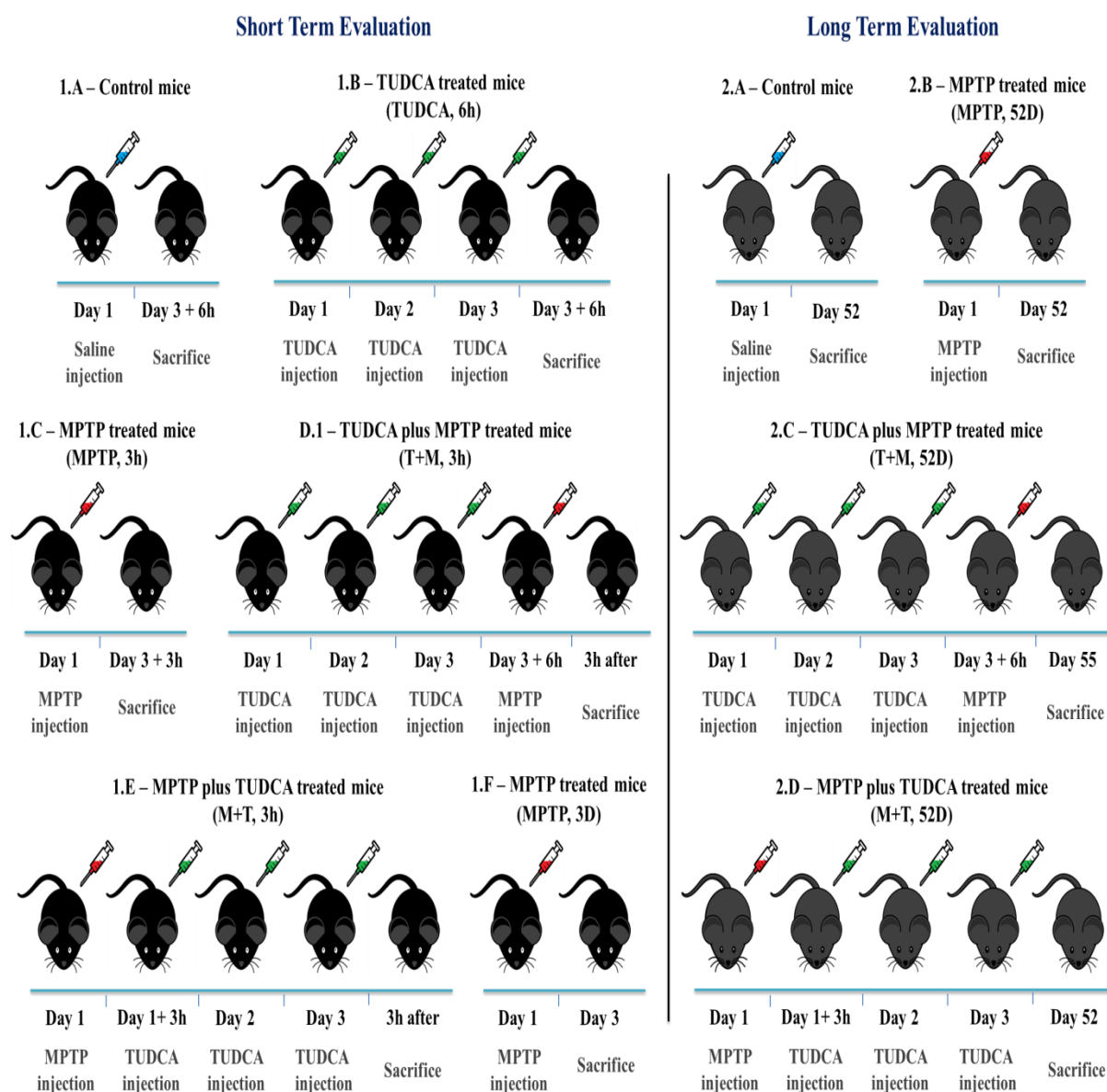


Figure II.1 – Schematic representation of mice treatments, short and term evaluation. C57BL/6 male mice were i.p injected with TUDCA (50mg/Kg body weight), for three consecutive days. MPTP was administered i.p at a single dose of 40 mg/Kg body weight. **Short term evaluation treatments** are comprised of: **1.A** – Control mice received saline. **1.B** – Mice were injected with TUDCA for three consecutive days, and were sacrificed 6h after TUDCA last injection. **1.C** – Mice were treated with a single dose of MPTP for 3h. **1.D** – MPTP injection was administered 6h after the last TUDCA injection, on day 3, and mice were sacrificed 3h after MPTP administration. **1.E** – TUDCA was administered for three consecutive days, 3h after MPTP injection on day 1. Mice were sacrificed 6h after the last TUDCA injection. **1.F** – Mice were injected on day 1 with MPTP and sacrificed 3Days after neurotoxin administration. **Long term evaluation treatments** consisted of: **2.A** – Control mice received saline. **2.B** – Mice were injected on day 1 with MPTP and sacrificed 52 Days after neurotoxin administration. **2.C** – MPTP injection was administered 6h after the last TUDCA injection, on day 3, and mice were sacrificed 52 days after MPTP administration. **2.D** – TUDCA was administered for three consecutive days, 3h after MPTP injection on day 1. Mice were sacrificed 52 Days after MPTP injection. 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 an immortalized murine microglial cell line, BV2 (kindly provided by E. Rodrigues, Faculdade de Farmácia, Lisboa). This cell line expresses properties of activated microglial cells and therefore, it can be used as alternative model system for primary microglia cultures or for animal experiments examining brain inflammation (Blasi *et al.*, 1990; Bocchini *et al.*, 1992; Henn *et al.*, 2009).

Cells were maintained in T75 flasks in RPMI 1640 medium supplemented with 10% FBS and 1% of Penicilin/streptomycin, at 37°C in a humidified atmosphere of 5% CO₂, 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 37°C and were then counted, with a hemocytometer, and diluted in RPMI 1640 medium, according to their initial concentration and specific assay.

Cells were plated in 60mm culture dishes, at a concentration of 1×10^6 cells/mL and left to stabilize for 24 h. BV2 cells were treated with 100 μ M of TUDCA for different time points (3, 6 or 24 h). In another set of experiments BV2 cells were treated with 100 μ M of TUDCA for 6 h after a pre-treatment for 1h with either 100nM of Wortmannin, 100nM of K252A or 5 μ M of H89. Controls were always included, and consisted of treating the cells with vehicle (control). Treatments were carried out in at least two independent experiments. The schematic representation of the experiences time course treatment is illustrated in Figure II.2.

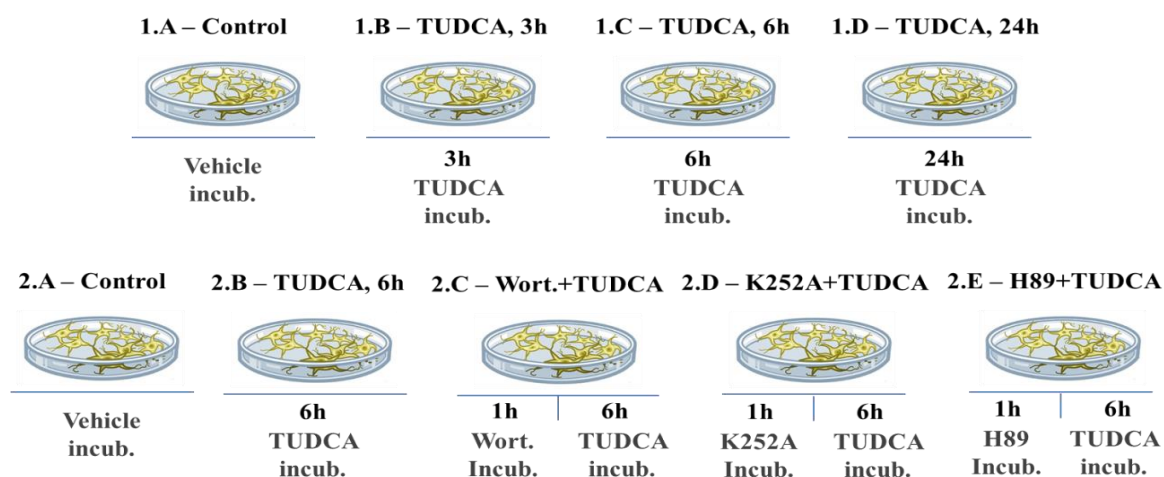


Figure II.2 – Schematic representation of three independent experiences in BV2 cells. Experience 1, BV2 cells were treated with TUDCA for 3h, 6h or 24h. Experience 2, BV2 treatments included TUDCA incubation for 6h or conjugated treatments of TUDCA for 6h, after 1h incubation with Wortmannin, K252A or H89 inhibitors. Controls were always included and treated with a vehicle. Incub. – Incubation; TUDCA - Tauroursodeoxycholic acid;

After treatments, BV2 cells were washed with ice-cold PBS supplemented with 1mM of EDTA in order to seize extracellular ANXA1 (Castro-Caldas *et al.*, 2003). Subsequently, cells were lysed in ice-cold lysis buffer (50mM Tris-HCl pH 7,4, 180 mM NaCl, 1mM EDTA and 1% Triton-X 100) plus Complete Mini protease inhibitor, 200mM Na₃VO₄, and incubated on ice for 30 min. Cells were collected and sonicated, in the Ultrasonic Processor UP100H (Hielscher-Ultrasound Technology, Teltow, Germany), three times for 5 sec each, on ice. Intracellular extracts were centrifuged at 13,000xg for 10 min, at 4°C, whereas extracellular extracts were centrifuged at 1,000xg for 5 min, at 4°C, the supernatants were collected and stored at -80°C. Total Protein concentration was determined by the Bradford method (Bradford, 1976), using Bio-Rad's Protein Assay Reagent.

Proteins were also extracted from primary mouse neurons cultures previously subjected to 0.5mM of MPP⁺ (Sigma Aldrich) and/or 100 μM of TUDCA. Neurons were treated with either MPP⁺ for 3 h or the conjugated treatment of a TUDCA 6 h incubation followed by MPP⁺ treatment for 3 h. Controls were included, and consisted of treating the cells with vehicle (control).

Primary cultures of C57BL/6 mice cortical neurons were prepared from 17 to 18 day old fetuses (Rosa *et al.*, 2017). Succinctly, pregnant mice were sacrificed in a CO₂ chamber and the fetuses were collected in Han's balanced salt solution (HBSS) (Sigma Aldrich, MO, USA) and rapidly decapitated. After removal of meninges and white matter, the brain cortex was collected in HBSS without Ca²⁺ and Mg²⁺ (HBSS-2). The cortex was then mechanically fragmented, transferred into a 0.05% trypsin solution (Sigma Aldrich, MO, USA), and incubated for 15 min at 37 °C. After trypsinization, cells were washed twice in HBSS-2 containing 10% FBS and resuspended in Neurobasal medium, supplemented with 0.5 mM L-glutamine, 25 μM L-glutamine acid, B-27 supplement and 12 mg/ml gentamicin (Gibco™, Thermo Scientific, MA, USA). Isolated neurons were plated in a density around 640 cells/mm² on culture plates pre-coated with poly-D-lysine (Sigma Aldrich), and maintained at 37°C in a humidified atmosphere of 5% CO₂. Half of the neuronal primary cultures medium was changed every 3-4 days. Glutamic acid was only added to the medium when plating the cells. All the media changes and cell treatments afterwards were free from glutamic acid.

2.3 Western Blot Analysis

Mice were decapitated, after being anesthetized with sodium pentobarbital (50 mg/Kg, i.p.), and the cerebral cortex was isolated at the level of the nigrostriatal axis (Bregma -3.20 to 1.00). Cortex region samples were then stored at -80°C until needed.

Mice cortex fragments were homogenized in cold PBS supplemented with Complete Mini Protease Inhibitor Cocktail, using a tissue grinder and centrifuged at 3.000 r.p.m. for 10 min, at 4°C. Pellets were then suspended in lysis buffer (20 mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM Na₂EDTA, 1mM EGTA, 1% Triton, 2.5mM sodium pyrophosphate, 1mM β -glycerophosphate, 1mM Na₃VO₄, 1 μ g/ml leupeptin) supplemented with Complete Mini Protease Inhibitor Cocktail, 200mM Na₃VO₄ and 1M NaF, and incubated on ice for 30 min. After sonication, in the Ultrasonic Processor UP100H (Hielscher-Ultrasound Technology, Teltow, Germany), six times for 5 sec each, on ice, samples were centrifuged at 13000 rpm for 15 min, at 4°C. Supernatants were collected and frozen at -80°C. Total Protein concentration was determined by the Bradford method (Bradford, 1976), using Bio-Rad's Protein Assay Reagent.

Protein extracts from both animal and cell treatments were added (5:1) to loading buffer 5x SDS-PAGE (0.25 mM Tris-HCl, 4% SDS, 40% glycerol, 0.2% 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 a fixed amperage of 35 mA per gel, for about 3 h. Subsequently, proteins were electrotransferred to an activated PVDF membrane (1 min in ethanol, 2 min in H₂O, 5 min in transfer buffer), in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol), during 2 h at a fixed amperage of 500 mA. Membranes were then stained with Amido black for 4 min, washed with destaining solution (25% isopropanol and 10% acetic acid) for 10 min, and dried at room temperature. After re-hydration with ethanol for 1 min, wash with distilled water for 2 min and equilibrate for 5 min in TBS-T (25 mM Tris Base, 137 mM NaCl, 2.7 mM KCl, 0.05% Tween 20, pH 7.4), membranes were 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 7.4), for at least, 1 h at room temperature, being then incubated with the specific primary antibodies: rabbit anti-Gpx1 (1:1000, 3% BSA), rabbit anti-HO-1 (1:1000, 3% BSA), rabbit anti-p-parkin (1:300, 3% BSA), mouse anti-parkin (1:1000, 5% non-fat dry Milk), rabbit anti-PINK1 (1:1000, 3% BSA), rabbit anti-p-AMPK (1:1000, 3% BSA), rabbit anti-AMPK (1:1000, 3% BSA), mouse anti-GFAP

(1:20000, 3% BSA), rabbit anti-ANXA1 (1:1000, 3% BSA), mouse anti- β -III-Tubulin (1:1000, 3% BSA), rabbit anti-DJ-1 (1:1000, 3% BSA), overnight, at 4°C, with agitation. Overnight incubation was 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 the membranes with TBS-T, the chemiluminescent immunocomplexes were detected using ECL or Femto reagents. The relative intensities of protein bands were analyzed with Image Lab TM analysis software, after being scanned 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 washed several times in TBS-T. Stripped membranes were then blocked as previously described and incubated with mouse anti- β -actin (1:80000, 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 intracellular ROS production

Intracellular ROS production was measured with the cell permeant and non-fluorescent reagent 2',7'-dichlorofluorescein diacetate (DCF-DA) that measures hydroxyl, peroxy and other ROS activity within the cell. After diffusion into the cell, the DCF-DA probe is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2',7'-dichlorofluorescein (DCF), a highly fluorescent compound.

Measurement of ROS was performed in cortex tissue extracts from long term evaluation experiments. Primarily, 50 μ g of protein from our samples were incubated with 10 μ M of DCF-DA probe, diluted in PBS, at 37°C for 1 h, in the dark. After incubation, the fluorescence intensity of the oxidized probe, DCF, was measured in the microplate reader GloMax® Multi Detection System Promega (Sunnyvale, CA, USA), at an excitation and emission wavelength of 485nm and 528nm, respectively. Blanks wavelengths, comprised of PBS incubated with DCF-DA probe were subtracted to our results. Each assay from three different experiments, was performed in duplicate and the results were normalized to the percentage of control.

2.5 Measurement of ATP levels

ATP levels were measured using with the ATP-Glo™ Bioluminometric Cell Viability Assay (Biotium, Hayward, CA, USA). The premise of this assay is based on a Bioluminescent reaction catalyzed by Firefly luciferase. This Protein uses the amount of ATP available in each sample to oxidize D-Luciferin and as a byproduct of the reaction producing light.

Measurement of ATP levels was performed in cortex tissue extracts from long term evaluation experiments, according to the manufacturer's instructions. The Luciferase activity in each sample was then measured in a luminometer (Berthold Systems). Negative controls were always included, and results were normalized to the total protein concentration in each well, as previously described (Rosa *et al.*, 2017).

2.6 Immunohistochemistry

The detection of reactive astrocytes and microglia activation markers (GFAP and Iba-1, respectively) was performed by immunohistochemistry, in cerebral tissue coronal sections from long term evaluated mice that were treated with MPTP and TUDCA as previously described.

Tissue sections stored at -80°C were left to dry 1 h, at room temperature being later fixated with 4% PFA in PBS for 10 min. Tissue sections were then washed three times and permeated with PBS supplemented with 0.2% of Triton X-100 for 30 min, at room temperature. After being washed three times with PBS for 10 min, sections were blocked with blocking solution (2% BSA, 0.05% Tween-20 in PBS), for 1 h at room temperature. Sections were then incubated overnight, at 4°C, with the specific primary antibodies rabbit anti-GFAP (1:200 in blocking solution) and rabbit anti-Iba-1 (1:100 in blocking solution), in a dark humidified chamber. Overnight incubation was followed by incubation with the secondary antibody goat anti-rabbit Alexa Fluor® 488 (1:200 in blocking solution) for 2 h, at room temperature. After staining cell nuclei with Hoechst 33258 dye (5µg/mL), the sections were mounted in glass slides with Mowiol anti-fading mounting medium.

Green (for both GFAP and Iba-1) and blue (for nuclei) fluorescence and UV images of, at least, fifteen random microscopic fields belonging to mice cortex region, were acquired per mice, 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 area occupied by GFAP and Iba-1 positive cells, measured by using the ImageJ software analysis (National Institutes of Health, USA).

2.7 Statistical analysis

All results are expressed as mean \pm SEM values. Data were analyzed by T-student test or one way ANOVA, where differences between groups were determined by post hoc Bonferroni's test and comparison of treatments to a single control by post hoc Dunnett's test, using GraphPad Prism 6.0 (San Diego, CA, USA). Means were considered statistically significant at a p value below 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 downstream targets in the cortex of C57BL/6 mice upon MPTP administration

Nrf2 is a key regulator of cellular redox status (Sandberg *et al.*, 2014). This transcription factor is responsible for promoting the expression of cytoprotective genes, including antioxidant enzymes, anti-inflammatory mediators and other transcription factors involved in mitochondrial biogenesis (Tufekci *et al.*, 2011). Therefore, Nrf2 system is widely expressed in the CNS and integral for a neuroprotective response in acute cerebral insults and in neurodegenerative diseases. Indeed it is reported that Nrf2 expression plays an important role in protecting against neurotoxin-induced oxidative stress (Jakel *et al.*, 2007; Sandberg *et al.*, 2014; Tufekci *et al.*, 2011). Consequently, Nrf2 pathway dysregulation has been deeply implicated in PD pathogenesis, making its activation and the up-regulation of antioxidant downstream targets a promising strategy for regulating oxidative damage in PD. As previously described, TUDCA antioxidant properties present a hopeful approach against oxidative stress in PD. In accordance, our group disclosed that TUDCA treatment prior to or after MPTP administration increased Nrf2 expression as well as an overall increase in GPx1 and HO-1 expression in mice striatum and midbrain (Moreira *et al.*, 2017). Thus, to evaluate the broad effect of TUDCA antioxidant role, we investigated its ability to modulate the expression of Nrf2 downstream targets, GPx1 and HO-1 in the cortex of MPTP-injected mice. The expression levels of these antioxidant enzymes were evaluated in the cortex of mice either subjected to short or long term evaluation (Figure III.1) by Western blot. In short term evaluation samples, both HO-1 and GPx1 expression were apparently increased in the cortex

of mice upon exposure to MPTP and/or TUDCA, still values did not reach statistical significance (Figure III.1- A.1; B.1). Interestingly, results from long term evaluation samples revealed a slightly decrease in these anti-oxidant enzymes expression, 52 days after exposure to MPTP (Figure III.1- A.2; B.2). However, when mice were treated with TUDCA prior to MPTP administration, the expression levels for both enzymes significantly increased as compared with MPTP ($p < 0,05$ vs MPTP). TUDCA treatment after MPTP injection only induced the expression of HO-1 ($p < 0.001$ vs MPTP).

These results revealed that TUDCA, when administered before MPTP, increases the expression of Nrf2 regulated cytoprotective enzymes, GPx1 and HO-1, in mice cortex. Furthermore, TUDCA when administered after the neurotoxic insult, also demonstrated sings of its antioxidant effect in cortex.

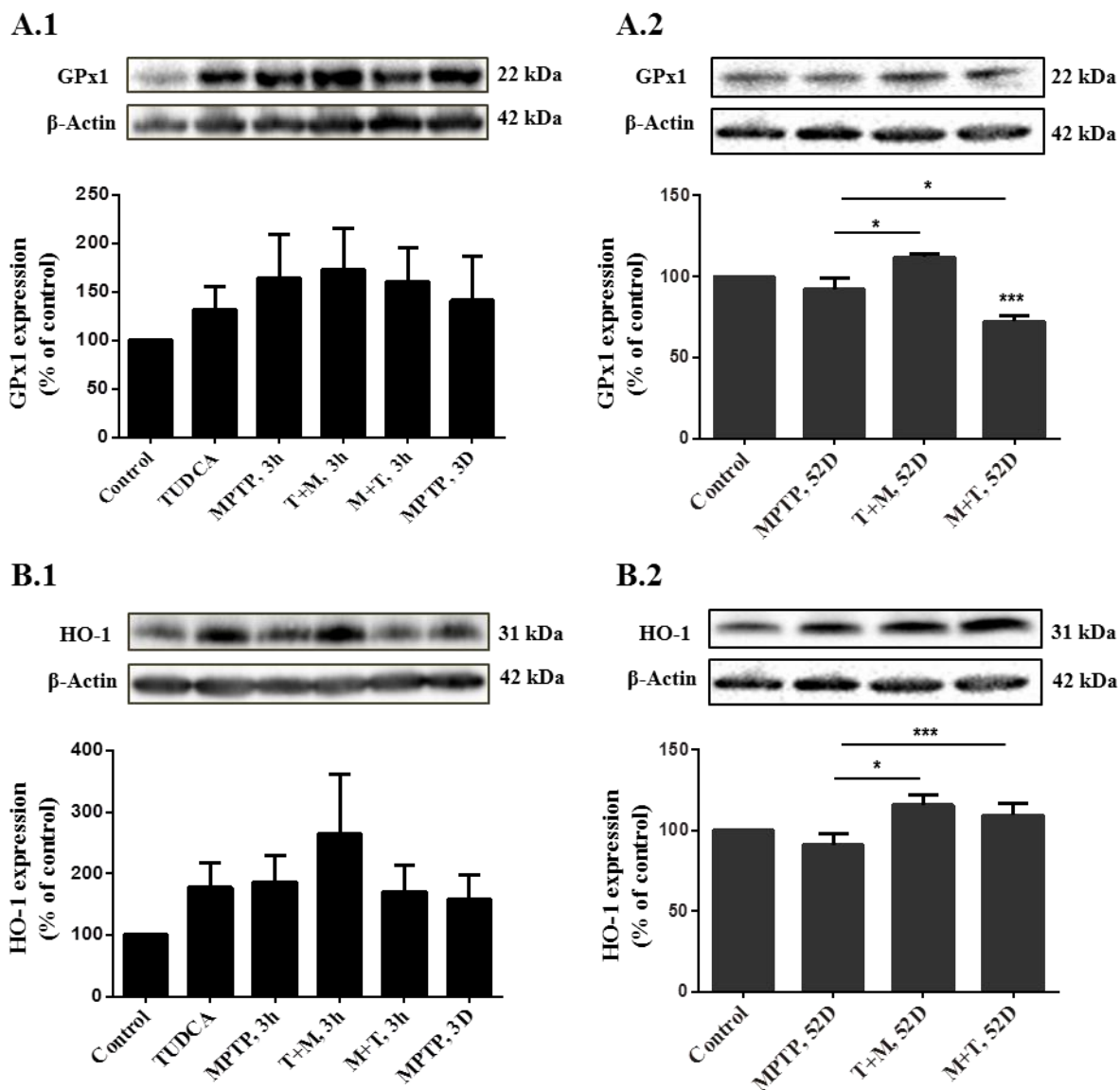


Figure III.1 – Effect of TUDCA on the expression of Nrf2 downstream target enzymes in the cortex of mice subjected to MPTP administration. C57BL/6 mice were treated with TUDCA and/or MPTP and were sacrificed after a short time treatment with MPTP (A.1 and B.1) or 52 days after MPTP injection (A.2 and B.2), as indicated in Methods. Tissue extracts from mice cortex were subjected to SDS-PAGE, and the blots were probed with antibodies against GPx (A.1, A.2) or HO-1 (B.1, B.2). β -actin was used as a loading control. The intensity of the bands was quantified using computerized image analysis (Image Lab). Representative immunoblots for each protein are shown over the correspondent graphs. Data are expressed as the mean values \pm SEM from four independent experiments, indicated as percentage of control. * $p < 0.05$, and *** $p < 0.001$ vs Control or MPTP using one-way ANOVA with Bonferroni's post hoc test.

1.2 Role of TUDCA on ROS generation in C57BL/6 mice cortex upon MPTP administration

As previously described above, oxidative stress derives from ROS generation coupled with a defective free radical scavenging system, causing a disturbance in the pro-

oxidant/antioxidant homeostatic state. ROS generation levels are not only increased in PD patients' brains but are also a by-product of MPTP toxicity. Our group previous studies show that MPTP induces an increase in ROS formation in mice midbrain and striatum that was abrogated by pre- and post-treatment with TUDCA (Moreira *et al.*, 2012). Therefore, we decided to investigate whether in the cortex MPTP and/or TUDCA would recapitulate the effects observed in the nigro-striatal axis, using a DCF-DA fluorescent probe and measuring ROS generation after 1 h of incubation. In contrast to our previous observations, intracellular ROS levels were not altered in any of the conditions tested, neither in short term nor in long term evaluations (Figure III.2).

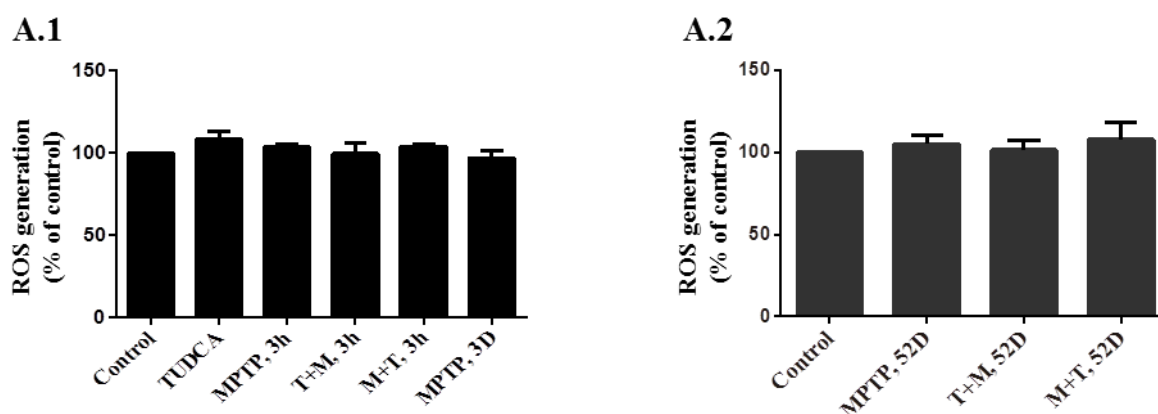


Figure III.2 – Role of TUDCA on ROS generation in the cortex of mice subjected to MPTP administration. C57BL/6 mice were treated with TUDCA and/or MPTP and were sacrificed after a short time treatment with MPTP (A.1) or 52 days after MPTP injection (A.2), as indicated in Methods. ROS generation in mice cortex subjected to short and long term evaluation was determined using DCF-DA probe. Fluorescence intensity of DCF was measured at an excitation wavelength of 485nm 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 \pm SEM from three independent experiments.

2. Evaluation of TUDCA effect on autophagy and mitophagy associated proteins in C57BL/6 mice cortex upon MPTP administration

2.1 TUDCA up-regulates the expression of PINK1 and parkin proteins in C57BL/6 mice cortex

PINK1 and parkin are key regulators of mitochondrial quality control, being essential to maintain a healthy pool of mitochondria. These proteins regulate the mitophagic process through PINK1/parkin pathway by recognizing damage mitochondria and promoting its

clearance (Scarffe *et al.*, 2014). In turn, mutations or impairments in PINK1 and/or parkin proteins can cause mitochondrial dysfunction and lead to PD pathogenesis and progression, being implicated in both familial and sporadic forms of the disease (Bose & Beal, 2016). Therefore, modulation of these proteins may represent a relevant therapeutic strategy for PD. To understand TUDCA neuroprotective mechanism of action our group focused on this bile acid ability to modulate mitophagy associated proteins. Previous studies showed that TUDCA treatment prior to MPTP administration significantly increased the expression of PINK1 and parkin, as well as parkin activation (phosphorylation) in mice striatum and midbrain (Rosa *et al.*, 2017). Thus to better understand TUDCA neuroprotective effect in the mice model of PD, we investigated its ability to modulate PINK1 and parkin in the cortex of MPTP administered animals.

The expression levels of p-parkin, parkin and PINK1 were evaluated by Western blot, in cortex of mice either subjected to short or long term evaluation treatments (Figure III.3). Regarding phosphorylated parkin expression in short term evaluation samples, (Figure III.3, A.1), there is a tendency for increase in all time points evaluated, especially when animals were pre-treated with TUDCA, although no significant results were obtained. Results from panel B.1 show that the levels of parkin were significantly increased in all conditions evaluated ($p < 0.001$ vs control). Importantly, pre-treatment with TUDCA induced an increase in parkin expression that was statistically different from MPTP-administered mice samples ($p < 0.001$ vs MPTP). In respect to full length PINK1 expression in samples from short term evaluation treatment, no significant differences were found in any condition tested. Unexpectedly, the expression levels of these proteins were not significantly affected in the cortex of animals subjected to long term evaluation protocol.

These results indicate that TUDCA treatment modulates parkin levels in the cortex of MPTP-intoxicated mice. Additionally, the regulation of parkin levels and activity by TUDCA may be transient, as previously described for other brain regions (Rosa *et al.*, 2017).

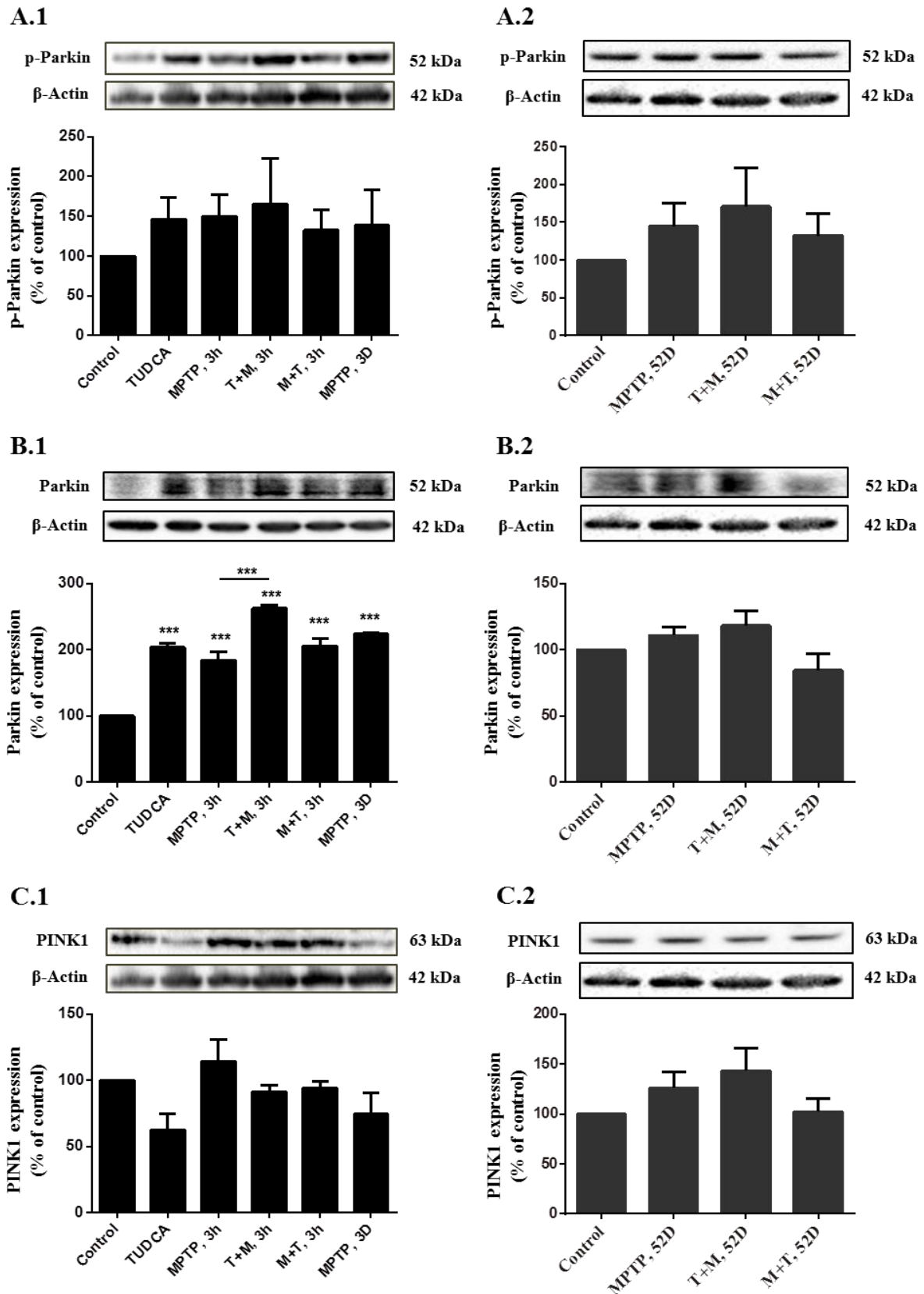


Figure III.3 – TUDCA induces expression of mitophagy related preteins parkin and PINK1 in mice administered with MPTP. C57BL/6 mice were treated with TUDCA and/or MPTP and were sacrificed after a short time treatment with MPTP (A.1, B.1 and C.1), or 52 days after MPTP injection (A.2, B.2 and C.2), as indicated in Methods. Tissue extracts from mice cortex were subjected to SDS-PAGE, and the blots were probed

with antibodies against p-parkin (**A.1, A.2**), parkin (**B.1, B.2**) or full length PINK1 (**C.1, C.2**). β -actin was used as a loading control. The intensity of the bands was quantified using computerized image analysis (Image Lab). Representative immunoblots for each protein are shown over the correspondent graphs. Data are expressed as the mean values \pm SEM from four independent experiments, indicated as percentage of control. *** $p < 0.001$ vs control or MPTP using one-way ANOVA with Bonferroni's post hoc test.

2.2 Neuroprotective role of TUDCA against MPTP-induced ATP depletion and AMPK phosphorylation in mice cortex

ATP depletion can be perceived as an evidence of mitochondrial dysfunction that ultimately leads to cell death. As already mentioned, MPTP actively participates in decreasing ATP, by directly inhibiting the Complex I of the ETC, contributing for a disruption in the oxidative phosphorylation process. Complex I impairment increases ROS formation and the subsequent damage to components of the respiratory chain and other mitochondrial factors, triggering a vicious cycle between mitochondrial dysfunction and oxidative stress that affects neighboring cells and increases neurodegeneration and PD progression.

The enzyme AMPK is essential for regulating mitochondrial homeostasis, being involved in biogenesis and autophagic processes. The autophagy pathway mediated by active (phosphorylated) AMPK is triggered upon energy stress conditions, increases in ROS and calcium concentration (Sundararaman *et al.*, 2016). Since we observed the ability of TUDCA to up-regulate the expression of antioxidant enzymes and the mitophagic associated protein parkin, we decided to explore TUDCA role in ATP depletion and AMPK activation as outcomes of its neuroprotective role in cortex. ATP levels were quantified using the ATP-Glo™ Bioluminometric Cell Viability Assay and expression levels of p-AMPK and AMPK were evaluated by Western blot, in the cortex of mice subjected to long term evaluation (Figure III.4). Results from Figure III.4 - A show that ATP levels had a notable decrease in the cortex of mice upon exclusive MPTP exposure, being significantly different from the control ($p < 0.01$ vs control). Interestingly, in mice pre-treated with TUDCA, ATP levels were increased, being statistically different from MPTP samples ($p < 0.001$ vs MPTP). In contrast, TUDCA administration after MPTP could not restore ATP levels, showing ATP levels similar to the ones found in MPTP-treated animals ($p < 0.01$ vs control). Accordingly, AMPK activation levels in the cortex from mice treated exclusively with MPTP, were found to be significantly lower than the control ($p < 0.05$ vs control), and pre-treatment with TUDCA raised the expression levels of p-AMPK ($p < 0.01$ vs MPTP). However, the levels of p-AMPK

were not different between control and animals treated with TUDA after MPTP administration.

In the cortex, at the time points evaluated, administration of this bile acid after MPTP exposure does not appear to be effective in restoring ATP levels, nor inducing AMPK. But together these results reveal that TUDCA, when administered before MPTP, prevents MPTP-dependent ATP depletion in mice cortex probably through multiple mechanisms involving antioxidant defense responses as well as activation of AMPK.

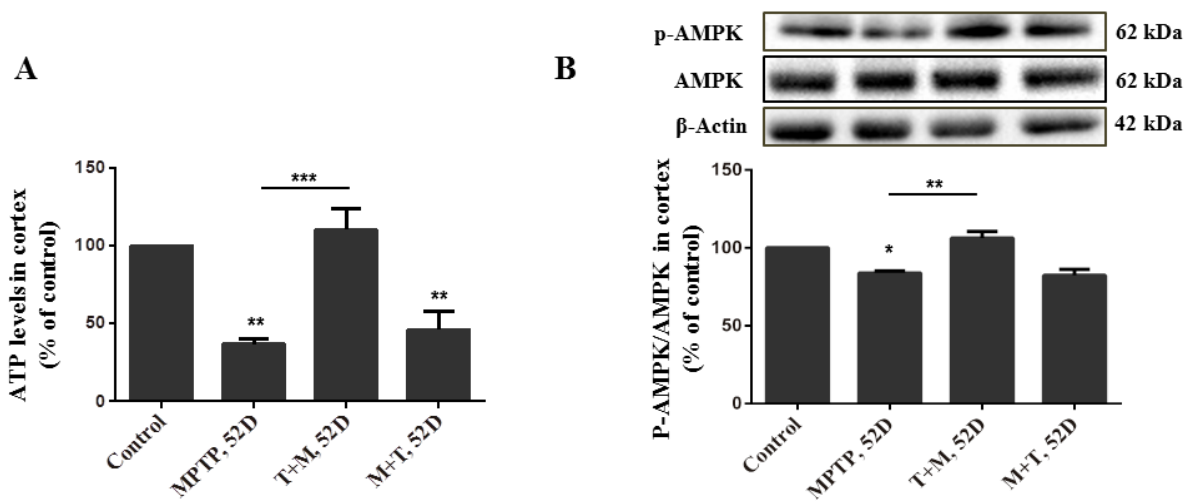


Figure III.4 – TUDCA protects against MPTP-induced ATP depletion and promotes AMPK activation in mice cortex. C57BL/6 mice were treated with TUDCA and/or MPTP and were sacrificed 52 days after MPTP injection as indicated in Methods for the long term evaluation group. **A** – ATP levels in cortex were quantified using ATP-Glo™ Bioluminometric Cell Viability Assay. The Luciferase activity was measured in a luminometer and the results are presented as a percentage of control. **B** – Tissue extracts from mice cortex were subjected to SDS-PAGE, and the blots were probed with antibodies against p-AMPK, AMPK or β -actin as a loading control. The intensity of the bands was quantified using computerized image analysis (Image Lab). Data are expressed as the mean values \pm SEM at least three independent experiments, indicated as percentage of control. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs control or MPTP using one-way ANOVA with Bonferroni's post hoc test.

3. Evaluation of the anti-inflammatory role of TUDCA in the MPTP mouse model of PD

3.1 TUDCA decreases reactive astrogliosis marker in C57BL/6 mice cortex in the presence of MPTP

Astrocytes are key players of the innate immune system in the CNS. These cells are capable of detecting harmful stimulus and respond by activating an adaptive immune defense. Reactive astrocytes are characterized by upregulation of GFAP protein and morphological

changes. Although the pro-inflammatory response can work as a protective mechanism, chronic exposure could become detrimental to the cell and lead to neuronal degeneration.

In addition to astrocytes, microglia also plays an important role in the immune response in the CNS. Activated microglia, characterized by Iba-1 expression, can either adapt a pro-inflammatory or a pro-resolving state in response to different stimulus. However, when the pro-resolving state cannot clear the debris, resolve the chronic inflammatory response and restore homeostasis it becomes harmful to the cell.

Our preliminary unpublished results show that MPTP significantly induces astro- and microgliosis in the midbrain and striatum from MPTP injected animals, which were significantly prevented by pre- or post-treatment with TUDCA (Rosa et. al, unpublished). For that reason, we explored whether the effects of MPTP on glia activation and neuroinflammation were restricted to the nigrostriatal axis, or could affect other brain regions. Additionally, we investigated the potential of TUDCA to modulate neuroinflammation in the cerebral cortex of animals exposed to MPTP.

The expression levels of GFAP in cortex were evaluated by Western blot, in mice either subjected to short or long term evaluation treatments, and by Immunohistochemistry in cortex samples of mice subjected to long term evaluation treatment, as depicted in Figure III.5. Western blot analysis revealed that in short and long term evaluations, GFAP expression was not considerably different in any experimental condition. In contrast, immunohistochemistry assay of animals sacrificed 52 days post-MPTP injection revealed that GFAP expression levels were significantly increased ($p < 0.001$ vs control). Importantly, astrocyte activation was partially prevented in animals treated with TUDCA prior to MPTP ($p < 0.001$ vs MPTP) and in animals treated with TUDCA after MPTP ($p < 0.01$ vs MPTP).

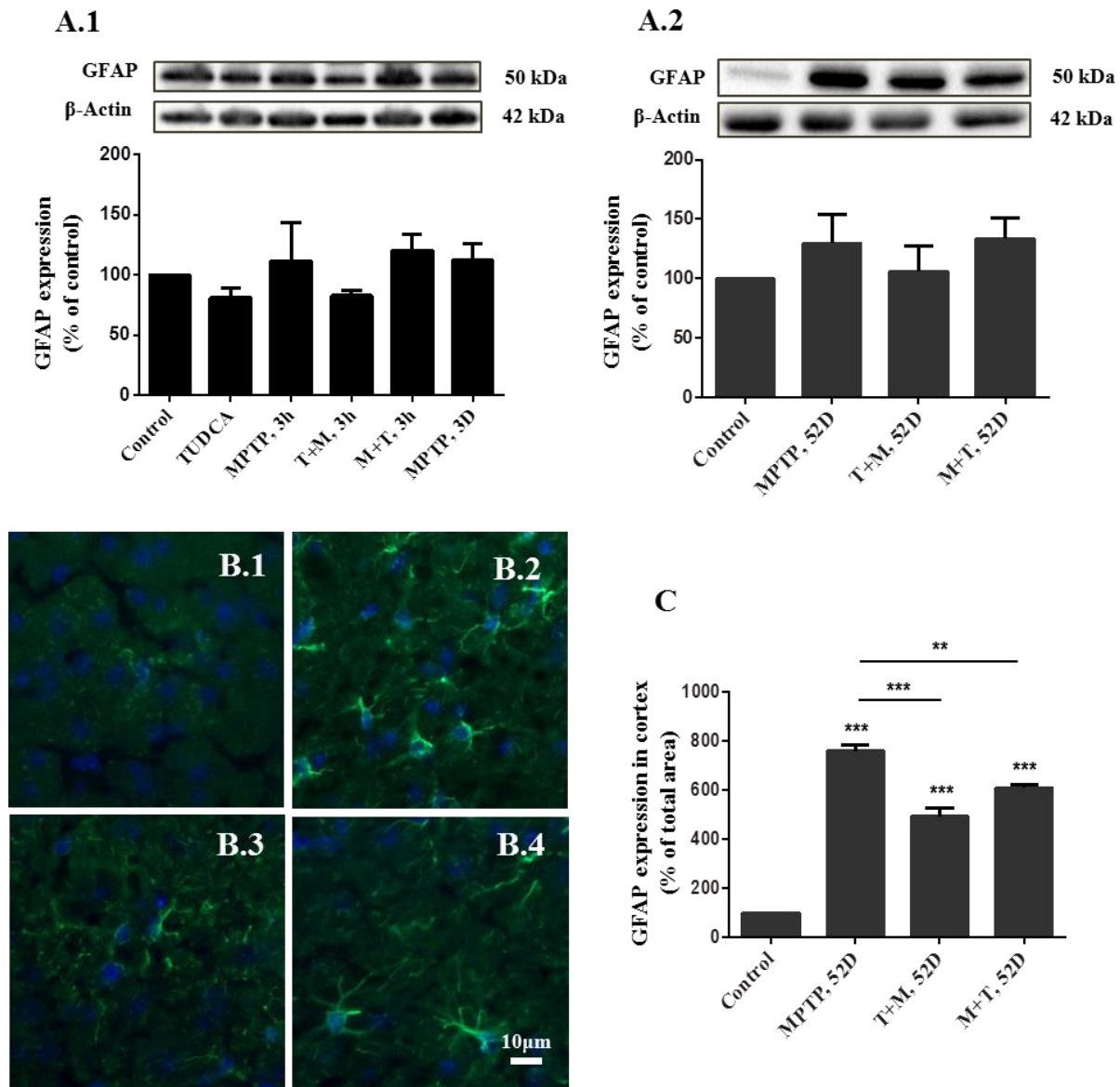


Figure III.5 – TUDCA prevents MPTP-induced astrocyte activation in mice cortex. C57BL/6 mice were treated with TUDCA and/or MPTP and were sacrificed after a short time treatment with MPTP (A.1), or 52 days after MPTP injection (A.2), as indicated in Methods. Tissue extracts from mice cortex were subjected to SDS-PAGE, and the blots were probed with antibodies against GFAP and β -actin, used as a loading control. The intensity of the bands was quantified using computerized image analysis (Image Lab). Representative immunoblots for each protein are shown over the correspondent graphs. Data are expressed as the mean values \pm SEM at least three independent experiments, indicated as percentage of control.

Fluorescence microscopy images show glial fibrillary acid protein (GFAP) staining of Control (B.1), MPTP (B.2), TUDCA prior to MPTP (B.3) and MPTP before TUDCA (B.4) treated mice. GFAP was stained in green and nuclei were counterstained with Hoechst dye 33258. Scale bar= 10 μ m. Graphic C represents the percentage of area occupied by Iba-1 positive cells, measured using the ImageJ software. Data shown are mean values \pm SEM of at least three independent experiences. ** p <0,01 and *** p <0,001 vs Control or MPTP using one way ANOVA with Bonferroni's post hoc test.

After evaluating TUDCA's neuroprotective effect in astrogliosis we decided to further characterize anti-inflammatory role of this bile acid in microglia activation. Iba-1 expression

levels were evaluated by Immunohistochemistry in cortex samples of mice subjected to long term evaluation treatments (Figure III.6). Treatment with MPTP in the presence or absence of TUDCA significantly increased Iba-1 expression in the cortex ($p < 0.001$). Importantly, pre-treatment with TUDCA efficiently prevented MPTP-induced Iba-1 expression ($p < 0.001$ vs MPTP).

Taken together, these results reveal that, when administered before MPTP, TUDCA exerts an efficient anti-inflammatory effect, by preventing the expression of these glia activation markers.

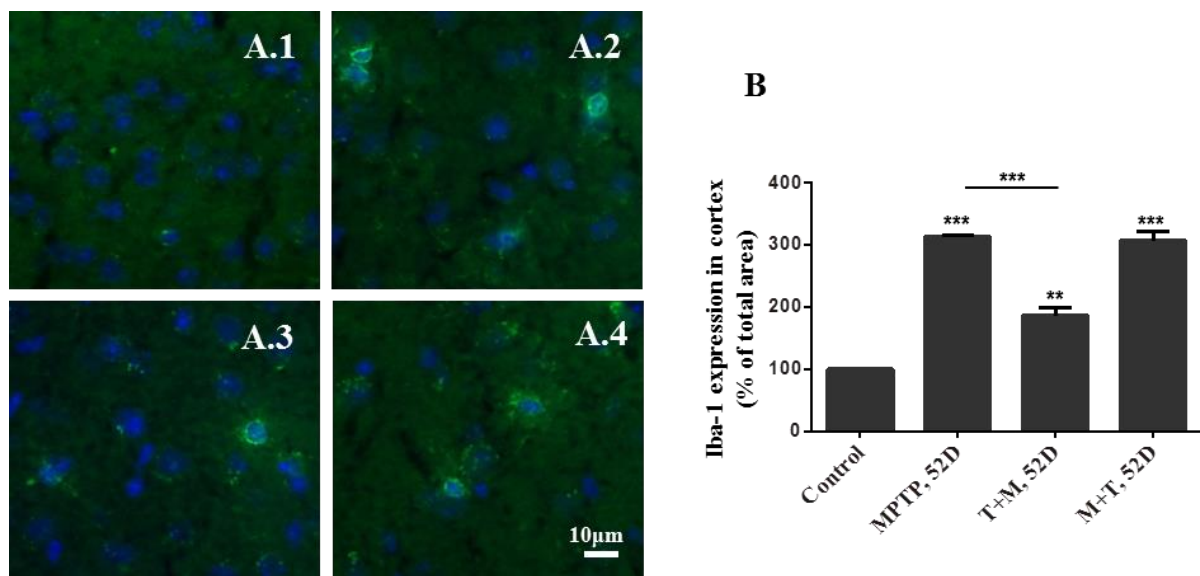


Figure III.6 – TUDCA prevents MPTP-induced glial activation in mice cortex. C57BL/6 mice were treated with TUDCA and/or MPTP and were sacrificed 52 days after MPTP injection as indicated in Methods for the long term evaluation group. Fluorescence microscopy images show ionized calcium binding adaptor molecule-1 (Iba-1) staining of Control (A.1), MPTP (A.2), TUDCA prior to MPTP (A.3) and MPTP before TUDCA (A.4) treated mice. Iba-1 was stained in green and nuclei were counterstained with Hoechst dye 33258. Scale bar= 10 μ m. Graphic B represents the percentage of area occupied by Iba-1 positive cells, measured using the ImageJ software. Data shown are mean values \pm SEM of at least three independent experiences. ** $p < 0,01$ and *** $p < 0,001$ vs Control or MPTP using one way ANOVA with Bonferroni's post hoc test.

After evaluating the effects of TUDCA in glial activation, we decided to explore the role of this bile acid on the expression of the anti-inflammatory protein ANXA1. ANXA1 is an anti-inflammatory protein that in inflammatory conditions is cleaved into an active state, essential for resolving the pro-inflammatory response and for helping restore tissue structure, function and homeostasis.

The expression levels of ANXA1 in cortex were evaluated by Western blot in cortex samples from mice either subjected to short or long term evaluation protocols (Figure III.7).

In the cortex of animals from the short term evaluation group MPTP and/or TUDCA did not significantly differed ANXA1 expression levels in any experimental condition. Interestingly, when animals were sacrificed 52 days post-MPTP TUDCA significantly increased ANXA1 expression when administered before ($p < 0.001$ vs control, $p < 0.01$ vs MPTP) or after ($p < 0.05$ vs control) the neurotoxin

Altogether, our results show that MPTP induces a pro-inflammatory response in the cortex that is efficiently ameliorated when animals are treated with TUDCA either before or after administration of the neurotoxin. The modulation of ANXA1 expression by TUDCA conveys a possible mechanism of action for this bile acid.

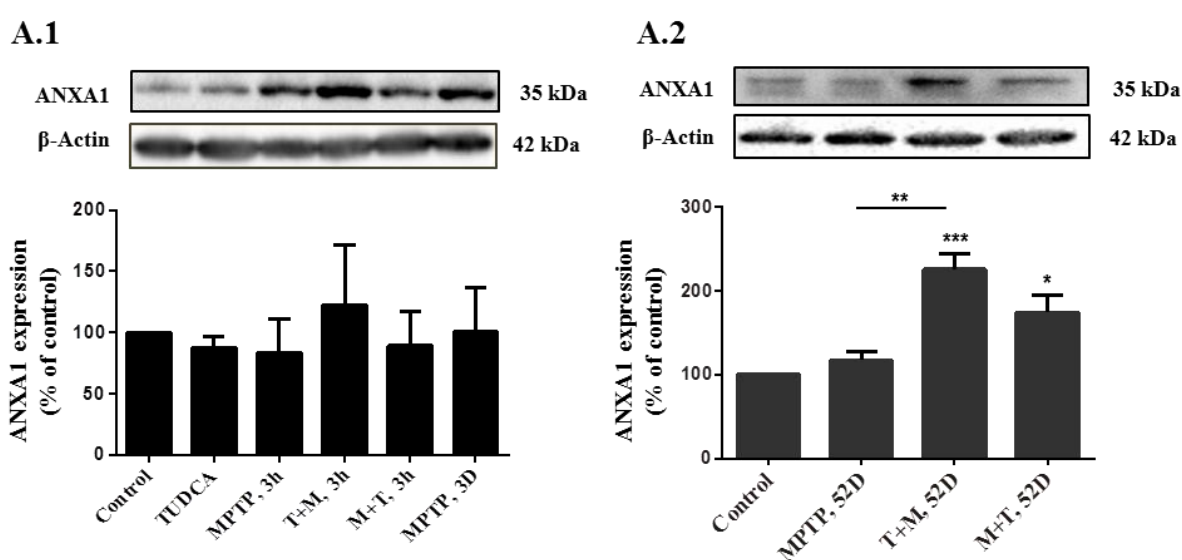


Figure III.7 – TUDCA induces the expression of ANXA1 in cortex from mice injected with MPTP. C57BL/6 mice were treated with TUDCA and/or MPTP and were sacrificed after a short time treatment with MPTP (A.1), or 52 days after MPTP injection (A.2), as indicated in Methods. Tissue extracts from mice cortex were subjected to SDS-PAGE, and the blots were probed with antibodies against ANXA1 and β -actin, used as a loading control. The intensity of the bands was quantified using computerized image analysis (Image Lab). Representative immunoblots for each protein are shown over the correspondent graphs. Data are expressed as the mean values \pm SEM from four independent experiments, indicated as percentage of control. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs control or MPTP using one-way ANOVA with Bonferroni's post hoc test.

4. Evaluation of TUDCA neuroprotective role in the MPTP mouse model of PD

Finally, to complete our study regarding the effect of MPTP and TUDCA effect in the mice cortex, we investigated the role of this compound in neuronal death. In order to examine neuronal loss, we assessed the expression levels of a microtubule element found in neurons,

β -III-tubulin in cortex by Western blot, in mice either subjected to short or long term evaluation protocols. Results presented in Figure III.8 show that the expression of β -III-tubulin was not significantly altered in any of the conditions tested.

These results suggest that although we have observed deleterious MPTP effects in mouse cortex, these effects do not culminate with neuronal cell death at the time points evaluated. However, cell death should be tested at longer time points and by other experimental approaches in order to exclude neurodegeneration in the cortex triggered by MPTP.

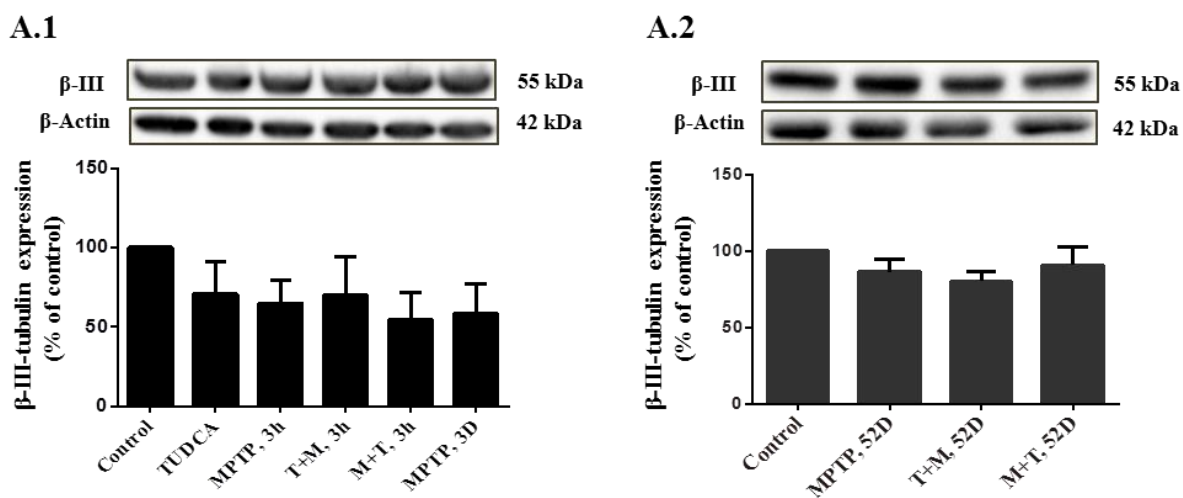


Figure III.8 – Evaluation of TUDCA effect in neuronal death, in mice cortex, after MPTP administration. C57BL/6 mice were treated with TUDCA and/or MPTP and were sacrificed after a short time treatment with MPTP (A.1), or 52 days after MPTP injection (A.2), as indicated in Methods. Tissue extracts from mice cortex were subjected to SDS-PAGE, and the blots were probed with antibodies against β -III-tubulin and β -actin, was used as a loading control. The intensity of the bands was quantified using computerized image analysis (Image Lab). Representative immunoblots for each protein are shown over the correspondent graphs. Data are expressed as the mean values \pm SEM from at least three independent experiments, indicated as percentage of control.

Collectively, the results obtained in this work, in mice cortex, emphasize the role of TUDCA as an antioxidant, anti-inflammatory and cytoprotective compound in a mice PD model and strengthened its promising therapeutic effects in PD.

5. Evaluation of the anti-inflammatory role of TUDCA in *in vitro* models

5.1 TUDCA modulation of ANXA1 expression in mice primary neurons treated with MPP⁺

Our group has previously explored the antioxidant role of TUDCA in experimental models of PD (Moreira *et al.*, 2017). Interestingly, in the present work we found that not only most of the mechanisms triggered by MPTP and TUDCA are recapitulated in the cerebral cortex, but we also demonstrate an important anti-inflammatory role of this bile acid. Here we show that TUDCA abrogates glia activation, and most notably induces the expression of the anti-inflammatory protein ANXA1 in the cortex, in this experimental model of PD. Therefore, we decided to further explore the ability of TUDCA to induce ANXA1 expression and/or secretion. For that, we used an *in vitro* approach starting by assessing ANXA1 expression in mice primary neuron cultures treated with MPP⁺, in the presence or absence of TUDCA. Results presented in Figure III.9 show that ANXA1 levels were increased about 3 times the control value when cells were incubated with MPP⁺, in the presence or absence of TUDCA. Still these values do not present statistical significance, due to a low number of experiments (n=3) and/or to the fact that probably ANXA1 expression in response to MPP⁺ happens in a wave-like response. The fact that ANXA1 is secreted upon its synthesis further increases the difficulty of this analysis. Unfortunately, in these cells secreted membrane-bound ANXA1 could not be detected due to low concentration, since these assays were performed in a 1.9 cm² plate that accommodates a low number of cells even in confluency.

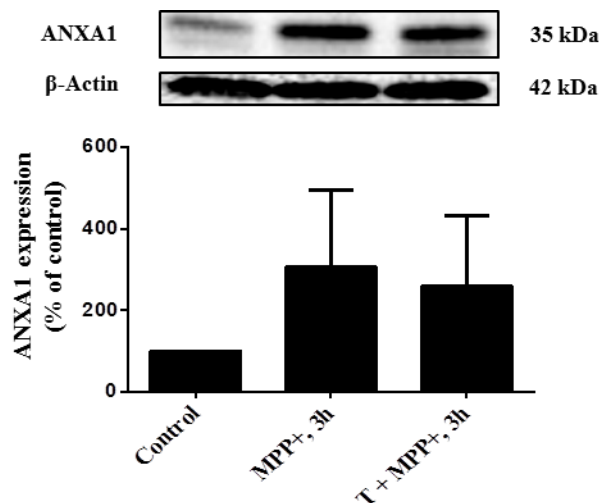


Figure III.9 – TUDCA effect in ANXA1 expression in mice primary neurons treated with MPP⁺. Primary neurons were treated with TUDCA and/or MPP⁺ as described in Methods. Cell extracts were subjected to SDS-PAGE, and the blots were probed with antibodies against ANXA1 and β -actin, was used as a loading control. The intensity of the bands was quantified using computerized image analysis (Image Lab). Representative immunoblots for each protein are shown over the correspondent graphs. Data are expressed as the mean values \pm SEM from three independent experiments, indicated as percentage of control.

5.2 TUDCA modulation of ANXA1 expression in BV2 microglia cell line

After evaluating the effect of TUDCA in ANXA1 expression levels, both in *in vivo* and *in vitro* PD models we decided to explore the mechanism by which this bile acid could modulate ANXA1 expression. Since this inflammatory protein, in the brain, is found to be almost exclusively expressed in glial cells, we focused our study in the BV2 microglia cell line (Solito *et al.*, 2008). In addition to evaluate intracellular levels of ANXA1 we also assessed its membrane-bound extracellular levels, considering the extracellular role of this protein in several inflammatory processes. The intra- and extracellular expression levels of ANXA1 in response to different time-points of treatment with TUDCA were evaluated by Western blot, and are shown in Figure III.10. Intracellular protein levels of ANXA1 (Figure III.10-A) do not seemed to change upon TUDCA administration. However, the extracellular levels of ANXA1 were significantly increased after 6 h exposure to TUDCA ($p < 0.05$). These results indicate that TUDCA induces the secretion of ANXA1 and replenishes the intracellular content of the protein, strongly suggesting *de novo* synthesis of ANXA1. Since the peak of extracellular protein was found at 6 h post-TUDCA addition, this time-point was used in the subsequent experiments aiming to determine the mechanisms underlying ANXA1 expression.

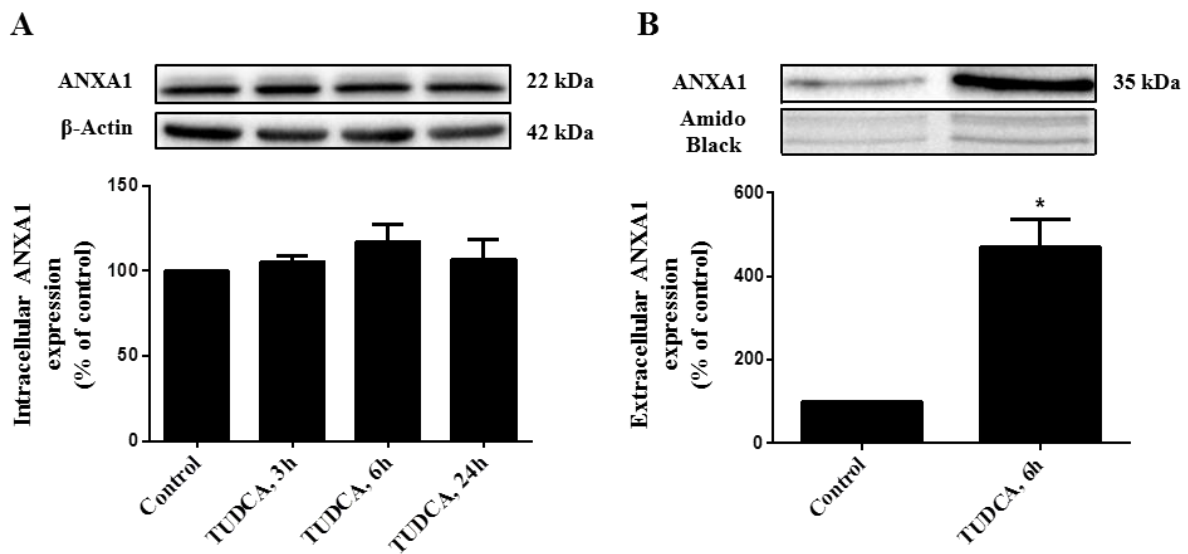


Figure III.10 – TUDCA increases ANXA1 expression and secretion in a mouse microglia cell line. BV2 cells were treated with TUDCA and extracellular ANXA1 was obtained after washing the cells with 1mM EDTA buffer, as described in Methods. Cell extracts (A) and products of EDTA buffer wash (membrane-bound extracellular protein) (B) were subjected to SDS-PAGE, and the blots were probed with antibodies against ANXA1 and β -actin (loading control). The intensity of the bands was quantified using computerized image analysis (Image Lab). Representative immunoblots are shown over the correspondent graphs. Data are expressed as the mean values \pm SEM from three independent experiments, indicated as percentage of control. * $p < 0.05$ using T-student test.

5.3 Unraveling the mechanism underlying the modulation of ANXA1 by TUDCA

The promotor region of *ANXA1* gene contains a cAMP responsive element (CRE) that requires an activated CRE binding protein (CREB) to induce ANXA1 mRNA synthesis (Antonicelli *et al.*, 2001). CREB activation occurs upon its phosphorylation, a process regulated by several signaling pathways. Among those, cAMP is thought to play a critical role, since production of this secondary messenger activates a protein kinase A (PKA) responsible for CREB phosphorylation (Hagiwara *et al.*, 1993). Besides PKA, protein kinase B (AKT) has also been implicated in CREB activation (Peltier *et al.*, 2007). Taking these reports in to account and together with the fact that TUDCA increases cAMP levels in primary cultures of microglia cells (Yanguas-Casas *et al.*, 2017), we evaluated whether PKA or AKT mediate TUDCA-induced ANXA1 synthesis, using specific pharmacological inhibitors of each kinase. Thus, BV2 cells were treated with K252A and H89, as PKA inhibitors, and Wortmannin has an AKT inhibitor, for 1 h prior to TUDCA treatment. The expression levels of intracellular ANXA1 were apparently decreased when cells were re-

treated with each of the inhibitors prior to TUDCA (Figure III.11). As expected, the more evident result was obtained when we used the PKA inhibitor H89. However, these results only represent data from two different experiments, therefore we were not able to test statistical significance in the experimental conditions tested. To confirm these results, corroborative experiments are ongoing. Additionally, extracellular membrane bound protein from these experimental conditions could not be detected due to technical problems, therefore these assays have to be repeated with another batch of samples. Taken these preliminary results in consideration, we decided to proceed our studies using H89.

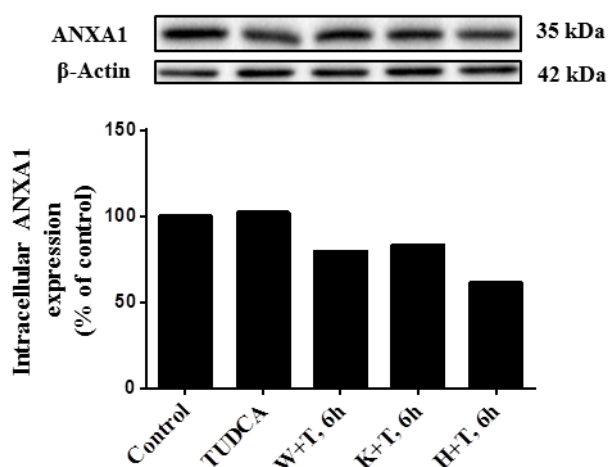


Figure III.11 - TUDCA effect in ANXA1 expression in a mouse microglia cell line treated with selective inhibitors. BV2 microglia cell line, were treated with TUDCA and/or Inhibitors as described in Methods. Cell extracts were subjected to SDS-PAGE, and the blots were probed with antibodies against ANXA1 and β -actin (loading control). The intensity of the bands was quantified using computerized image analysis (Image Lab). Representative immunoblots for each protein are shown over the correspondent graphs. Data are indicated as percentage of control. H – H89; K – K252A; T – TUDCA; and W – Wortmannin;

Taken together, these results indicate that TUDCA up-regulates ANXA1 synthesis through a PKA mediated mechanism. Although, this pathway has already been described for other ANXA1 inducers, further assays are needed to confirm the obtained data with TUDCA.

5.4 TUDCA modulation of DJ-1 expression in BV2 microglia cell line

Evidences of the ability of TUDCA to modulate DJ-1 expression in midbrain and striatum from a mice PD model were previously discussed by our group in Moreira *et al.*, 2017. Interestingly, DJ-1 is reported to modulate activation and cleavage of a member from

the Annexin family, Annexin 2 (Yamane *et al.*, 2015). Since neuroinflammation and oxidative stress are intimately related, and both DJ-1 and ANXA1 proteins are noted to be modulated by TUDCA in mice brain, we decided to verify DJ-1 expression in microglia BV2 cells upon treatment with TUDCA. Results depicted in Figure III.12, show that DJ-1 expression in response to TUDCA increased over time, being significantly different from control at 24 h of exposure to TUDCA ($p < 0.05$).

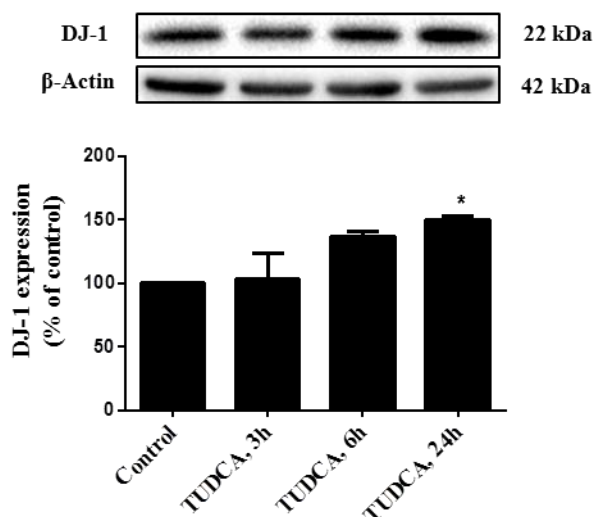


Figure III.12 - TUDCA effect in DJ-1 expression in a mouse microglia cell line. BV2 microglia cell line, were treated with TUDCA as described in Methods. Cell extracts were subjected to SDS-PAGE, and the blots were probed with antibodies against DJ-1 and β -actin (loading control). The intensity of the bands was quantified using computerized image analysis (Image Lab). Representative immunoblots for each protein are shown over the correspondent graphs. Data are expressed as the mean values \pm SEM from at least three independent experiments, indicated as percentage of control. * $p < 0.05$ using one-way ANOVA with Dunnett's post hoc test.

Together these results show that TUDCA modulates synthesis of ANXA1 *de novo* in microglia cells, reinforcing the results obtained in mice cortex, suggesting that the anti-inflammatory role of TUDCA is, at least partially, mediated by ANXA1. In accordance, in microglia cells TUDCA also up-regulates the expression of DJ-1, a protein that is involved in several cellular processes including anti-oxidant and anti-inflammatory mechanisms, that may also serve as a mediator of the cytoprotective effects of TUDCA.

However, our preliminary results in BV2 cells were insufficient to provide a clear explanation for TUDCA mechanism of action and effects in ANXA1, being necessary to acquire supplementary data.

IV. Discussion & Conclusion

Over this last 200 years PD research and knowledge has greatly advanced. Nonetheless, the main cause for neurodegeneration is still elusive. This may explain why there are still no effective therapies to delay, stop or prevent neurodegeneration in PD. However, throughout the years several mechanisms such as mitochondrial dysfunction, oxidative stress and neuroinflammation have been deeply implicated in the pathogenesis of PD (Blesa *et al.*, 2015; Hirsch *et al.*, 2012; Winklhofer & Haass, 2010). Therefore, the search for novel therapeutic strategies that help maintain mitochondria homeostasis, counteract ROS mediated damage and promote pro-resolving anti-inflammatory mechanisms is of utmost importance. In that matter TUDCA, an endogenous bile acid that has been reported to have antioxidant, anti-inflammatory and neuroprotective properties in several experimental models of neurodegenerative diseases (Keene *et al.*, 2001; Sola *et al.*, 2006), including PD (Castro-Caldas, Carvalho, Rodrigues, Henderson, Wolf, Rodrigues, *et al.*, 2012; Moreira *et al.*, 2017; Rosa *et al.*, 2017) is a promising therapeutic agent for PD. Additionally, although the most vulnerable region in PD is considered to be the nigro-striatal axis (Fahn, 2003) the spread of neurologic insult to other brain regions is still not completely unraveled. However, with PD progression damage in regions such as cerebral cortex is unavoidable (Poewe *et al.*, 2017). Therefore, understanding the broader damage, as well as the effects of therapeutic strategies in those areas is essential.

Thus, in this thesis we investigated whether the deleterious effects of MPTP in the mouse brain were also evident in the cerebral cortex. In parallel, we investigated the effect of TUDCA on key mediators implicated in the down-regulation of oxidative stress and mitochondrial dysfunction. We have also discovered the anti-inflammatory potential of TUDCA in the mouse cortex of mice exposed to MPTP. Finally, we have conducted *in vitro*

experiments to further dissect a possible mechanism underlying the anti-inflammatory potential of TUDCA in the brain.

A major endogenous cellular defense mechanism against oxidative stress involves the activation of the Nrf2 pathway (Nguyen *et al.*, 2009). This transcription factor is responsible for inducing the expression of several cytoprotective enzymes including GPx1 and HO-1 (Brigelius-Flohe, 2006; Choi & Alam, 1996). Under pathological conditions, including experimental models of PD (Cook *et al.*, 2011; Jakel *et al.*, 2007; Ramsey *et al.*, 2007; Xiao *et al.*, 2011) the protective role of Nrf2 against oxidative stress highlights the importance of activating this pathway and upregulating Nrf2-driven antioxidant enzymes (de Vries *et al.*, 2008). So we decided to evaluate the effect of TUDCA on Nrf2 downstream targets expression in the cortex of C57BL/6 mice upon MPTP administration. Our results revealed that in a short term evaluation protocol exposure to MPTP and/or TUDCA increased protein levels of both HO-1 and GPx1 enzymes in cortex. The fact that MPTP by itself up-regulates the expression of these enzymes may indicate a possible compensatory mechanism in response to MPTP-induced oxidative stress. Indeed, in previous studies our group found that Nrf2 was activated with concomitant increase in its downstream targets in striatum and midbrain of mice exposed to MPTP (Carvalho *et al.*, 2016; Moreira *et al.*, 2017). Accordingly, other groups also suggested that Nrf2 activation is part of an adaptive response by the cells against oxidative stress, a reported repercussion of MPTP exposure (Chen *et al.*, 2009; Cleeter *et al.*, 1992; Wang *et al.*, 2014). Notably, results from long term evaluation protocol showed that pre-treatment with TUDCA further enhanced the expression of GPx1 and HO-1. The differences observed in protein expression levels of both enzymes when animals were treated with TUDCA after MPTP exposure could be due to different regulatory mechanisms, and different kinetics of induction by Nrf2. In fact, GPx1 is a primary defense network enzyme, whereas HO-1 is an inducible detoxifying enzyme (Bajpai *et al.*, 2017; Loboda *et al.*, 2016; Lubos *et al.*, 2011). To have a better idea of the antioxidant role of TUDCA we should not only have analyzed protein expression levels, but also we should have determined the biological enzymatic activity of these proteins. Although we have these data for midbrain and striatum (Carvalho *et al.*, 2016; Moreira *et al.*, 2017) it would have been interesting to understand the ability of TUDCA and/or MPTP to modulate the activity of these enzymes in the cortex. Nevertheless, the differences obtained in protein expression levels for HO-1 and GPx1 upon TUDCA treatment do not undermine the antioxidant properties of TUDCA in the cortex and its effectiveness before and after the toxicity onset.

To further explore the antioxidant role of TUDCA we analyzed the ability of this bile acid to modulate ROS generation in the cerebral cortex in the presence of MPTP. As mentioned in the Chapter I of this thesis, oxidative stress derives from excessive ROS production, which can result from the inhibition of ETC Complex I, PD associated mutations, protein aggregation, among others (Gautier *et al.*, 2008; Junn & Mouradian, 2002; Schapira *et al.*, 1990; Taira *et al.*, 2004). Oxidative stress is, indeed, an important hallmark of sporadic and familial PD, and is recapitulated in models of the disease in response to neurotoxins, such as MPTP (Bose & Beal, 2016; Hwang, 2013; Mizuno *et al.*, 1987; Schapira *et al.*, 1990). Interestingly, our results showed that intracellular ROS levels were invariable in all the experimental conditions tested, for both short- and long-term treatments, contradicting our initial predictions and previous studies (Castro-Caldas, Carvalho, Rodrigues, Henderson, Wolf, Rodrigues, *et al.*, 2012; Cleeter *et al.*, 1992; Sriram *et al.*, 1997). Probably this could be due to lack of ROS induction by MPTP in the cerebral cortex, yet our results regarding ATP determination are in contradiction with this hypothesis. Moreover, we have previously found that in MPTP-exposed mice midbrain or striatum determination of ROS generation by this experimental approach shows waves with transient peaks. For example, we detected significant ROS generation at 1 h post-MPTP injection, but failed to determine any increase at 3 or 6 h post MPTP administration (Moreira *et al.*, 2017, and unpublished data). Additionally, evidences for ROS generation in waves were also reported in *in vitro* MPP⁺ models (Zawada *et al.*, 2011). Therefore, we probably failed to detect the peak of ROS production in cerebral cortex. Different time-points of MPTP exposure together with experimental approaches, such as Electron Spin Resonance (ESR) or the utilization of different fluorescent probes (Dihydrorhodamine 123 or Dihydroethidium), should be used in order to clarify this result.

Mitochondrial dysfunction has been deeply associated with PD, therefore in parallel to evaluation of the antioxidant effects of TUDCA we also analyzed its role in the expression of proteins involved in mitochondrial homeostasis and mitophagy pathways. PINK1 and parkin are key players in a major mitophagy pathway which is essential for ensuring mitochondrial quality control (Truban *et al.*, 2017). Besides parkin role in the elimination of damaged and dysfunctional mitochondria, this protein is also implicated in other mitochondrial-turnover mechanisms such as mitochondrial biogenesis (Shin *et al.*, 2011). In fact, parkin mediates parkin-interacting substrate (PARIS) ubiquitination, which prevents PARIS from binding to the peroxisome proliferator-activated receptor γ coactivator - 1 α (PGC-1 α) and suppressing its expression (Shin *et al.*, 2011). In turn, PGC-1 α regulates the expression of transcription factors such as nuclear regulatory factors (NRF-1) and promotes mitochondrial biogenesis,

serving as a key player in mitochondrial and energy homeostasis (Scarpulla, 2011; Wu *et al.*, 1999). Since PINK1 and parkin are involved in mitophagy, mitochondrial biogenesis and energy metabolism, dysregulation or dysfunction of these proteins are deeply implicated in PD, highlighting the importance of a therapeutic approach based on enhancing mitochondria turnover in order to halt the progression of the disease (Nardin *et al.*, 2016; Ryan *et al.*, 2015). Our results reveal that in both short and long term evaluation the expression levels of active parkin (p-parkin) and full length PINK1 were not significantly altered in any condition tested. Results show a tendency with lack of statistical significance, indicating that further studies should be performed in different animal samples to increase data. In contrast, short term evaluation results showed a significant increase in parkin expression levels in the presence of MPTP, indicating a possible compensatory mechanism for MPTP-induced mitochondrial dysfunction. Interestingly, pre-treatment with TUDCA increased significantly parkin protein expression when compared to MPTP only, evidencing TUDCA neuroprotective effect by mediating this protein expression in cortex, and corroborating the results obtained by our group in the striatum and midbrain region (Rosa *et al.*, 2017). These results also highlight the potential of TUDCA as an inducer of parkin expression in different brain areas and cells.

The capacity of TUDCA to modulate important endogenous targets as well as activate downstream pathways, as a neuroprotective strategy makes this bile acid a powerful therapeutic molecule to be used in neurodegenerative conditions.

As an outcome of the neurotoxic/neuroprotective effects of MPTP/TUDCA in the cerebral cortex we investigated ATP levels and AMPK activation. ATP levels are extremely important for intracellular homeostasis and take part in the majority of the metabolic events. Most intracellular ATP derives from glycolysis and oxidative phosphorylation processes. The last process comprises the oxidation of reduced cofactors through the ETC, in the mitochondria, which culminates in ATP synthesis by Complex V of the ETC, the ATP synthase (Brown, 1992). In neurons, MPTP inhibits the Complex I of the ETC, contributing to mitochondrial dysfunction in neurons, and leading to a decrease in ATP production (Chan *et al.*, 1991; Nicklas *et al.*, 1985). Consequently, severe depletion of ATP triggers a cascade of events that culminates in cellular death (Moon & Paek, 2015). In turn, AMPK is a highly conserved energy sensor that regulates energy metabolism, autophagic degradation and stress resistance (Salminen & Kaarniranta, 2012). Deregulation of AMPK can be attributed to protein aggregates, arisen from defective clearance mechanisms, excitotoxicity, metabolic alterations and oxidative stress, present in neurodegenerative disorders like PD (Domise & Vingdeux, 2016). AMPK active form (p-AMPK) not only has been reported to be expressed

in PD patients brains but also being induced by PD neurotoxins in *in vivo* and *in vitro* studies, ultimately triggering the autophagic pathway (Choi *et al.*, 2010; Jiang *et al.*, 2013; T. W. Kim *et al.*, 2013; Xu *et al.*, 2014). Even though, some studies view AMPK activation detrimental in PD, by contributing to neurodegeneration, others highlight its neuroprotective role by enhancing autophagy (Dulovic *et al.*, 2014; Hou *et al.*, 2015; Wu *et al.*, 2011). Moreover, studies also reveal the beneficial role of AMPK activation in the regulation of mitochondrial function in PD, including the participation of this protein in mitochondrial biogenesis in disease models (Ferretta *et al.*, 2014; Ng *et al.*, 2012). Although, the advantages of AMPK activation are still controversial, this protein can be an interesting therapeutic target to approach mitochondrial turnover (Hang *et al.*, 2015).

Our results show that ATP levels, exhibit a significant decrease in MPTP treated mice evidencing a neurotoxic detrimental effect in cortex, coincident with previous observations in PD patients brains and MPTP models of the disease (Chan *et al.*, 1991; Przedborski *et al.*, 2000). Notably, pre-treatment with TUDCA prevented the impairment of intracellular ATP levels, proving the neuroprotective effects of this bile acid *in vivo*, confirming our previous observation in an *in vitro* cellular model of PD (Rosa *et al.*, 2017). In this experimental condition, post-treatment with TUDCA had no effect on MPTP-triggered ATP depletion.

In respect to AMPK activation, our results revealed that in the presence of MPTP, the expression of active kinase (p-AMPK) decreased. The same results were not confirmed in similar studies using the MPTP model of PD (Choi *et al.*, 2010). The reason for this difference could be the distinct assays used to investigate AMPK activation, as well as the brain region studied, the MPTP dosage used (twice the concentration used in this work), and/or the time-point of evaluation. Since AMPK is involved in several regulatory processes its active form might be tightly regulated and have transient levels of activation (Castro-Caldas, Carvalho, Rodrigues, Henderson, Wolf, & Gama, 2012; Salminen & Kaarniranta, 2012) Interestingly, p-AMPK expression increased when animals were treated with TUDCA prior to MPTP administration, highlighting the neuroprotective effects of this bile acid, already described in other PD models since this protein is involved in important cellular survival pathways (Hang *et al.*, 2015). Moreover, TUDCA administration after toxic onset did not altered p-AMPK expression, which is in accordance with the results regarding ATP determination. Together these results indicate a concomitant activation of AMPK and preservation of ATP levels when animals are pre-treated with TUDCA, but not when TUDCA is administered after MPTP.

To evaluate the susceptibility of cortical neurons to MPTP, we assessed neurodegeneration by detection of β -III-tubulin expression. This protein is expressed exclusively in neurons, and can be used as a neuronal marker, being helpful in recognizing disease-related alterations in brain neuronal composition (Geisert & Frankfurter, 1989; Sullivan, 1988). In contradiction to other studies and our initial expectations, our results showed unaltered β -III-tubulin expression levels upon MPTP administration. Even though, results regarding ATP pool indicate a cytotoxic effect of MPTP that may not be solely directed to neurons. Moreover, the time-point of actual cell death can occur later after MPTP administration. In order to have a better understanding of neurodegeneration in cerebral cortex in the conditions tested, different experimental approaches, should have been performed including immunohistochemistry analysis of β -III-tubulin expression or the employment of different neuronal markers such as microtubule-associated protein 2 (MAP2) (Izant & McIntosh, 1980). Moreover, in conjugation with specific neurons and/or glia markers, methods to evaluate general cell death should have been used, such as TdT-mediated dUTP nick end labeling (TUNEL) assay or assessment of apoptotic markers (like cleaved caspase 3 and/or downstream targets), to evaluate whether other brain cells are being affected by MPTP (Korzhevskiy & Kirik, 2015; Lyck *et al.*, 2008; Mangili *et al.*, 1999; Regan, 1988; Yang *et al.*, 2002).

To further explore the role of TUDCA in this PD model, we decided to investigate anti-inflammatory effect of this bile acid in cerebral cortex. The neuroinflammation process, although initially beneficial can rapidly become detrimental after chronic exposure, being often linked to propagation of the initial trigger in neurodegenerative disorders (Kempuraj *et al.*, 2016). Chronic inflammation is characterized by lasting glia activation which results in a sustained release of inflammatory mediators. This leads to an increase in ROS, perpetuating the inflammatory cycle and further prolonging inflammation (Chen *et al.*, 2016). Therefore, a useful and promising therapeutic strategy relies on targeting mechanisms that drive this process (Heneka *et al.*, 2014; Tansey *et al.*, 2007).

Collectively, our results showed an increased expression of astro- and microglial markers, namely GFAP and Iba-1 proteins, respectively, in the presence of MPTP, consistent with the toxic effects of this compound described in other studies (Khan *et al.*, 2013; Lofrumento *et al.*, 2011). Notably, TUDCA treatment prevented or decreased the expression levels of both these indicators of glia activation, having a more effective role when administered before MPTP. These results support the anti-inflammatory effect of TUDCA described by Yanguas-Casás and colleagues (Yanguas-Casas *et al.*, 2014, 2017), which states

that TUDCA decreases pro-inflammatory cytokines attenuating the inflammatory response and can activate G protein-coupled bile acid receptor 1/Takeda G protein-coupled receptor 5 (GPBAR1/TGR5), increasing cAMP and mediating anti-inflammatory effects on microglia. To better understand the mechanism underlying the anti-inflammatory effects of TUDCA we explored how this bile acid modulated ANXA1 expression. Our results revealed that in the presence of MPTP, expression levels of ANXA1 were lower, possibly suggesting an endogenous inflammatory state with depiction of microglia M1-like phenotype instead of a pro-resolving/anti-inflammatory M2-like phenotype. Under inflammatory condition, in this case triggered by MPTP, NF- κ B dissociates from its inhibitor in the cytosol and translocates to the nucleus in its free active form. There, NF- κ B mediates the production of pro-inflammatory cytokines such as IL-1 β , IL-6, TNF- α , cyclooxygenase 2 (COX-2), iNOS and ROS (Kiani-Esfahani *et al.*, 2016; Tang & Le, 2016). These cytokines not only target the source of inflammation but also nonspecific neurons leading to neurodegeneration, creating a vicious cycle between M1-like phenotype and neuronal death, and preventing an effective pro-inflammatory response (Heneka *et al.*, 2014; Rocha *et al.*, 2015). Notably, results showed an increase of ANXA1 expression, when animals were treated with TUDCA either before or after the neurotoxic insult. These results not only reinforce the anti-inflammatory effect of TUDCA but also could describe a possible mechanism of action. The ability of TUDCA to modulate ANXA1 expression has never been described, but is in accordance with the anti-inflammatory potential of TUDCA and the mechanisms already described to modulate ANXA1 expression in several cell types. Other studies showed that the promotor region of ANXA1 gene contains a CRE that can be induced by increased levels of cAMP (Antonicelli *et al.*, 2001; Castro-Caldas *et al.*, 2003; Lima *et al.*, 2017), and that TUDCA was been shown to upregulate cAMP levels through activation of TGR5 (Yanguas-Casas *et al.*, 2017). Therefore, we decided to further explore the ability of TUDCA to modulate ANXA1 synthesis and secretion using a microglia cell line.

ANXA1 is an anti-inflammatory protein, that in the brain is mostly expressed in microglia cells, whose intracellular function range from playing a role in differentiation to apoptosis (de Coupade *et al.*, 2000; McArthur *et al.*, 2010; Solito *et al.*, 2001). Moreover, extracellular ANXA1 has also been reported to play a role in anti-inflammatory processes, making the secretion of this protein a possible indicator of a pro-resolving response mediated by ANXA1 (Buckingham & Flower, 1997; Fan *et al.*, 2004; Flower, 1988).

We began our *in vitro* studies by analyzing MPP⁺ and/or TUDCA modulation of ANXA1 expression levels, in mouse primary neuronal cultures. Our results revealed that in cells

incubated with MPP⁺, in the presence or absence of TUDCA, expression levels of ANXA1 were not significantly altered, despite presenting an increase 3 times superior to the control. Moreover, due to technical problems, which could not be overcome in the course of this thesis, secreted membrane-bound ANXA1 was not detected. Thus, resulting in insufficient data to draw conclusions regarding MPP⁺ and TUDCA modulation of ANXA1 protein, in this experimental model of PD.

Nonetheless, in a microglia cell line, TUDCA significantly promoted ANXA1 secretion, without changes in the intracellular pool of the protein. The up-regulation of ANXA1 secretion, together with *de novo* synthesis of this protein possibly reveals one mechanism by which TUDCA exerts its anti-inflammatory effects. In dissecting the mechanisms underlying TUDCA modulation of ANXA1 expression our results, even though preliminary (n=2), showed a decrease in protein expression upon pre-incubation with the PKA inhibitor, H89. Since PKA is a downstream target of cAMP, and also an upstream regulator of CREB activation, our data could possibly unveil the pathway responsible for TUDCA regulation of this anti-inflammatory protein expression.

Finding new factors for inflammation relief has become more and more significant, especially in neurodegenerative disorders. Our work, despite being in the initial stages, shows a potential role in ANXA1 upregulation as an anti-inflammatory strategy induced by TUDCA. However, much more work needs to be done in order to understand the ability of TUDCA to regulate this protein.

Neuroinflammation and mitochondrial dysfunction are common features of PD. Both can increase oxidative stress by excessive release of ROS, further promoting neuronal damage and subsequent inflammation, resulting in a vicious cycle of chronic neurodegeneration (Fischer & Maier, 2015). DJ-1 is a stress sensor, whose mutations and deficiency have been associated with PD pathogenesis (Ariga *et al.*, 2013; Bonifati *et al.*, 2003; Lev *et al.*, 2006). Besides DJ-1 antioxidant role, this protein has also been implicated in the inflammatory process, being postulated that DJ-1 deficiency increases microglia neurotoxicity. Since neuroinflammation and oxidative stress are intimately related, and DJ-1 is noted to be modulated by TUDCA in mice brain (Moreira *et al.*, 2017), we explored this protein expression in microglia cells, upon treatment with TUDCA. Our results revealed a significant up-regulation of DJ-1 expression over time, which suggests that together with ANXA1, DJ-1 may serve as a mediator of TUDCA cytoprotective effects. Moreover, further investigation of the relation between ANXA1 and DJ-1 would be interesting to investigate, not only because DJ-1 is reported to modulate activation and cleavage of a member from the Annexin family,

Annexin 2 (Yamane *et al.*, 2015), but also in hopes to obtain a better insight of a possible TUDCA mechanism of action.

Shortly, results discussed here not only confirm the effects of MPTP in cerebral cortex, but also validate TUDCA broad antioxidant, neuroprotective and anti-inflammatory effects in this brain region. In addition, results from cellular models reinforce TUDCA anti-inflammatory and antioxidant effects, presenting ANXA1 as a possible direct target of this bile acid, paving the way for future work.

Altogether, our work in association with promising results from previous studies, reinforce the potential benefits of using TUDCA as a therapeutic strategy in the treatment of PD.

V. Future Perspectives

The main question left unanswered in this work was the discovery of the molecular mechanisms involved on ANXA1 synthesis and secretion in response to TUDCA. Our preliminary results helped us to establish a possible pathway dependent on cAMP/PKA but there is still a lot to be unraveled.

Therefore, our future goals are:

- **Understand how TUDCA modulates ANXA1 expression and/or secretion in the presence of an inflammatory stimulus.** For that, we could analyze ANXA1 expression in response to lipopolysaccharide (LPS), in the presence or absence of TUDCA. Pharmacologic inhibitors of PKA, such as H89, as well as agonists/antagonists of cAMP and antagonists of TGR5 could be used in order to study the involvement of this kinase on TUDCA effects in inflammatory conditions. The expression levels of p-CREB, p-AKT and pro-inflammatory cytokines should also be assessed, to further evaluate TUDCA mechanism of action.
- **Investigate if ANXA1 mediates the anti-inflammatory effects of TUDCA.** For that we should carry experiments using microglia cells transfected with ANXA1 small interfering RNA to analyze the importance of ANXA1 in TUDCA anti-inflammatory role.

Ultimately, the prosecution of this work would give us more insights into TUDCA mechanisms of action and would help us to explore ANXA1 as a potential target of this bile acid, with implications in several neurodegenerative diseases that have an inflammatory component.

VI. Bibliography

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