Carolina Leça de Azevedo Pereira Tavares

Licenciada em Bioquímica

Interplay of Endoplasmic Reticulum Stress and Autophagy in Parkinson's Disease

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

Orientador: Maria João Gama, PhD,
Faculdade de Farmácia da Universidade de Lisboa

Co-orientador: Andreia Neves Carvalho, PhD
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Part of the results discussed in this thesis were presented in the following meetings:

Tavares C.L.A., Rodrigues E., Castro-Caldas M., Neves Carvalho A., Gama M.J. Interplay of Endoplasmic Reticulum Stress and Autophagy in Parkinson's disease. Role of Glutathione S-Transferase pi. Jornadas intercalares das Dissertações Anuais dos Mestrados, Universidade Nova de Lisboa, 2nd February 2017, Caparica [Oral Communication]

Tavares C.L.A., Rodrigues E., Castro-Caldas M., Neves Carvalho A., Gama M.J. Expression Levels of ER Stress and Autophagy Markers in Mice Brain and N2a Cells Under MPTP/MPP^+ - Induced Oxidative Stress. 9th iMed.UL Postgraduate Students Meeting, 13th July 2017, Lisbon, Portugal [Abstract and Poster]

The results regarding mRNA quantitative analysis of UPR factors were obtained in the setting of an active collaboration with W. Scheper, J.J. Hoozemans and J. van Horssen, VU University Medical Center, Amsterdam, The Netherlands.

This work was supported by National Funds (Fundação para a Ciência e Tecnologia – FCT, Portugal) with the project PTDC/NEU-OSD/0502/2012 and SFRH/BPD/98023/2013 (to ANC).

iMed.ULisboa Research Institute for Medicines

FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

GOVERNO DE PORTUGAL

AGRADECIMENTOS

As minhas primeiras palavras de agradecimento não poderiam deixar de ser para a Professora Doutora Maria João. Não por se tratar do politicamente correto e do que seria esperado, mas sim por ter sido a melhor orientadora que eu poderia ter desejado para a minha tese. Obrigada por todas as palavras de incentivo, compreensão e coragem. Obrigada por perceber todos os imprevistos pessoais e laboratoriais que foram surgindo pelo caminho. Obrigada pela boa disposição e dedicação constantes e por nunca me ter feito duvidar de ter escolhido o caminho certo. De um modo geral, obrigada por ser um exemplo para mim e um marco muito importante na minha vida científica.

Andreia, se existisse uma palavra superior ao "obrigada" era para ti que a guardava. Ao longo desta jornada foste a minha parceira de alegrias e frustrações (temos de admitir que mais de frustrações do que alegrias) e desde o início que soube que podia sempre contar contigo. Obrigada por me tentares ensinar que "há sempre o dia de amanhã" apesar de muitas vezes eu achar que aquele dia é o fim do mundo. Obrigada também por me teres feito perceber que antes de ser mãe tenho de comprar um bloco de notas gigante para apontar todos os detalhes e mais alguns. Mais do que tudo, obrigada por nunca me fazeres sentir a tua aluna, mas sim fazeres de mim uma companheira. Admiro imenso tudo o que fazes e tornaste-te para mim um exemplo a seguir.

Agradeço também à Professora Doutora Cecília Rodrigues a oportunidade de fazer parte do seu grupo "Cellular Function and Therapeutic Targeting", enriquecendo os meus conhecimentos e preparando-me para o mundo da investigação.

Á Maria, um grande obrigada por estares sempre disponível a ajudar e esclarecer toda e qualquer dúvida e por seres o maior exemplo de organização que conheço. O laboratório sem ti não é o mesmo. Á professora Elsa tenho de agradecer a sua boa disposição e diversão e por me ter mostrado o seu livro de "experiências falhadas". Sei que provavelmente este parágrafo entra nos agradecimentos de todas as pessoas que passam pelo CellFun, mas de facto é muito importante para quem está do lado de cá. À professora Margarida quero agradecer todo o interesse mostrado ao longo da elaboração da minha tese e por toda a disponibilidade e espirito de entreajuda.

Tenho de agradecer aos restantes membros do CellFun, Vanda, Maria, Diane, Dionísio, Pedro, André, Alexandra, Marta, Sara, Simão, Hugo e Sofia por todos os almoços de descontração que tornavam o dia um bocadinho melhor. Obrigada por me ajudarem sempre que eu precisei e me mostrarem que um laboratório é muito mais do que um local de trabalho.

Marta e Mariana, este ano não teria sido o mesmo sem vocês. Companheiras de queixas, cafés e sorrisos foram um dos meus grandes escapes dentro do CPM. Estou muito orgulhosa da tese que conseguiram desenvolver. Mariana, para ti um grande obrigada por seres a melhor companheira que podia ter tido e, como não poderia faltar, obrigada por todas as vezes que puseste as minhas membranas em primário.

Durante este ano, o apoio que tive fora do laboratório tornou-se tão crucial para o desenvolvimento desta tese como todo o apoio que me proporcionaram dentro das paredes do CPM. Um enorme obrigada aos meus "Grandes Babes Lindões" por tornarem as minhas idas ao Porto tão especiais e por sempre me terem feito sentir que a distância se trata apenas de um número. Ao João,

um obrigada por nunca dizeres “não” e por estares sempre disponível para mim. Mostraste-me que os amigos não precisam de concordar a 100% para que a amizade seja especial. Maria, obrigada por seres sempre a minha melhor amiga, estando eu no Porto, em Lisboa ou na China. És dos meus maiores apoios na vida e quero que a nossa amizade continue sempre assim. Gosto muito de ti.

Cláudia e Rita, foram a melhor coisa que a capital me proporcionou. Nunca pensei que fosse possível sentir uma amizade tão forte por pessoas que conheço há tão pouco tempo. Obrigada por todas as idas ao cinema, ao ginásio e ao laréu. São ambas um enorme exemplo para mim e não podia estar mais orgulhosa. Gosto muito de vocês! Tiago, obrigada por todas as boleias e boa disposição. Obrigada por me fazeres sempre rir. Joana, Filipe e João obrigada pela amizade e pelos almoços na “casa do povo”, sempre tão especiais.

Filipa, Teresa e Patrícia, as melhores colegas de casa que poderia ter tido, saiu-me a sorte grande. Obrigada por todas as vezes que compreenderam a minha ausência de tempo e me alimentaram. Obrigada por todas as conversas até de madrugada e por me fazerem sempre sentir melhor. Nunca pensei sentir-me em casa tão fora de casa.

Os meus agradecimentos mais especiais estão guardados para a minha família. Mãe e Vitor, obrigada por apostarem na minha educação e por nunca me recusarem nada. Obrigada por perceberem todos os finais de semana em que não pude voltar a casa e também todos aqueles em que precisava do vosso conforto. Obrigada por me apoiarem não só ao longo deste ano, mas também ao longo de toda a minha vida. Helena, obrigada por me mostrares que a família não é só sangue. Obrigada por teres sido uma segunda mãe para mim hoje e sempre, principalmente no pior ano das nossas vidas. Maria, obrigada por me mostrares o que é amor de irmã. Tenho muito orgulho em ti e sei que te estão reservadas grandes coisas no futuro. Adoro-te, e podes contar comigo sempre.

Silvestre, obrigada por me tentares sempre fazer ver que a vida é muito mais para além desta tese. Obrigada por seres o meu saco de boxe nos dias menos bons e por (quase) nunca te queixares. Obrigada pela amizade e companheirismo, mas sobretudo obrigada pelo amor. És o que de mais especial tenho na minha vida.

Pai, esta tese é para ti. Dava tudo para que pudesses lê-la com todo o interesse que demonstravas em tudo na minha vida. És a minha estrelinha e espero que fiques orgulhoso de mim, onde quer que estejas. Amo-te.

Com carinho,
Carolina

ABSTRACT

Parkinson's disease (PD) is characterized by the selective loss of dopaminergic neurons of the *substantia nigra pars compacta*, and by the accumulation of misfolded proteins. Evidence from studies in human PD brain indicates that endoplasmic reticulum (ER) stress and consequent unfolded protein response (UPR) are common features of the disease, placing ER dysfunction as an early component of PD pathogenesis.

It is well established that autophagy is up-regulated in response to ER stress, probably as a compensatory mechanism. However, in the face of impaired UPR and autophagy, there is inefficient clearance, leading to protein accumulation that will result in development and progression of neurodegeneration.

The main objective of this study is to evaluate the expression levels of ER stress and autophagy markers in C57BL/6 wild-type mice and N2a cells, a mouse neuroblastoma cell line, upon treatment with the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 1-methyl-4-phenylpyridinium (MPP^+), respectively. In parallel, mice and cells were also treated with Tauroursodeoxycholic acid (TUDCA), a chemical chaperone that modulates ER adaptive capacity, in order to assess if the neuroprotetive effect of this molecule results from modulation of autophagy. Finally, we seek to understand if TUDCA modulates the expression levels of glutathione-S-transferase pi (GSTp), an antioxidant enzyme.

Expression of ER stress and autophagy markers was analysed using Western blot analysis and qPCR. Our results show that MPTP/MPP^+ administration increases the expression of ER stress responsive genes and that this effect is attenuated with administration of TUDCA prior or after MPTP/MPP^+ administration, namely by stimulation of autophagy. We also demonstrated by Western blot and immunocytochemistry that TUDCA increases the levels of GSTp.

Together, our results suggest that TUDCA modulates ER stress by stimulation of UPR pathways as an early response and by stimulation of autophagy when ER stress is persistent, therefore TUDCA remains a promising therapeutic agent to be implemented in PD treatment.

Keywords: ER stress; Unfolded Protein Response; Autophagy; Parkinson's disease; TUDCA

RESUMO

A doença de Parkinson (DP) é caracterizada pela perda seletiva de neurónios dopaminérgicos da *substantia nigra pars compacta* e pela acumulação de proteínas *misfolded*. Estudos feitos em cérebro de pacientes com DP mostram fortes evidências que o stress do reticulo endoplasmático (RE) e consequente *unfolded protein response* (UPR) são características típicas da doença, acentuando o papel da disfunção do RE como um componente inicial na patogénese da DP.

Sabe-se que a autofagia é induzida em resposta ao stress do reticulo, provavelmente como um mecanismo compensatório. No entanto, na presença de UPR e autofagia comprometidos não existe remoção eficiente de proteínas *misfolded*, levando à sua acumulação e consequente desenvolvimento e progressão de degenerescência neuronal.

Assim, o principal objetivo desta tese é a avaliação dos níveis de expressão de marcadores do stress do RE e de autofagia em ratinhos C57BL/6 e em células N2a, uma linha celular de neuroblastoma de ratinho, após tratamento com a neurotoxina 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP) e com 1-metil-4-fenilpiridina (MPP^+), respetivamente. Em paralelo, os ratinhos e as células foram tratados com o ácido tauroursodeoxicólico (TUDCA), um chaperone químico que influencia a capacidade adaptativa do RE, de maneira a perceber se o efeito neuroprotetor desta molécula resulta da modulação da autofagia. Por fim, pretendemos também perceber se o TUDCA altera os níveis de expressão de glutationo-S-transferase pi (GSTp), um enzima antioxidante, nas células N2a.

Os níveis de expressão de marcadores do stress do RE e de autofagia foram analisados por Western blot e análise por reação em cadeia da polimerase por método quantitativo (qRT-PCR). Os resultados obtidos mostram que a administração de MPTP/MPP^+ aumenta a expressão de genes suscetíveis ao stress do RE e que este efeito é atenuado pela administração de TUDCA antes ou após MPTP/MPP^+, provavelmente por estimulação da autofagia. Demonstramos também por Western blot e imunocitoquímica que os níveis de expressão de GSTp aumentam na presença de TUDCA.

Estes resultados sugerem que o TUDCA influencia o stress do RE por estimulação da UPR como primeira resposta e por estimulação da autofagia em casos de stress do RE persistente. Sendo assim, o TUDCA é um agente terapêutico promissor no tratamento da DP.

Palavras-chave: stress do RE; Unfolded protein response; Autofagia; Doença de Parkinson, TUDCA

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ABBREVIATIONS

6-OHDA	6-hydroxydopamine
ADP	Adenosine diphosphate
AMPK	Adenosine monophosphate activated protein kinase
ASK1	Apoptosis signal-regulating kinase 1
ATF4	Activating transcription factor-4
ATF6	Activating transcription factor-6
Atg	Autophagy-related proteins
ATP	Adenosine triphosphate
BBB	Blood brain barrier
Bcl-2	B-cell lymphoma 2
BSA	Albumin bovine serum
Ca^{2+}	Calcium
cDNA	Complementary DNA
CHOP	C/EBP-homologous protein
DA	Dopamine
DAT	Dopamine transporter
DJ-1	Parkinson protein 7
DNA	Deoxyribonucleic acid
eIF2α	Eukaryotic initiation factor 2
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
ETC	Electron transport chain
$FADH_2$	Flavin adenine dinucleotide
FBS	Fetal bovine serum
GBA	Glucocerebrosidase
GRP78/BiP	Immunoglobulin-heavy-chain-binding protein
GSH	Glutathione
GST	Glutathione S-transferase
GSTP	Glutathione S-transferase Pi
i.p.	Intra-peritoneally

IP3R	Inositol triphosphate receptor
IRE1α	Inositol-requiring protein 1α
JNK	c-JUN NH2-terminal kinases
KEAP1	Kelch ECH associating protein
KO	Knockout
LBs	Lewy Bodies
LC3	Microtubule-associated light chain
LRRK2	Leucine-rich repeat kinase 2
MAO-B	Monoamine oxidase type B
$MPDP^+$	1-methyl-4-phenyl-2,3-dihydropyridinium
MPP^+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex one
NADH	Nicotinamide adenine dinucleotide
NMS	Non-motor symptoms
Nrf2	Nuclear factor erythroid 2-related factor 2
PBS	Phosphate buffered saline
PD	Parkinson's Disease
PERK	Protein kinase RNA-like ER kinase
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PINK1	Phosphatase and tensin (PTEN) induced putative kinase 1
PRKN	Parkin
PVDF	Polyvinyl difluoride
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
Raptor	Regulatory-associated protein of mTOR
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S1P	Site 1 protease
S2P	Site 2 protease

SDS	Sodium dodecyl sulphate
SNCA	α-synuclein gene
SNpc	Substantia nigra pars compacta
ST	Striatum
TBS-T	Tris-buffered saline-Tween 20
TRAF2	Tumor necrosis factor-α receptor-associated factor 2
TUDCA	Tauroursodeoxycholic acid
Tun	Tunicamycin
UDCA	Ursodeoxicholic acid
ULK1	UNC-51-like kinase
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome system
VMAT2	Vesicular monoamine transporter-2
VPS35	Vacuolar sorting protein 5
XBP1	X-box-binding protein 1

I . INTRODUCTION

1. Parkinson's Disease

Parkinson's disease (PD) is estimated to affect more than 10 million individuals worldwide representing the most common neurodegenerative movement disorder (Abdullah et al., 2015). PD is an idiopathic neurodegenerative movement disorder mainly characterized by the selective loss of dopaminergic (DA) neurons in the *substantia nigra pars compacta* (SNpc), that project their terminals into the *striatum* (ST), leading to the subsequent depletion of dopamine and loss of signalling in the nigrostriatal pathway (Martin et al., 2011; Moore et al., 2005). Loss of DA neurons leads to the major clinical symptoms of PD, but there is widespread neuropathology and the SNpc only becomes involved towards the middle stages of the disease (Braak et al., 2003). Lewy bodies (LBs) – abnormal aggregates of α-synuclein protein- and dystrophic neurites (Lewy neurites) in the surviving neurons are classical pathologic hallmarks of PD (Kalia & Lang, 2015).

The etiology of PD is multifactorial, which probably results from an interplay of mostly unknown factors: several genes modifying effects by susceptibility alleles, environmental exposures and gene-environment interactions (e.g., influence of environmental agents on gene expression), and their direct impact on the developing and aging of the brain (Lill, 2016). Therefore PD is considered a sporadic disorder with occult causes in 95% of the cases (designated as sporadic PD) with only the remaining 5% of the cases being due to genetic factors (referred as familial PD) (Ranjita Betarbet et al., 2000; Piccini et al., 1999).

In the case of Parkinson's, a clear Mendelian inheritance is rarely seen, and the familial PD comprises the autosomal dominant form and autosomal recessive form (Bonifati, 2014). Dominant forms manifest in the presence of mutations on genes *SNCA*, *LRRK2*, *VPS35* and *GBA*. Mutations in the *alpha-synuclein gene* (*SNCA*) are rare and include point mutations and whole-locus multiplications (duplications or triplications) and are believed to cause PD through a toxic gain of function and Lewy Bodies may represent the attempt to purge the cell of toxic damaged alpha-synuclein (Bertoncini et al., 2005; Chen & Feany, 2005). The most common known cause of autosomal dominant PD results from mutations in *leucine-rich repeat kinase 2* (*LRRK2*) a large protein with many domains capable of protein–protein interactions, and thus it is plausible that changes in these domains would influence the *LRRK2*'s relationship with other proteins, although its pathogenic mechanism is still uncertain (Lill, 2016). Mutations in the *vacuolar protein sorting 35* (*VPS35*) were identified as a cause of dominant autosomal disease in 2011 but the brain pathology in carriers of this mutation remains unknown (Vilarino-Guell et al., 2011). The gene encodes a subunit of the retromer complex linked to the trafficking and recycling of proteins and synaptic vesicles (Bonifati, 2014). Loss of function mutations in *glucocerebrosidase* (*GBA*) cause impairment in glycolipid metabolism and are a frequent and strong risk factor for PD (Sidransky et al., 2009).

Autosomal recessive forms can result in an early-onset parkinsonism or juvenile atypical parkinsonism. Mutations in *parkin* (*PRKN*) are the most common known cause of these PD forms and

explain up to half of familial PD. Parkin protein functions as an ubiquitin ligase in the process of ubiquitination (Bonifati, 2014). *Phosphatase and tensin* (*PTEN) induced putative kinase 1* (*PINK 1*) codes for a protein with a kinase domain located in the mitochondria. PINK1 and parkin proteins interact with each other, with PINK1 acting upstream of parkin. Together they are important to tag damaged mitochondria for degradation by autophagy (Narendra et al., 2010). *Parkinson protein 7* (*DJ-1*) gene codes for a ubiquitous protein and functions as a cellular sensor of oxidative stress (Junn et al., 2005). *PRKN*, *PINK1* and *DJ-1* are all implicated in mitochondrial health, however the pathology of *DJ-1* PD patients remains unknown. Main mutations involved in PD are described in Table I.1.

Despite the intensive search in the field of PD it remains unknown if the disease results from either environmental factors, genetic causes or a combination of both. Loss of dopaminergic neurons is shown to be associated with mitochondrial dysfunction with related oxidative stress and deficient adenosine triphosphate (ATP) production, neuroinflammation and impaired proteolytic degradation. These seem to be observed in both familial and sporadic forms of PD (Dauer & Przedborski, 2003).

Table I.1 – PARK designated PD related loci. Adapted from (Klein & Westenberger, 2012)

Symbol	Gene	Inheritance	Disorder	Status and remarks
PARK1	*SNCA*	AD	EOPD	Confirmed
PARK2	*PARKIN*	AR	EOPD	Confirmed
PARK3	*Unknown*	AD	Classical PD	Unconfirmed; may represent a risk factor; gene not found since first described in 1998
PARK4	*SNCA*	AD	EOPD	Erroneous locus (identical to PARK1)
PARK5	*UCHL1*	AD	Classical PD	Unconfirmed (not replicated since described in 1998)
PARK6	*PINK1*	AR	EOPD	Confirmed
PARK7	*DJ-1*	AR	EOPD	Confirmed
PARK8	*LRRK2*	AD	Classical PD	Confirmed; variations in LRRK2 gene include risk-conferring variants and disease-causing mutations
PARK9	*ATP13A2*	AR	Kufor-Rakeb syndrome; atypical PD with dementia, spasticity, and supranuclear gaze palsy	Confirmed; but complex phenotype that would not be mistaken for early-onset or classical parkinsonism
PARK10	Unknown	Risk factor	Classical PD	Confirmed susceptibility locus; gene unknown since first described in 2002
PARK11	Unknown; not *GIGYF2*	AD	Late-onset PD	Not independently confirmed; possibly represents a risk factor; gene not found since first described in 2002
PARK12	Unknown	Risk factor	Classical PD	Confirmed susceptibility locus; possibly represents a risk factor; gene not found since first described in 2003
PARK13	*HTRA2*	AD or Risk factor	Classical PD	Unconfirmed
PARK14	*PLA2G6*	AR	Early-onset dystonia-parkinsonism	Confirmed
PARK15	*FBX07*	AR	Early-onset parkinsonian-pyramidal syndrome	Confirmed
PARK16	Unknown	Risk factor	Classical PD	Confirmed susceptibility locus
PARK17	VPS35	AD	Classical PD	Confirmed
PARK18	EIF4G1	AD	Classical PD	Unconfirmed

1.1. Pathophysiology, clinical presentation, diagnosis and treatment

PD is a progressive disease which incidence increases markedly with age, with a mean age at onset of 60 years old. PD affects more men than women, with both incidence and prevalence 1.5 to 2.0 higher in men (A. Lee & Gilbert, 2016). Symptoms tend to worsen overtime with most PD patients suffering considerable motor disability after 5-10 years of disease, even when treated with symptomatic medications (Dauer & Przedborski, 2003). However, in about 10% of the times the onset occurs earlier, between 20 and 50 years of age, being classified as young onset (Dexter & Jenner, 2013).

Loss of dopaminergic neurons, also enriched in neuromelanin, in the SNpc, is the main neuropathological feature of PD. Cell bodies of dopaminergic neurons are located in the SNpc, sending their projections to the caudate and putamen nucleus, in the striatum, creating the nigrostriatal pathway, key to motor function and voluntary movement control. The subsequent loss of striatal dopamine and neuromelanin content is accepted as being responsible for the classical motor features of PD and complex depigmentation (Dexter & Jenner, 2013). Remaining neurons develop intraneuronal inclusions, known as LBs and Lewy neurites, composed mostly of aggregated pre-synaptic α-synuclein together with phosphorylated neurofilaments and ubiquitin, that impair optimal neuronal functioning (Braak et al., 2003). The neuropathology observed in PD extends well beyond dopaminergic neurons as neurodegeneration and LBs formation are found in noradrenergic, serotonergic and cholinergic systems, as well as in the cerebral cortex, olfactory bulb and autonomic nervous system. Degeneration of hippocampal structures and cholinergic cortical inputs contribute to the high rate of dementia that accompanies PD, particularly in older patients (Dauer & Przedborski, 2003).

At the onset of symptoms, putamenal dopamine is depleted in about 80%, and approximately 60% of SNpc dopaminergic neurons have already been lost (Dauer & Przedborski, 2003). Symptoms of PD can be divided into two main groups: motor symptoms and non-motor symptoms. The earliest symptoms might be unnoticed or misinterpreted for a long time. Usually impaired motor function is used to make a clinical diagnosis of PD, as it is secondary to progressive loss of dopaminergic neurons. The main motor symptoms are bradykinesia (slowness of initiating movement), rigidity (resistance of muscles to passive movement around a joint), resting tremor (present in about 70% of PD patients) and postural instability with an asymmetric onset spreading to become bilateral with time (Dexter & Jenner, 2013; Hess & Okun, 2016). Non-motor symptoms (NMSs) are broadly classified as autonomic nervous system dysfunction, cognitive changes, pain, cognitive abnormalities and sleep disturbances. NMSs represent some of the challenges to life quality in PD since they usually do not respond to dopamine therapy as well as motor symptoms (Beitz, 2014; H. M. Lee & Koh, 2015). Table I.2 summarizes the main motor and non-motor symptoms of PD. Whereas the causes of motor dysfunction in PD are reasonably well understood, the cause of NMS in PD remains poorly researched and they may largely relate to pathology outside of the basal ganglia (Dexter & Jenner, 2013).

Table I.2 – Clinical features of Parkinson Disease. Motor and non-motor symptoms. Adapted from (Beitz, 2014; Garcia Ruiz et al., 2011; Hess & Okun, 2016; H. M. Lee & Koh, 2015)

Motor symptoms	The onset is insidious where individuals may attribute the symptoms to aging processes. PD symptoms are progressive but rates of motor progression are highly variable
• Resting tremor (initially unilateral) • Bradykinesia (slowness, clumsiness, and difficulty in making voluntary and automatic semi voluntary movements) • Rigidity • Shuffling gait (sensation of heavy legs, slowness of walking, shorter and insecure steps) • Loss of postural reflexes • Hypomimia (masked facial expression) • Dystonia • Stooped posture	
Non-motor symptoms	Affect all patients with PD, the frequency of which increases with disease severity. They are often poorly diagnosed and treated. Later in the disease, the disability from nonmotor symptoms often overshadows that from motor symptoms
• Autonomic dysfunction (sexual dysfunction, swallowing disorder, incontinence, constipation, excessive salivation, orthostatic hypotension) • Sensory disorders (pain syndromes, abnormal sensations, olfactory dysfunction) • Integumentary (malignant melanoma, drug rashes, skin denervation, flush) • Visual (diplopia, blurred vision) • Neurobehavioral (anxiety, depression, psychosis, cognitive dysfunction, dementia, apathy) • Sleep problems (insomnia, REM sleep disorder, restless leg syndrome)	

Diagnosis of PD is based upon the presence or manifestation of resting tremor, rigidity, postural instability and bradykinesia. Differential diagnosis is challenging given the fact that the classic PD symptoms can be present in other neurodegenerative disorders (Beitz, 2014). Despite diagnosis of PD being made on clinical grounds the gold standard for diagnosis of Parkinson's disease is the neuropathological assessment. However, there are no generally accepted standard pathological diagnostic criteria for Parkinson's disease (Klein & Westenberger, 2012). There is no definitive test able to confirm the diagnosis during life, except for gene testing in a reduced number of cases. Therefore, a definite diagnosis of PD requires a post-mortem confirmation (Massano & Bhatia, 2012).

PD is still an incurable progressive disease. Drugs like levodopa, dopamine agonists, monoamine oxidase type B (MAO-B) inhibitors, and, less commonly, amantadine enhance the intracerebral dopamine concentrations or stimulate the dopamine receptors and are still the standard treatment for motor symptoms (Kalia & Lang, 2015). Other targets include mitochondrial dysfunction and oxidative stress, calcium channel activity, among others (AlDakheel et al., 2014). When symptoms

cannot be managed using medication, surgical interventions, namely live targeted gene therapy or deep brain stimulation of bilateral subthalamic nuclei, may be the answer (Kordower & Bjorklund, 2013; Li et al., 2017). Nowadays, a major goal of PD research is the development of disease-modifying drugs that slow or stop the inevitable neurodegeneration phenomenon.

1.2. Role of oxidative stress in Parkinson's disease

Oxidative stress can be defined as a condition in which the cell is not able to keep the levels of reactive oxygen species (ROS) below a toxic threshold, caused either by failure of cell-buffering mechanisms or over-production of ROS (Gaki & Papavassiliou, 2014). The production of ROS requires the activation of molecular oxygen, being continuously produced *in vivo* as a result of oxygen metabolism. To achieve cellular energy, within the mitochondria occurs the transport of electrons resulting from oxidation of reduced nicotinamide adenine dinucleotide (NADH) or flavin adenine dinucleotide ($FADH_2$) along the electron transport chain (ETC) until there is a reduction of oxygen to water at complex IV (Winklhofer & Haass, 2010). The transport of electrons generates a proton flow, creating an electrochemical gradient resulting in ATP production from adenosine diphosphate (ADP), through the ATP synthase located in complex V of ETC (Keane et al., 2011). This process, known as oxidative phosphorylation, provokes leakage of electrons that can react with molecular oxygen originating ROS such as superoxide anion, hydrogen peroxide and nitric oxide that can produce oxidative damage by reacting with deoxyribonucleic acid (DNA), lipids and proteins (Kirkinezos & Moraes, 2001).

The brain consumes about 20% of the oxygen supply of the body, and a significant portion of that oxygen is converted to ROS (W. M. Johnson et al., 2012). The presence of oxidative damage to lipids, proteins and DNA in the SNpc of sporadic PD patients' brains have implicated oxidative damage in the pathogenesis of the disease, once it contributes to neuronal degeneration of sensitive neurons (Jenner, 2003). Also, the oxidation of dopamine by monoamine oxidase is a reaction known to cause production of superoxide and hydrogen peroxide (Miller et al., 2009). Deficits in the subunits and activity of mitochondrial complex I of the ETC are a prominent phenomenon in blood platelets and SNpc of PD patients, as well as reduced complex I activity in cytoplasmic hybrid cell lines containing mitochondrial DNA from PD patients (Beal, 2005; Swerdlow et al., 1996). These findings suggest an important and determinant role of oxidative stress in the pathogenesis of PD.

The source of the oxidative stress remains contested, with both neuronal and glial sources being implicated, but there seems to exist little doubt that the most likely contributor is increased radical formation originating from mitochondria and endoplasmic reticulum (Dexter & Jenner, 2013). Considerable studies show that ROS may accumulate due to dopamine metabolism, low glutathione (GSH) and high levels of iron and calcium in the SNpc. The brain also contains high levels of polyunsaturated fatty acids, which under oxidative stress conditions result in lipid peroxidation and generation of toxic products (Vera Dias, 2014). Also, studies suggest that pesticides, such as rotenone, and other environmental toxins that inhibit complex-I are involved in the pathogenesis of sporadic PD. One of these toxins is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), that replicates most

features of PD in humans and animals, through inhibition of complex-I, in a mechanism explained in section 6.1.1 (Moore et al., 2005).

1.3. Autophagy and Parkinson's disease

Clearance of misfolded proteins is done through the ubiquitin-proteasome and autophagy-lysosome pathways in healthy cells, however, in PD patients the process is compromised leading to the accumulation of misfolded and damaged proteins. Phenomena's that induce protein alterations induce autophagy to minimize errors. Stimuli such as ROS, nutritional deprivation and chemicals are among these (Abdullah et al., 2015).

Previous studies showed that overexpression of wildtype α-synuclein inhibits autophagosome synthesis in mammalian cells, compromising autophagy as well as enhances the onset of tremors and weight loss, whereas deletion of α-synuclein increases autophagy, thereby significantly delaying the onset of disease phenotypes (Sarkar, 2013). Also, the downregulation of proteins involved in autophagy contributes to the accumulation of SNCA protein, and the pathogenic mutations on this gene can block the uptake of damaged proteins by lysosomes causing inhibition of autophagy (Alvarez-Erviti et al., 2013; Cuervo et al., 2004). It appears that as neurons age there is a decrease in autophagy and in the ability of clearing misfolded proteins, such as SNCA, therefore leading to an increase in the susceptibility to Parkinson's (Abdullah et al., 2015). Last, studies showed an autophagosome accumulation in the brain of PD patients and in MPTP-induced PD animal models (Su et al., 2015).

Studies on rapamycin and the mammalian target of rapamycin (mTOR) pathway help to highlight the link between PD and autophagy. The mTOR pathway is a major negative regulatory axis of autophagy so rapamycin, an mTOR inhibitor, leads to autophagy induction and protein clearing (Abdullah et al., 2015). However, studies also show that L-DOPA – precursor of dopamine - administration to PD mice results in dopamine D1 receptor-mediated activation of the mTOR pathway (Santini et al., 2009). Regardless of this, it is clear that compromised autophagy contributes to PD susceptibility as the brain ages.

2. The Endoplasmic Reticulum and Oxidative Stress

Although the molecular mechanisms of DA neuronal demise are still unclear, solid evidence has been implicating oxidative stress, mitochondrial dysfunction and failure of protein degradation pathways with consequent accumulation of protein aggregates as critical players in the pathogenesis of PD (Henchcliffe & Beal, 2008; Jenner, 2003; McCoy & Cookson, 2012). All of the mentioned processes are associated with ROS and reactive nitrogen species production, that are important for execution of physiological functions of the cell but become detrimental to cell membranes and can cause cell death when produced in excess (Loh et al., 2006). In order to cope with the many oxidative reactions, cells produce antioxidants such as glutathione as well as antioxidants enzymes like superoxide dismutase and catalase to maintain the balance between ROS formation and antioxidant capacity. However, when the production of ROS overwhelms the antioxidant defense, damage can be done in cellular proteins, lipids and DNA and apoptotic pathways can be activated (Carvalho et al., 2017; Miller et al., 2009).

The brain consumes about 20% of the oxygen supply of the body and brain cells are quite susceptible to oxidative damage due to high levels of polyunsaturated fatty acids in their membranes and relatively low activity of endogenous antioxidant enzymes (Mariani et al., 2005). Further it is also known that oxidation of dopamine via monoamine oxidase, causes production of superoxide and hydrogen peroxide and consequently DA neurons are in a continuous state of oxidative stress (Miller et al., 2009). *Post-mortem* studies in patients suffering from PD also showed increased levels of 4-hydroxyl-2-nonenal, a by-product of lipid peroxidation, carbonyl modifications of soluble proteins and DNA and ribonucleic acid (RNA) oxidation products (Vera Dias, 2014). Finally, the link between oxidative stress and PD is further supported by the reduction in mitochondrial complex-I activity (Mythri et al., 2011). The increase in oxidative stress together with the decline in endogenous antioxidants are important underlying risk factors for older people to develop neurodegenerative diseases like PD (Cardoso et al., 2005).

Mitochondrial oxidative phosphorylation is the primary source of high energy compounds in the cell and the dysfunction of mitochondrial metabolism leads to reduced ATP production, impaired calcium buffering and generation of ROS (Surmeier et al., 2011). The endoplasmic reticulum (ER) is composed of a single continuous phospholipid membrane that is comprised of the outer nuclear envelope, flattened peripheral sheets with ribosomes (rough ER), and a complex network of smooth tubules (smooth ER) that extend throughout the cell (Deegan et al., 2013). ER enables protein and lipid synthesis, ion homeostasis, quality control of newly synthesized proteins and organelle communication (Borgese et al., 2006). It is involved in several metabolic processes like gluconeogenesis and is the major intracellular calcium reservoir in the cell (Chaudhari et al., 2014). Rough ER is mainly responsible for protein synthesis and secretions as smooth endoplasmic reticulum, lacking ribosomes, is important for fatty acid and phospholipid synthesis, carbohydrate metabolism, lipid bilayer assembly and regulation of calcium homeostasis (Chaudhari et al., 2014).

Being responsible for quality control of new proteins, the ER needs to be able to degrade misfolded proteins, therefore it contains an array of chaperone systems as glycosidases, calcium (Ca^{2+})-dependent chaperones, and members of the protein disulfide isomerase family that are responsible for the correct folding of proteins under normal physiological conditions (Deegan et al., 2013). These

processes contribute to the levels of ROS in the cell, with its production increasing with the increase of ER stress by protein overload, impaired redox homeostasis and calcium release, which in turn can augment the production and accumulation of mitochondrial ROS, influencing their functions (Malhotra & Kaufman, 2011).

The correct folding of proteins is under the vigilance of ER resident molecular chaperones and folding enzymes that secure appropriate conformation and maturation of proteins. The Endoplasmic Reticulum Quality Control System is composed by glucose-regulated proteins and ER lectin-like chaperone system among others and has a vital role in converting a protein from nascent to native state (Araki & Nagata, 2011; Kampinga & Craig, 2010; Rutkevich & Williams, 2011). However, these mechanisms fail in the presence of accumulation of unfolded or misfolded proteins, disturbances in cellular redox regulation and endogenous ROS production, hypoxia, hyperglycaemia and hyperlipidaemia, alterations in calcium regulation and viral infections, that alter the ER homeostasis, causing ER stress (Chaudhari et al., 2014). In response to such diverse signals, ER elicits a protective or adaptive response called the unfolded-protein response (UPR) with an aim to restore ER homeostasis. However, if the stress signal is severe and/or prolonged, cell death pathways can be triggered (Benbrook & Long, 2012).

2.1. The Unfolded Protein Response

One consequence of accumulating misfolded proteins is the generation of ER stress, which triggers a coordinated and rapid response known as the UPR (Hetz & Saxena, 2017). The UPR is a protective or adaptive response provided by the ER to cope with protein-folding alterations with the final aim to restore homeostasis (Hetz, 2012). UPR induction involves a shift towards a more reductive environment in the ER transducing information about the protein-folding status in the ER lumen to the nucleus and cytosol in order to buffer fluctuations in unfolded protein load through several pro-survival mechanisms, including the expansion of the ER membrane, the selective synthesis of key components of the protein folding and quality control machinery and the attenuation of the influx of proteins into the ER (Hetz, 2012; Pani et al., 2009).

The UPR is responsible for controlling the stability of RNAs and the rate of proteins synthesis, activating the transcription of genes involved in the secretory pathway. Usually UPR target genes encode for proteins associated with protein folding, ER-associated degradation (ERAD), vesicular trafficking, autophagy, ER redox control, amino acid metabolism and lipid synthesis (Hetz & Saxena, 2017; M. Wang & Kaufman, 2016). Two main components are involved in the UPR: stress sensors at the ER membrane and downstream transcription factors that reprogramme gene expression toward stress mitigation or the induction of proapoptotic programmes (Chow et al., 2015) . The activation of different genes varies with the physiological perturbation that causes ER stress and tissue context and this variation might result from the formation of different heterodimers between transcription factors, post-translational modifications and epigenetic changes (Hetz et al., 2015).

Three main type-I transmembrane proteins initiate the UPR: protein kinase RNA-like ER kinase (PERK/EIF2AK3), inositol-requiring protein 1α (IRE1α/ERN1) and activating transcription factor-6

(ATF6, α and β) (Hetz & Saxena, 2017) (Figure I.1). Each of these transmembrane proteins has an ER-luminal domain that senses unfolded proteins, a transmembrane domain by which it is targeted to the ER membrane, and a cytosolic domain that transmits signals to the transcriptional or translational apparatus (K. Zhang & Kaufman, 2008). Under normal physiological conditions all three effectors are negatively regulated by the ER chaperone GRP78/BiP (immunoglobulin-heavy-chain-binding protein), which supresses their activity by binding to their luminal ends (Bertolotti et al., 2000). In the presence of misfolded proteins, complex GRP78/BiP releases PERK, IRE1α and ATF6, these become activated and GRP78 binds to misfolded proteins (Hoozemans et al., 2007). These three sensors transmit signals from the ER to the cytoplasm or nucleus and activate the following three pathways: suppression of protein translation to stop the production of more unfolded proteins, induction of genes encoding ER molecular chaperones to ease protein folding, and activation of ERAD to decrease the accumulation of unfolded proteins in the ER (R. J. Lee et al., 2004).

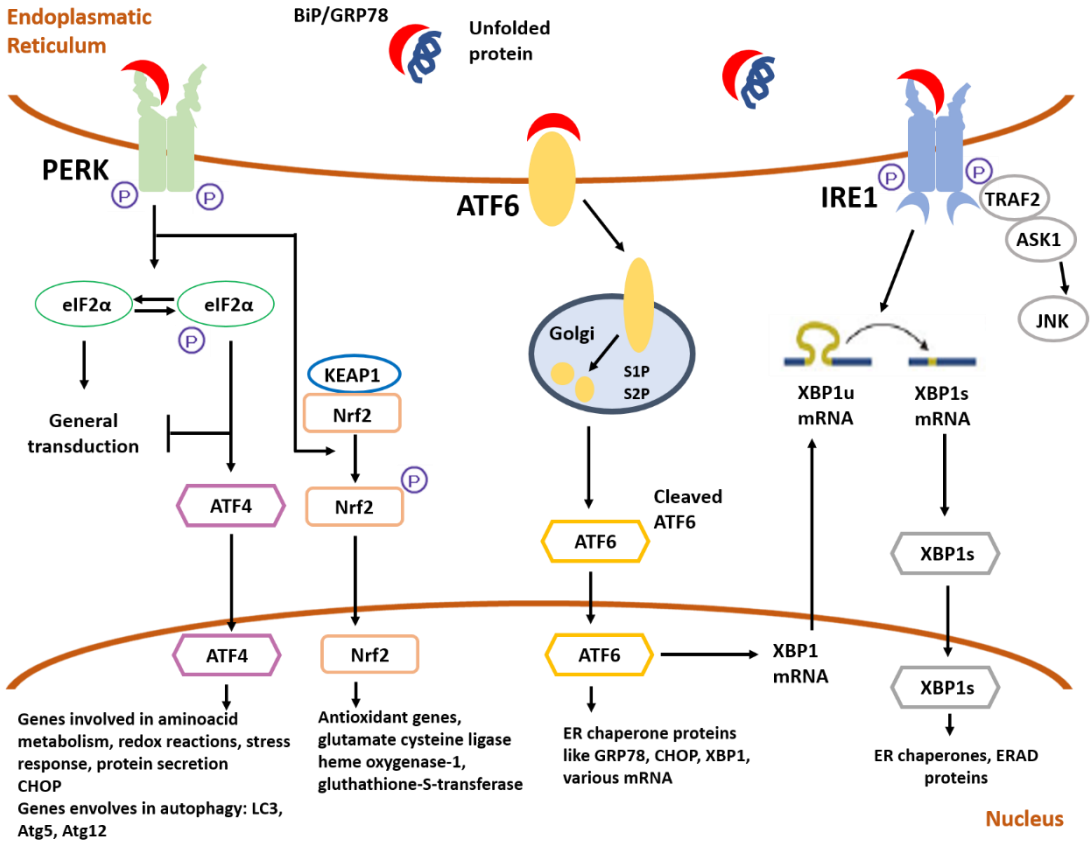

Figure I.1 – Schematic representation of the UPR. Accumulation of unfolded proteins in the ER lumen results in the binding of GRP78/BiP to those proteins, with consequent release of UPR stress sensors PERK, ATF6 and IRE1α. PERK, after self-phosphorylation, inactivates eIF2α through phosphorylation, inhibiting general proteins synthesis permiting non-canonical translation of ATF4 mRNA. Activated PERK also acts in Nrf2, breaking the complex with KEAP1. Activated ATF6 is processed in the Golgi by site 1 (S1P) and site 2 proteases (S2P), resulting in the transcription of XBP1 mRNA. IRE1α has a domain responsible for processing unspliced XBP1 mRNA, translating it into an active transcription factor. Activation of IRE1α, similar to PERK, results from dimerization and autophosphorylation. Last, IRE1α recruits TRAF2 and ASK1 leading to the activation of JNK. ASK1 – Apoptosis signal-regulating kinase 1; ATF4 – Activating transcription factor-4; ATF6 – Activation transcription factor-6; eIF2α

- Eukaryotic initiation factor 2; BiP/GRP78 - Immunoglobulin-heavy-chain-binding protein; IRE1α - Inositol-requiring protein 1α; JNK - c-JUN NH2-terminal kinases; KEAP1 - Kelch ECH associating protein 1; Nrf2- Nuclear factor erythroid 2-related factor 2; PERK - Protein kinase RNA-like ER kinase; S1P – Site 1 protease; S2P – Site 2 protease; TRAF2 - tumor necrosis factor-α receptor-associated factor 2; XBP1 - X-box-binding protein 1. Adapted *from (Deegan et al., 2013).*

2.1.1. PERK/EIF2AK3 signalling

PERK is the major protein responsible for lessening of mRNA translation under ER stress, preventing the influx of newly synthesized proteins into the stressed ER compartment. It is a type I ER transmembrane protein with serine/threonine kinase activity, with its N-terminus in the ER lumen, involved in the regulation of its dimerization and interaction with BiP, while the C-terminus is cytosolic and harbours its autophosphorylation sites and the kinase domain (Deegan et al., 2013; Song et al., 2017). When sensing ER stress, the cytosolic kinase domain of PERK oligomerizes and is activated by *trans*-autophosphorylation. The activated cytosolic kinase domain of PERK then recruits and phosphorylates the α subunit of eukaryotic initiation factor 2 (eIF2α), inactivating it (P. Wang et al., 2016). The inactivation of eIF2α results in a decrease of protein load and a translation attenuation of mRNAs with 5' cap which usually encode for proteins involved in cell growth and proliferation and also results in non-canonical translation of ATF4 mRNA via an open reading frame in its 5'-untranslated region that is bypassed only when eIF2α is inactivated (Deegan et al., 2013). ATF4 mRNA encodes for a cAMP response element binding transcription factor which activates genes important for amino acid metabolism, redox balance, protein folding, autophagy and apoptosis (Deegan et al., 2013). The role of ATF4 in apoptosis is associated with its cooperation with C/EBP-homologous protein (CHOP/GADD153), that is reported to downregulate B cell lymphoma 2 (Bcl-2) and up-regulate transcription of proteins that lead to mitochondrial outer-membrane permeabilization and initiation of the intrinsic apoptotic cascade (H. Kim et al., 2009; McCullough et al., 2001).

PERK also phosphorylates nuclear factor erythroid 2-related factor 2 (Nrf2), causing its nuclear translocation after release from complex with Kelch ECH associating protein 1 (KEAP1). Once activated, Nrf2 binds the antioxidant response element (ARE), located in the promoter or enhancer regions of antioxidant and cytoprotective genes (M. Zhang et al., 2013). Nrf2 is involved in free radical scavenging, detoxification of xenobiotics and maintenance of redox potential (Chan & Kwong, 2000). Known targets of Nrf2 include glutathione S-transferase (GST) isoforms that will be described in section I-4.

2.1.2. IRE1α/ERN1 signalling

The IRE1 branch is the most conserved and sole branch of the UPR in lower eukaryotes (Mori, 2009). IRE1 is also a type I ER transmembrane protein containing a serine/threonine kinase domain and a site-specific endoribonuclease (RNase) that, in response to ER stress, is autophosphorylated. IRE1 is divided in two isomers in humans: IRE1α that is ubiquitously express, and IRE1β, which expression is restricted to epithelial cells of the intestine and the lungs (Deegan et al., 2013). After

initiation of the signalling cascade it has been observed a self-assembly of the cytosolic region followed by IRE1α oligomerization, leading to its RNase activation. It was also described autophosphorylation at serine 724 and ADP binding (Hocking et al., 2010; Korennykh et al., 2009).

The cytoplasmic part of IRE1α forms a complex with tumor necrosis factor-α (TNF-α) receptor-associated factor 2 (TRAF2) and apoptosis signal-regulating kinase-1 (ASK1), resulting in an activation of c-JUN NH_2-terminal kinases (JNK), promoting autophagy or apoptosis (Ron & Hubbard, 2008). The signal of this branch of the UPR is transmitted through excision of a 26-base nucleotide intron from X-box-binding protein 1 (XBP1) mRNA, which is then ligated by an uncharacterized RNA ligase and translated to produce XBP1s, that has been reported to be important for the activation of unfolded protein response element, controlling the expression of the ERAD, thus helping to degrade unfolded proteins in the ER (Korennykh et al., 2009; Yamamoto et al., 2004). The ERAD pathway is constituted by different components including chaperones, protein transporters, and ubiquitin-related enzymes that sense, deliver, and retro translocate misfolded proteins to the cytoplasm for proteasome mediated degradation (Vembar & Brodsky, 2008).

Overall, IRE1α has been suggested to have a role in development, metabolism, immunity, inflammation, and neurodegeneration (Kaufman & Cao, 2010). In addition, IRE1 can selectively degrade mRNAs encoding for proteins that are predicted to be difficult to fold and micro-RNAs (Hetz, 2012).

2.1.3. ATF6 signalling

ATF6 is a type II transmembrane receptor and a member of the leucine zipper protein family that is synthesized as an ER membrane-tethered precursor, with its C terminal domain located in the ER lumen and its N-terminal DNA binding domain facing the cytosol (Haze et al., 1999). There are two isoforms of ATF6 – α and β – that are ubiquitously expressed in the ER. Upon ER stress, BiP dissociates from ATF6, unravelling its two Golgi localization signals, allowing ATF6 to interact with the protein trafficking complex COPIII, which causes translocation of ATF6 to the Golgi for processing, to become active (Shen et al., 2002). The 90-kDA protein is cleaved by site 1 protease (S1P) and site 2 protease (S2P), originating a 50-kDa cleaved N-terminal cytosolic domain that translocates to the nucleus where it acts as a transcriptional factor, activating target genes such as GRP78/BiP, CHOP and XBP1 (Adachi et al., 2008). Then ATF6 indirectly regulates autophagy or apoptosis via XBP1 and CHOP. It has also been reported that ATF6 regulates an array of miRNAs to alleviate ER stress (Belmont et al., 2012). Moreover, it is thought that the ATF6 and IRE1α pathways merge through the regulation of XBP1 activity: ATF6 increases the amount of XBP1 mRNA whereas IRE1α removes the 26-nucleotide intron, increasing XBP1 activation potential (K. Lee et al., 2002).

2.2. ER stress and UPR in Parkinson's Disease

A lot of neurodegenerative disorders share a common neuropathology associated with the accumulation of abnormal protein aggregates or inclusions in the brain containing specific misfolded proteins. These diseases include PD, amyotrophic lateral sclerosis, Alzheimer's disease, Huntington's disease, and many others (Selkoe, 2003; Taylor et al., 2002). The presence of aggregates of misfolded proteins indicates that protein quality control mechanisms have been compromised or are incompetent to restore protein homeostasis and provides potential targets for therapeutic intervention (Hoozemans & Scheper, 2012).

Accumulating evidence shows an important role of UPR and ER stress in PD. The involvement of the UPR in PD has been shown in cellular and *in vivo* models using 6-hydroxydopamine (6-OHDA), MPTP and rotenone (Hoozemans et al., 2007). Generation of ROS by these toxins lead to a rapid accumulation of oxidized proteins that can activate the UPR (Blesa & Przedborski, 2014). Presence of ER stress in human tissue derived from PD patients has been reported, showing the UPR as an early event of the disease (Hoozemans & Scheper, 2012). Previous studies show the activation of PERK-eIF2α pathway of the UPR in dopaminergic neurons in the *substantia nigra* of PD patients, and the neurons presenting activated PERK were positive for αSyn inclusions (Hoozemans et al., 2007). Studies also showed that XBP1 deletion in DA neurons produce different outcomes, related with the age of the animals and reflecting compensatory changes in UPR-related factors. During development, the lack of XBP1 induced mild ER stress with protection of DA neurons against neurotoxicity of 6-OHDA. Down-regulation of XBP1 in adult mice nigral dopaminergic neurons triggered chronic ER stress, with CHOP induction, which caused spontaneous degeneration (Valdes et al., 2014).

Recent evidence from both toxicological and genetic models of PD indicates that activation of the UPR has a beneficial effect on the survival of dopaminergic neurons. The loss of DA neurons and the accumulation of ubiquitin-positive inclusions induced by MPTP are enhanced in ATF6α-deficient animals, showing the importance of this stress sensor as it controls the levels of BiP, ERAD components and promotes astrogial activation under resting conditions in DA neurons (Egawa et al., 2011; Hashida et al., 2012). Similarly, deletion of the gene encoding for CHOP protects DA neurons against exposure to 6-OHDA and MPTP in different experimental settings (Silva et al., 2005).

Given the dual role of the UPR in maintaining cell viability and the engagement of cell death, it is predicted that low levels of ER stress during the early stages of PD may actually protect dopaminergic neurons against proteostasis defects. Results showed that low concentrations of tunicamycin (Tun), an ER stress inducing agent, increased neuronal surviving in genetical and pharmacological models of the disease, since Tun triggered a preconditioning effect in which sub lethal levels of ER stress selectively engaged adaptive UPR signalling events involving the expression of XBP1s in the brain but not the proapoptotic factor CHOP (Mercado et al., 2016).

Despite these results, characterization of ER markers is still very poor and the precise mechanisms of interplay between oxidative stress and ER stress in dopaminergic neurons have been sparsely described. The mechanisms leading to ER stress in PD and the impact of the UPR on the degeneration cascade in the disease are just starting to be uncovered (Mercado et al., 2013).

3. The autophagy machinery

Autophagy is now considered a major survival mechanism of the cell, being involved in the removal of misfolded proteins and turnover of organelles for cellular homeostasis during starvation by recycling of cytosolic components. The autophagic flux comprises the fusion of autophagosomes with late endosomes to form amphisomes which subsequently fuse with lysosomes forming autolysosomes for degrading its load (Ravikumar et al., 2010). Basal levels of autophagy are very low but, in the presence of stress or extracellular cues, an efficient mechanism of autophagy is crucial to regulate the turnover of long-lived proteins and getting rid of damaged structures (He & Klionsky, 2009). Regulation of autophagy can be done by the mTOR pathway, the classical regulation which negatively controls this process, or by mTOR independent pathways, like the inositol signalling pathway (Sarkar, 2013). Here, we will focus of the mTOR dependent signalling pathways and diverse upstream and downstream signals impinging on it.

3.1. mTOR signalling pathways

The mammalian target of rapamycin is a serine threonine kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family that is involved in cell metabolism, growth, proliferation and survival and is conserved throughout evolution, nucleating at least two distinct multi protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (Laplante & Sabatini, 2009). Studies have shown that mTORC1 inhibition increases autophagy, whereas stimulation of mTORC1 reduces this process (Codogno & Meijer, 2005). mTORC1 is a complex formed by five proteins, being the catalytic subunit regulatory-associated protein of mTOR (Raptor) one of them. Previous studies shown that Raptor binds directly to mTOR, functioning as a scaffold protein that regulates complex assembly in addition to substrate recognition, determining downstream signalling of mTORC1. Therefore, in order to inhibit autophagy, mTOR needs to be linked to Raptor (L. Wang et al., 2009). Importantly, mTORC1 can be phosphorylated in S2488 by one of the downstream targets of mTOR, p70S6 kinase, an important reaction for the correct signalling of mTORC1 (Chiang & Abraham, 2005).

In conditions of low ATP/ADP ratio, AMP-activated protein kinase (AMPK), a master-sensor of intracellular energy status, is activated through phosphorylation in residue T172 and directly phosphorylates Raptor at residues S792 and S722, inhibiting mTOR (L. Wang et al., 2009). Also, the activated AMPK inhibits mTORC1 through phosphorylation and activation of tuberous sclerosis complex 2 (TSC2), activating its GAP activity towards Rheb, to inhibit mTORC1 signalling. Thus, there are two separate pathways that transmit AMPK signalling to mTORC1 (Jung et al., 2010).

The initiation, elongation, maturation and fusion states of autophagy's pathway are orchestrated by several autophagy-related (Atg) proteins. Atg13 is involved in the biogenesis of autophagosomes, namely through binding to UNC-51-like kinase (ULK1) and FIP200, leading to the formation of a ULK1–Atg13–FIP200 stable complex that signals to the autophagic machinery downstream of mTORC1 (Sarkar, 2013). Under physiological conditions, mTOR inhibits Atg13 and ULK1 through phosphorylation. Under stress conditions, mTOR dissociates from mTORC1, resulting in the inhibition of mTORC1-mediated phosphorylation of Atg13 and ULK1. This leads to dephosphorylation dependent

activation of ULK1 and ULK1-mediated phosphorylation of Atg13, FIP200 and ULK1 itself, which regulates autophagosomes synthesis and triggers autophagy (Laplante & Sabatini, 2009). Besides phosphorylating Raptor and inhibiting mTORC1 activation, activated AMPK is also able to decrease phosphorylation levels of ULK1 in residue S757 (via inactivation of mTOR), therefore promoting the phosphorylation of ULK1 in other sites like S555 and S777, although there is not much consensus about the residues of ULK1 phosphorylated by AMPK (J. Kim et al., 2011). A simplified scheme of mTORC1 signalling pathway is represented in figure I.2.

Two ubiquitin-like conjugation systems function in the initiation of autophagy. The first one involves several Atg as the second one involves the conjugation of microtubule associated protein 1 LC3 (light chain 3) to phosphatidylethanolamine. LC3 undergoes post-translational modifications originating the cytosolic LC3-I form, that conjugated with phosphatidylethanolamine generate the autophagosome associated LC3-II form. Therefore, the levels of LC3-II correlate with the steady-state number of autophagosomes (Sarkar, 2013). The polyubiquitin-binding protein p62/SQSTM1 acts as a scaffold to facilitate aggregation of ubiquitylated mutant proteins and is also a specific autophagy substrate that targets the mutant proteins for autophagic degradation, anchoring substrates of autophagy to LC3I (Sarkar, 2013).

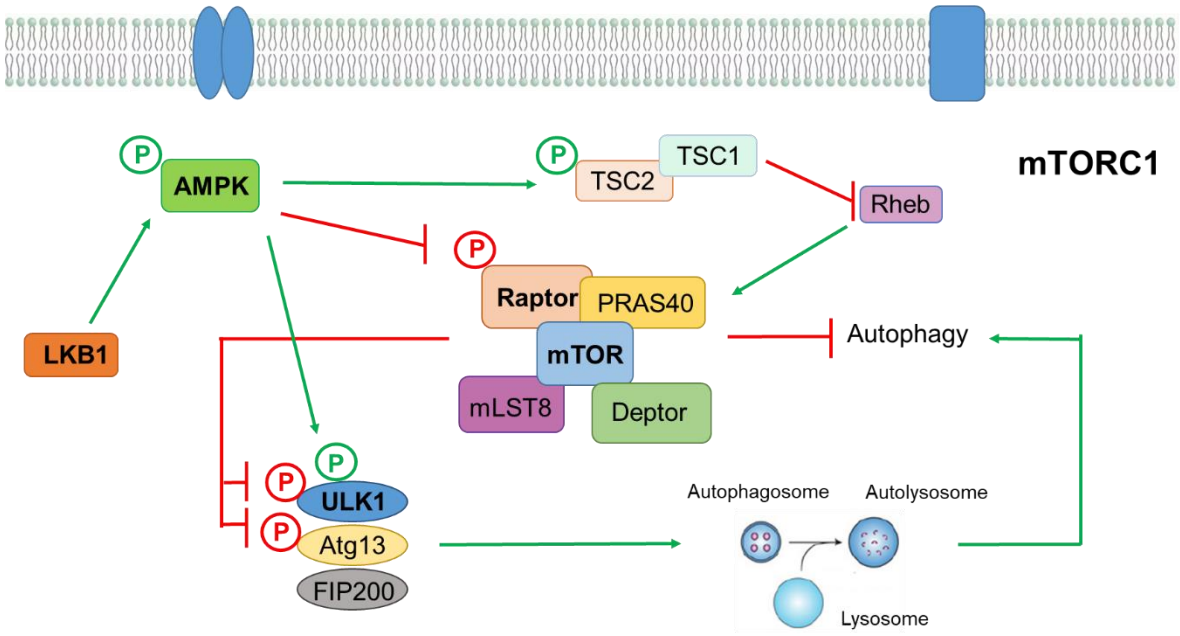

Figure I.2 - Simplified schematic representation of mTORC1 autophagy signalling pathway. Diverse signals like amino acids, growth factors, energy status and stressors activate mTORC1, which negatively regulates autophagy. mTORC1 complex is formed by five proteins that interact with each other – Raptor, mTOR, PRAS40, mLST8 and Deptor. To inhibit autophagy, mTOR needs to be linked to Raptor, that regulates complex assembly. Even more, mTOR inhibits ULK1 and Atg13 through phosphorylation, preventing formation of autophagosomes. In conditions of low energy levels, LKB1 activates AMPK by phosphorylation and AMPK can inhibit mTORC1 in two ways – it phosphorylates and activates TSC2, that through its GTPase-activating protein activity, inactivates the small GTPase Rheb, that is necessary to activate mTORC1 complex; and inactivates Raptor through phosphorylation. Furthermore, AMPK can phosphorylate ULK1, activating it, therefore promoting autophagy. AMPK - AMP-activated protein kinase; Atg13 – Autophagy related gene 13; FIP200 – focal adhesion kinase family-

interacting protein of 200 kDa; LKB1 – Serine-threonine kinase 11; mLST8 – mammalian lethat with Sec13 protein 8; mTOR – mammalian target of rapamycin; PRAS40 – proline-rich AKT substrate 40 kDa; Raptor – regulatory-associated protein of mTOR; Rheb – Ras homolog enriched in brain; TSC1/2 – tuberous sclerosis complex; ULK1 – UNC 51 like kinase.

3.2. Autophagy and ER stress

The UPR is one of the first cellular responses in order to restore homeostasis within the ER in cases of accumulation of misfolded proteins, namely in the central nervous system. However, if the UPR fails to rescue neurons from ER stress, ensuing ER stress culminates in activation of apoptosis (Cai et al., 2016). Importantly, neurons exhibit higher basal levels of autophagy, when compared to all the other cell types, probably because neurons are extremely sensitive to accumulation of toxic aggregates and require high energy for execution of normal functions. However, once the UPR and autophagy responses are unable to eliminate the damaged proteins or organelles effectively, neurons become highly susceptible to protein aggregation that occurs in neurodegenerative diseases (Cai et al., 2016).

It is well established that autophagy is up-regulated in response to ER stress, however, very little emphasis is given to its importance as a mediator in relieving ER stress (Deegan et al., 2013). A link between autophagy and ER stress can be done through Ca^{2+}. The release of Ca^{2+} from the ER lumen to the cytosol can be both an inducer of ER stress or/and a result of ER stress, however, an increase in cytosolic Ca^{2+} has been shown to lead to initiation of autophagy (Deegan et al., 2013). This process is mediated by Ca^{2+}/calmodulin-dependent kinase (kinase-β) which is activated in response to increased cytosolic Ca^{2+} and subsequent activation of AMPK, that in turn is involved in autophagy activation through inhibition of mTORC1 and direct phosphorylation of ULK1 (Hoyer-Hansen et al., 2007). Another link between these two processes comes through the PI3K-Akt signalling pathway, a positive regulator of mTORC1. ER stress results in the inactivation of the Akt pathway, contributing to the decrease of mTOR activity and subsequent autophagy induction by a mechanism still unclear (Qin et al., 2010).
Besides promoting the initiation of autophagy, ER stress and the UPR also play a role in vesicle nucleation and elongation of the phagophore (Deegan et al., 2013). In response to ER stress, IRE1 kinase domain recruits TRAF2, forming a complex that consequently recruits ASK1 which mediates signalling by several proteins, namely JNK. JNK activation results in phosphorylation of pro-apoptotic proteins enhancing their activity, and also phosphorylates anti-apoptotic Bcl-2 inhibiting its activity. Autophagy is the response activated in an early phase when JNK-mediated phosphorylation of Bcl-2 results in the dissociation of Bcl-2, however, with sustained activation of JNK by prolonged ER stress apoptosis is initiated (Song et al., 2017). The elongation of the phagophore requires the conversion of LC3I to LC3II and the covalent binding of ATG12 to ATG5. During prolonged stress-induced autophagy genes of ATG5, ATG12, and LC3I must be transcriptionally up-regulated in order to maintain flux through the pathway. PERK activation results in the transcriptional up-regulation of ATG5, ATG12, and LC3. ATG12 is transcriptionally up-regulated in response to ER stress in a PERK-eIF2α-dependent manner but the mechanism is not yet fully understood. LC3 and ATG5 are also transcriptionally up-regulated

through the PERK–eIF2α arm, however, LC3 is up-regulated by ATF4 whereas ATG5 is up-regulated by CHOP (Deegan et al., 2013).

When it comes to neurodegenerative diseases, it is clear that autophagy dysregulation is a contributory factor and that ER stress clearly plays an integral role in autophagy regulation. A certain degree of autophagy can remove the ubiquitinated unfolded/misfolded proteins and therefore reduce ER stress. However, excessive activation of autophagy induced by the heightened duration/degree of ER stress, can lead to cell self-digestion and even apoptosis, and further aggravate cell injury. The effects of autophagy induced by ER stress on cell survival are different and it has dual role including pro-survival and pro-death, which may be dependent on the extent of ER stress (Song et al., 2017).

Taken together, these examples support a complex scenario where UPR signalling converges in the modulation of autophagy process at both the transcriptional and post-translational level, suggesting a dynamic interplay between both pathways to maintain global proteostasis (Antonucci et al., 2015).

4. Glutathione-S-transferases

Glutathione S-transferases (GSTs) are phase II drug metabolizing enzymes that belong to a multigene family of isoenzymes responsible for the detoxification of electrophiles through conjugation with the nucleophilic thiol-reduced GSH (Mannervik & Danielson, 1988). The conjugation of reduced glutathione to electrophilic groups on substrate molecules, like by-products of oxidative stress, makes them more soluble therefore easier to eliminate from the cell (Hayes et al., 2005). In addition to their role in detoxification, GSTs isoenzymes are involved in functions such as the regulation of mitogen-activated protein kinases and participation in steroid synthesis, tyrosine degradation and dehydroascorbate reduction (Tew & Townsend, 2012; Wu & Dong, 2012).

GSTs can be grouped in four structurally distinct enzyme families: the Kappa class mitochondrial GSTs (soluble enzymes that appear to be expressed in mitochondria and peroxisomes in mammals and in *Caenorhabditis elegans)*; the MAPEG enzymes (membrane associated proteins in eicosanoid and glutathione metabolism); the fosfomycin resistance proteins (manganese and potassium dependent glutathione-S-transferases that catalyse nucleophilic addition of GSH to carbon-1 of fosfomycin) and cytosolic GSTs (found in all cellular life forms, with mammalian cytosolic GSTs having great importance in drug and xenobiotic metabolism) (Board & Menon, 2013).

The human cytosolic GSTs are typically dimeric proteins composed of 25-30 kDa that include an N-terminal domain, with the glutathione binding site, and the C-terminal domain, composed entirely of a helical bundle. They can be classified into 7 distinct classes of catalytically active enzymes termed Alpha, Mu, Pi, Sigma, Theta, Zeta and Omega (Board & Menon, 2013). In the brain, active GSTs are composed of dimers containing alpha, mu, or pi class GST, with GSTmu being the most highly expressed isoform in the brain in terms of abundance, followed by GSTpi, then GSTalpha (Smeyne & Smeyne, 2013). GSTpi and GSTmu were shown to be expressed in both neurons and astrocytes and the distribution of GSTs in the brain appears to be age related, with GSTpi being the only GST isoform that expressed in human fetal brain, while the alpha, mu and pi classes are found in adult (Abramovitz et al., 1988; Carder et al., 1990; Smeyne et al., 2007).

4.1. Glutathione S-transferase Pi

Humans have a single functional glutathione S-transferase Pi (GSTP) gene termed *GSTP1,* located in chromosome 11q13, in contrast to the mouse that has two Pi class GST genes (Board et al., 1989). Single nucleotide polymorphisms in the *GSTP1* gene have been found and associated with several malignancies such as Hodgkin's lymphoma (Lourenco et al., 2009). GSTP is mainly found in the cytoplasm and widely distributed in a range of tissues (Suzuki et al., 1987).

GSTP has been shown to protect cells from ROS by modulating S-glutathionylation of proteins following oxidative and nitrosative stress, a post-translational modification that consists of the addition of GSH to low pka cysteine residues of target proteins (Tew, 2007). S-glutathionylation usually occurs when a cysteine within a protein forms a disulphide bond with GS^- in response to endogenous oxidative or nitrosative stress mediated signalling events or from exposure to external environmental drug

treatments, like hydrogen peroxide (Townsend, 2007). GSTP itself is a subject of S-glutathionylation, reducing its enzymatic activity against chemical substrates and promoting multimerization (Dalle-Donne et al., 2007). Furthermore, it has been demonstrated that upon MPTP induced oxidative stress, GSTP potentiates S-glutathionylation of KEAP1 (Carvalho et al., 2016).

Besides being involved in cellular redox homeostasis, GSTP was shown to participate in reactions involving stress kinases. It was demonstrated that GSTP acts as a ligand-binding protein controlling the catalytic activity of JNK (Adler et al., 1999; Castro-Caldas et al., 2012a). GSTP exists predominantly in a monomeric form and the sequences of the C-terminus bind to JNK, inhibiting the interaction of JNK with c-Jun, reducing downstream apoptotic signalling. Under the presence of low levels of free radicals, GSH levels alone are sufficient to maintain redox balance. As the number of free radicals within the cell increases GSTP subunits dimerize, interfering with the C-terminus interaction with JNK, allowing progression of cell death signalling. Therefore, GSTP may serve as an endogenous regulator of the cellular stress response, eliciting protection against cell death induced by ROS by controlling JNK activity (Adler et al., 1999; Castro-Caldas et al., 2012a; Yin et al., 2000).

The presence of GSTP has been reported in mouse brain, being constitutively and predominantly expressed in glial cells, namely oligodendrocytes and astrocytes, and also in DA neurons from SNpc (Castro-Caldas et al., 2009; J. A. Johnson et al., 1993). Previous studies showed that sub-acute administration of MPTP to C57BL/6 mice induced GSTP expression in the midbrain and striatum and that MPTP-induced dopaminergic neuronal degeneration is an earlier event when comparing GSTP null and wild-type mice, suggesting it has a protective role against oxidative stress and the associated ER stress (Castro-Caldas et al., 2012c; Castro-Caldas et al., 2009). Interestingly, in the SNpc, the structure most affected in the central nervous system of PD patients, only GSTP, but not GSTmu, is found in the A9 DA neurons, a finding that may provide a clue to why these neurons are particularly sensitive to oxidative stress (Smeyne et al., 2007). Studies that compared ventricular cerebrospinal fluid from PD and normal control subjects, have shown differences in protein expression in PD patients, namely in *GSTP1*. Epidemiological studies have shown that decreased GSTpi expression is a significant risk factor for developing PD and that GSTpi wild type allele is an individual protective genetic trait in idiopathic PD (Golbe et al., 2007; Maarouf et al., 2012). All of these studies suggest as important role of GSTP in development and progression of PD.

5. Tauroursodeoxycholic acid

The search for novel therapeutic targets and strategies for PD is of utmost importance, once the most effective treatment (levodopa) targets the lack of DA in ST and is only symptomatic (Kalia & Lang, 2015). New therapeutic approaches to slow or prevent neurodegeneration might involve the targeting of oxidative stress, using pharmacological agents that possess either antioxidant or free radical scavenging properties.

Tauroursodeoxycholic acid (TUDCA) is a taurine conjugate of the hydrophilic endogenous bile acid, ursodeoxicholic acid (UDCA) (Duan et al., 2002). Bile acids are hydrophilic molecules synthesized in the liver and secreted into the intestine where they play crucial roles, like lipid solubilisation. Due to chemical structure alterations, some bile acids are cytotoxic molecules, while others are cytoprotective (Amaral et al., 2009). UDCA is synthesized in the liver and has been used for several decades in the treatment of a wide variety of liver diseases, particularly those associated with cholestasis (Beuers et al., 1998). UDCA which also plays a role in apoptosis, modulating the apoptotic threshold in several cell types, has been recently used in clinical trials for treatment of amyotrophic lateral sclerosis and is an FDA approved drug for the treatment of liver diseases, like primary biliary cirrhosis, not showing any relevant side effects during chronic treatment (Min et al., 2012; Rodrigues et al., 1998a; Yanguas-Casas et al., 2014). After oral administration, UDCA can be conjugated, in the liver, with glycine or taurine, originating respectively glycoursodeoxycholic acid or TUDCA (Vaz et al., 2015).

TUDCA is usually produced endogenously at very low levels in humans and easily crosses the blood brain barrier (BBB) with no associated toxicity (Castro-Caldas et al., 2012c). Importantly, it was demonstrated that TUDCA acts as an antioxidant molecule, preventing the formation of ROS as well inhibiting apoptosis through different cellular mechanism, namely by interfering with the mitochondrial pathway of cell death, as well as with targets upstream of mitochondria, including cell cycle related proteins E2F-1, p53 and endoplasmic reticulum stress (Ramalho et al., 2004; Rodrigues et al., 1998b; Viana et al., 2011). Indeed, TUDCA has been shown to exhibit antioxidant properties, maintain mitochondrial membrane potential, and prevent cytochrome c release, Bax translocation, caspase activation, and apoptosis in hepatocytes exposed to apoptotic stimuli (Rodrigues et al., 1998a; Rodrigues et al., 1998b; Rodrigues et al., 1999). Growing evidence also point to an important protective role of TUDCA in both *in vitro* and *in vivo* models of neurologic disorders, such as Huntington's disease, Alzheimer's disease, and stroke (Keene et al., 2002; Ramalho et al., 2006; Rodrigues et al., 2003).

Interestingly it was discovered by our group that pre-treatment with TUDCA was also able to prevent the MPTP-induced JNK phosphorylation protecting GSTP KO mice against MPTP toxicity (Castro-Caldas et al., 2012c). Although known to have all this beneficial effects, the molecular mechanisms underlying TUDCA elicited neuronal protection remain elusive. However, it is plausible to think that the properties of this bile acid should be considered in the search for new treatments for PD.

6. Experimental models of PD

Like previously mentioned, precise information about the mechanism and progression of PD is not yet fully understood. However, in the last few years, important advances were made in this area, both in the study of disease progression and discovery of possible treatments, due to the use of experimental models of the disease. So far, experimental models can be divided in two major categories: genetic models, which do not represent the typical degeneration of dopaminergic neurons, and neurotoxin-based models, that remain the most popular tool to produce selective neuronal death in both *in vitro* and *in vivo* systems (Bove et al., 2005).

6.1. Neurotoxin-based models of PD

To become an optimal model for PD, the model should conceive the pathological and clinical features of PD, it should involve both dopaminergic and nondopaminergic systems, the central and peripheral systems as well as motor and non-motor symptoms (Tieu, 2011). However, it is well known that none of the current models display all of these PD features. Nonetheless, validation of the animal model used is based on the possibility to translate the results obtained with the model into a clinical application to treat PD patients.

Among the neurotoxins used to induce dopaminergic neurodegeneration, 6-OHDA, MPTP, paraquat and rotenone are the ones that received more attention and all of them presumably provoke the production of ROS. Despite all toxins available, 6-OHDA and MPTP are the best characterized and the most widely used agents, with only MPTP being clearly linked to a human form of parkinsonism, and being thus the most widely studied model (Dauer & Przedborski, 2003). Since the discovery of MPTP a lot of valuable insights have been made in PD investigation, namely in the discovery of potential pathogenesis and mechanisms for cell death in PD. Studies using this model have led to concepts such as environmental toxicity as a potential culprit in sporadic PD, and mitochondrial dysfunction as a potential pathogenic mechanism (Tieu, 2011).

6.1.1. MPTP mechanism of action

MPTP is a lipophilic molecule that easily crosses the BBB after systemic administration, entering the brain (Tieu, 2011). Once in the brain, the pro-toxin MPTP is oxidized to 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) by MAO-B in glia and serotonergic neurons, the only cells containing this enzyme. Probably by spontaneous oxidation it is then converted to the active toxic molecule, 1-methyl-4-phenylpyridinium (MPP^+), and released by an unknown mechanism into the extracellular space (Dauer & Przedborski, 2003). MPP^+ is a polar molecule, therefore it cannot enter cells freely, depending on plasma membrane carriers to enter the cell (Javitch et al., 1985). Therefore, it was described that MPP^+ enters dopaminergic neurons due to its high affinity for dopamine transporter DAT. Importantly, pharmacological inhibition or genetic deletion of DAT prevents MPTP-induced dopaminergic damage demonstrating the obligatory character of this step in MPTP neurotoxicity (Javitch et al., 1985). However,

uptake by DAT does not entirely explain the selectivity of the nigrostriatal dopaminergic lesion caused by MPTP. Figure I.2 – A shows a representation of MPTP metabolism.

Inside neurons, MPP^+ can follow three different routes (Figure I.2 – B): it can be translocated into synaptosomal vesicles, through binding to the vesicular monoamine transporter-2 (VMAT2); once into the mitochondria, it can block complex I of the ETC, by a mechanism that relies on the mitochondrial transmembrane potential; or it can remain in the cytosol to interact with cytosolic enzymes, especially those carrying negative charges (Dauer & Przedborski, 2003). Importantly, the deleterious events observed in PD patients' brains are mimicked by the impairment of complex I by MPP^+, namely the rapid decrease of ATP tissue content, particularly in striatum and ventral midbrain, the stimulation of the production of ROS, especially superoxide and the elevated intracellular calcium concentration (Dauer & Przedborski, 2003; Watanabe et al., 2005).

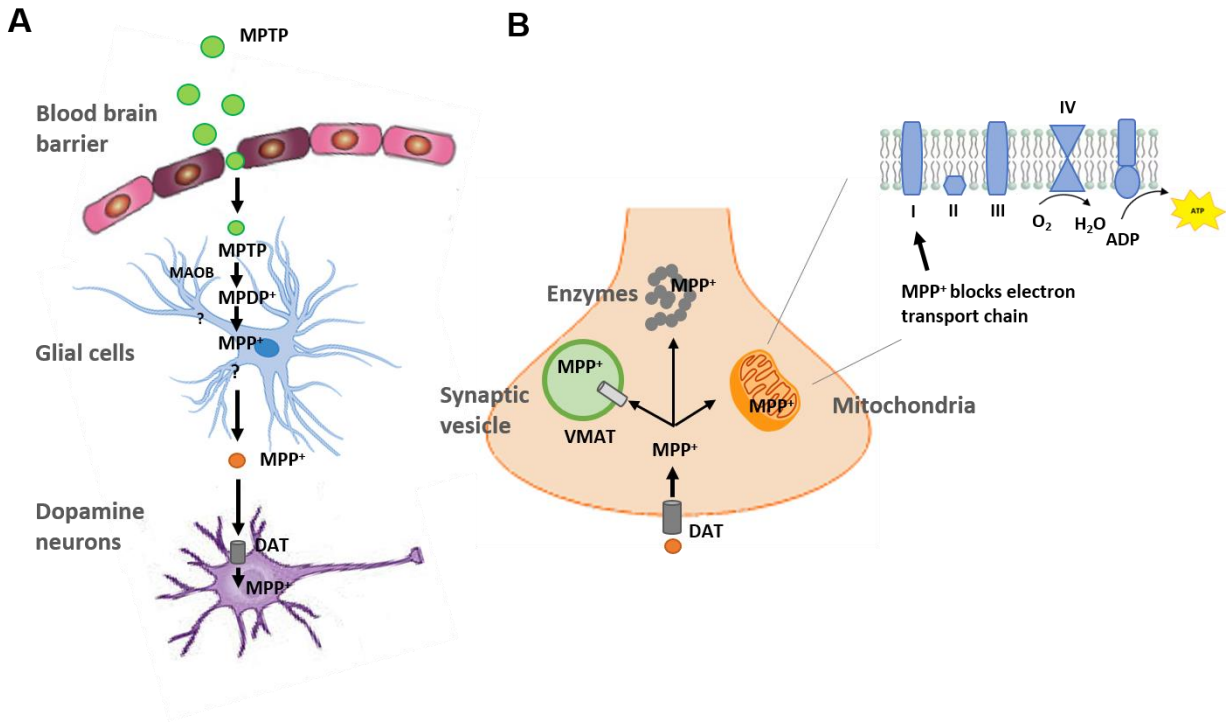

Figure I.3 – Schemactic representation of MPTP metabolism and intracelular pathways. A – *MPTP metabolism.* After crossing the blood-brain barrier MPTP is metabolized to its first product, $MPDP^+$ by MAO-B within glial cells or dopaminergic neurons (not shown) and is converted to its active metabolite MPP^+, probably by spontanuous oxidation. MPP^+ then enters dopaminergic cells via dopamine transporters. **B –** *Intracellular pathways of* MPP^+. Inside the cell, MPP^+ can be sequestered into synaptic vesicles via VMAT2, it can interact with cytosolic enzymes or finally, it can block the complex I of mitochondrial ETC. DAT – Dopamine transporter; MAO-B – Monoamine oxidase; $MPDP^+$ - 1-methyl-4-phenyl-2,3-dihydropyridinium; MPP^+ - 1-methy-4-phenylpyridinium; MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; VMAT2 - vesicular monoamine transporter-2. Adapted from (Dauer & Przedborski, 2003).

6.2. PD Animal Models

As noted, animal models may be useful for studying pathogenic mechanisms, for testing therapeutic strategies, or both. As such, no single model is likely to be suitable for all studies. To be considered a good animal model of PD the neurodegeneration of dopaminergic cells should not present any unprompted recuperation, the model should have reproducible nigral damage and there should be possible to establish a neuroprotective strategy (Emborg, 2004). The best animal models should mimic the pathophysiology of the disease such as the formation of Lewy bodies, the loss of neurons in the SNpc and behavioural symptoms that arise during the disease (Potashkin et al., 2010). At this time, none of the currently available models phenocopy the disease, mainly because they lack some specific neuropathological and/or behavioural feature of PD.

Rodents have been used over time to study this disease because they are easily available, reproduce easily and at a large scale, are genetically malleable, and have relatively low cost as compared to larger animals (Dawson et al., 2010) *C. elegans* and *drosophila* are models used to rapidly screen for pharmacologic and genetic interventions that may modify neurodegeneration, however, they lack the expression of α-synuclein. Studies with dogs, cats and nonhuman primates have also been successfully done but their utility has been limited by high costs and ethical concerns (Dawson et al., 2010).

6.2.1. The MPTP rodent model

Diverse susceptibility to neurotoxins exists between species. Mice present higher intracerebral levels of MAO-B, therefore they are more sensible to MPTP than rats. Furthermore, different strains of mice can exhibit distinct sensitivity to MPTP. Gender, age and body weight also affect MPTP sensitivity: female mice, mice under 8 weeks, and mice smaller than 25 g are less sensitive and their lesions are more variable (Emborg, 2004). It was described that C57BL/6 mice show an optimal reproducibility of MPTP-lesioning when they have 25-30 g in weight (Przedborski et al., 2001).

MPTP can be administrated in different manners, like oral and intracerebral stereotaxic injections, but the most reliable lesions are induced by systemic or intracarotid artery injections of freshly prepared MPTP solution (Emborg, 2004). Usually, MPTP is administered to mice in two ways: acute or chronic. The acute administration consists in four injections at 2h interval in 1 day (20 mg/kg i.p.) and induces nigral cell death mainly be necrotic mechanisms, it has higher mortality but behavioural deficits can be detected with specific tests (Przedborski & Vila, 2003). Chronic administration consists in one injection a day, during 5 days (30 mg/kg i.p.) and induces cell death mainly by apoptotic mechanisms but almost undetectable behavioural deficits (Tatton & Kish, 1997). Following MPTP exposure, changes include decreased levels of dopamine and its metabolites in the striatum, increased oxidative damaged proved by increased lipid peroxidation and decreased concentrations of antioxidants, like GSH. Also the presence of α-synuclein aggregates was observed in baboons, but with different characteristics from Lewy Bodies (R. Betarbet et al., 2002).

6.3. Cellular models of PD

In vitro models, like cell lines, primary cultures and stem cells are also used as an experimental model for PD, and became an important tool in the field of neurological diseases. These models present a controlled environment that helps investigations of molecular and cellular pathophysiological mechanisms of dopaminergic degeneration in PD, dissecting the molecular function of the genes and proteins implicated, and the screening of potential therapeutics. Also, cellular models have the advantage of being fast and reproducible, therefore tests can be done relatively quickly and robustly using molecular, biochemical and pharmacologic approaches (Alberio et al., 2012; Lopes et al., 2017). By contrast, these models also present limitations, such as the difficulty in mimicking the central nervous system complexity, differences in genotype and phenotype, natural function and responsiveness to stimuli alterations, causing heterogeneity in cultures and also, possible contaminations with other cell lines and mycoplasma (Kaur & Dufour, 2012).

Because dopaminergic degeneration is a common hallmark in PD cases it is very important the development of new dopaminergic cell models as well as a better understanding of the existing models. Models made of cell lines are suitable for evaluation of neuroprotective compounds for PD treatment, namely via high throughput screening approaches, using either genetic or toxin-based models of the disease (Lopes et al., 2017). Cell lines originate from a population of cells from a multicellular organism and are immortalized, leading to loss of some cell cycle checkpoint pathways and normal cellular senescence (Maqsood et al., 2013).

The mouse neuroblastoma cell line N2a is classified as a neural crest-derived cell line and was previously shown to be a candidate model for neurotoxicological studies (De Girolamo et al., 2000; Nagatsu et al., 1981). N2a cells have been also used to study neuronal differentiation, neurite growth, synaptogenesis and signalling pathways. N2a cells have the advantage of responding quickly to serum deprivation and other stimuli in their environment by expressing signalling molecules that lead to neuronal differentiation and neurite growth, being capable of originating dopaminergic neurons (Tremblay et al., 2010). Previous studies using MPTP to treat N2a cells showed sub cytotoxic concentrations of MPTP can induce neurotoxic changes in differentiating N2a cells characterized by an inhibition of axon outgrowth well before cell death (De Girolamo et al., 2000). Therefore, N2a cells might be a good cellular model for the study of the neurotoxicity of PD.

7. Main goals

The main goal of this thesis is to evaluate the modulation of ER stress and autophagy signalling pathways upon MPTP/MPP^+-induced neurotoxicity. Moreover, we seek to understand if the neuroprotective effect of TUDCA might be due to modulation of ER stress response, autophagy pathway or both. The characterization of these cellular pathways is important for discovery of new strategies of treatment of PD, namely the characterization of novel therapeutic targets. To accomplish the aims of this thesis, both *in vitro* and *in vivo* experimental models of the disease were used.

The specific objectives are:

- Evaluate the expression levels of ER stress markers and autophagy markers upon MPTP-induced neurotoxicity in C57BL/6 mice brain and N2a mouse neuronal cells;

- Characterize the effect of TUDCA in both experimental models in order to understand the molecular mechanisms underlying its neuroprotective role;

- Investigate the putative relation between UPR/ER stress and autophagy signalling pathways in the presence or absence of TUDCA;

- Evaluate GSTP expression levels in response to ER stress.

II . MATERIALS AND METHODS

1. Materials

1.1. Supplements and chemicals

Tauroursodeoxycholic acid (TUDCA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1-methyl-4-phenylpyridinium (MPP^+), Bovine Serum Albumine (BSA) (fraction V), Complete Mini Protease Inhibitors, DAPI, Mowiol mounting medium, Tunicamycin, Triton X-100, phosphate buffered saline (PBS) were purchased from Sigma Aldrich (St Louis, MO, USA); Dulbecco's Modified Eagle Medium (DMEM), Reduced Serum Medium (Opti-MEM® I), fetal bovine serum (FBS), Penicillin/Streptomycin, L-glutamine and TriplE Express were obtained from GIBCO ® (Life Technologies, Inc., Grand Islands, USA); Izol-RNA lysis reagent was secured from 5 Prime (Hamburg, Germany); Random primers for reverse-transcribed complementary DNA (cDNA) were obtained from Promega (Sunnyvale, CA, USA); SuperScript II reverse-transcriptase kit was purchased from Invitrogen (Grand Island, NY, USA); SensiFAST™ SYBR® Hi-ROX kit was acquired from Bioline (London, UK); ECL Western blotting detection reagent was secured from GE Healthcare (Buckinghamshire, UK); SuperSignal® West Femto Maximum Sensitivity was acquired from Thermo Scientific (Rockford, USA); Bio-Rad's Protein Assay Reagent was purchased from Bio-Rd Laboratories (Hercules, CA, USA); Polyvinyl difluoride (PVDF) membrane was obtained from Millipore (Bedford, MA, USA).

1.2. Antibodies

The antibodies used in western blot and immunofluorescence assays are listed in Table II.1.

Table II.1 – List of the primary antibodies used in western blot and immunofluorescence assays.

Primary antibody (antigen)	Host	Supplier	Reference	Dilution
AMPK	Rabbit	Cell Signaling	#2532	1:1000
ATF4	Rabbit	Cell Signaling	#11815	1:250
ATF6	Mouse	Novus Biologicals	NBP1-40256	1:1000
β-actin	Mouse	Sigma-Aldrich	A2228	1:8000
CHOP	Rabbit	EnoGene	E11-0863B	1:200
eIF2α	Rabbit	Cell Signaling	#9722	1:1000
GST-π	Mouse	BD Transduction Laboratories	610719	1:50
IRE1α	Rabbit	Novus Biologicals	NB110-59971	1:1000
LC3	Rabbit	Thermo Scientific	PA1-16931	1:2000
mTOR	Rabbit	Cell Signaling	#2972	1:1000
PERK	Rabbit	Cell Siganilling	#3192	1:200
p-AMPK	Rabbit	Cell Signaling	#2535	1:1000
p-eIF2α	Rabbit	Cell Signaling	#9721	1:1000
p-IRE1α	Rabbit	Novus Biologicals	NB100-2323	1:1000
p-mTOR	Rabbit	Cell Signaling	#2971	1:1000
p-PERK	Rabbit	Santa Cruz Biotechnology	Sc-32577	1:500
p-Raptor	Rabbit	Cell Signaling	#2083	1:500
p-ULK1 (Ser 555)	Rabbit	Cell Signaling	#5869	1:500
p62	Mouse	Abcam	Ab56416	1:2000
Raptor	Rabbit	Cell Signaling	#2280	1:500
ULK1	Rabbit	Cell Signaling	#8054	1:1000

2. Methods

2.1. Animal treatments

All animal experiments were carried out in accordance with the institutional, Portuguese, and European guidelines (*Diário da República, 2.ª série N.° 121* of 27 June 2011; and 2010/63/EU European Council Directive) and methods were approved by the Direção Geral de Alimentacão e Veterinária (DGAV, reference 021944) and the Committee for Animal Welfare (ORBEA) of the Faculty of Pharmacy, University of Lisbon. Twelve-week-old male C57BL/6 wild-type mice, purchased from Harlan, and the C57BL/6 *Gstp1/p2* null mice lineage were used. The *Gstp1/p2* null mice lineage is a double-knockout line, since in the mouse both Gstp genes (Gstp1 and Gstp2) are arranged in tandem on chromosome 1 and were deleted by homologous recombination (Henderson et al., 1998). All animals were maintained at the FFULisboa Animal House - Campus Lumiar and housed under standardized conditions on a 12 h light–dark cycle, with constant temperature (22–24 °C) and humidity (50–60%) with free access to a standard diet and water *ad libitum.*

Both wild-type (WT) and knockout *Gstp1/p2* (KO) mice were intraperitoneally (i.p.) injected with MPTP, TUDCA or saline and divided in different groups according each treatment – control mice injected with saline (CT) and sacrificed on the last day of treatments; mice treated with only TUDCA that received injections for three consecutive days and were sacrificed 6 hours after the last injection (TUDCA); mice injected with only MPTP that were sacrificed 3 hours (M3) or 6 hours (M6) after MPTP administration; mice treated with TUDCA followed by i.p administration of MPTP on day 3 and sacrificed 3 hours after injection (T+M3) or 6 hours after injection (T+M6); mice injected with MPTP on day one, followed by TUDCA administration after 3 hours (M+T3) or after 6 hours (M+T6) and mice were sacrificed 6 hours after the last TUDCA injection, on day three. The schematic administration of TUDCA and MPTP is represented in figure II.1.

TUDCA and MPTP were dissolved in PBS (pH 7.4) and administrated at a dose of 50 mg/kg and 40 mg/kg body weight, respectively. Mice were anesthetized with isoflurane, decapitated, and brains were removed and placed in fresh PBS. Brains were then placed on their ventral surface in a mouse brain matrix (Agar Scientific) and a slice between Bregma -2.5 and Bregma -3.8 was isolated. The entire cortex region, midbrain and striatum were dissected and frozen under liquid nitrogen and kept in -80ºC until further use. All animal experiments were designed with commitment to refinement, reduction, and replacement, minimizing the numbers of mice and suffering via emphasis on humane end points. For statistical validity, we used n = 3 to 5 for biochemical analysis (with three replicates).

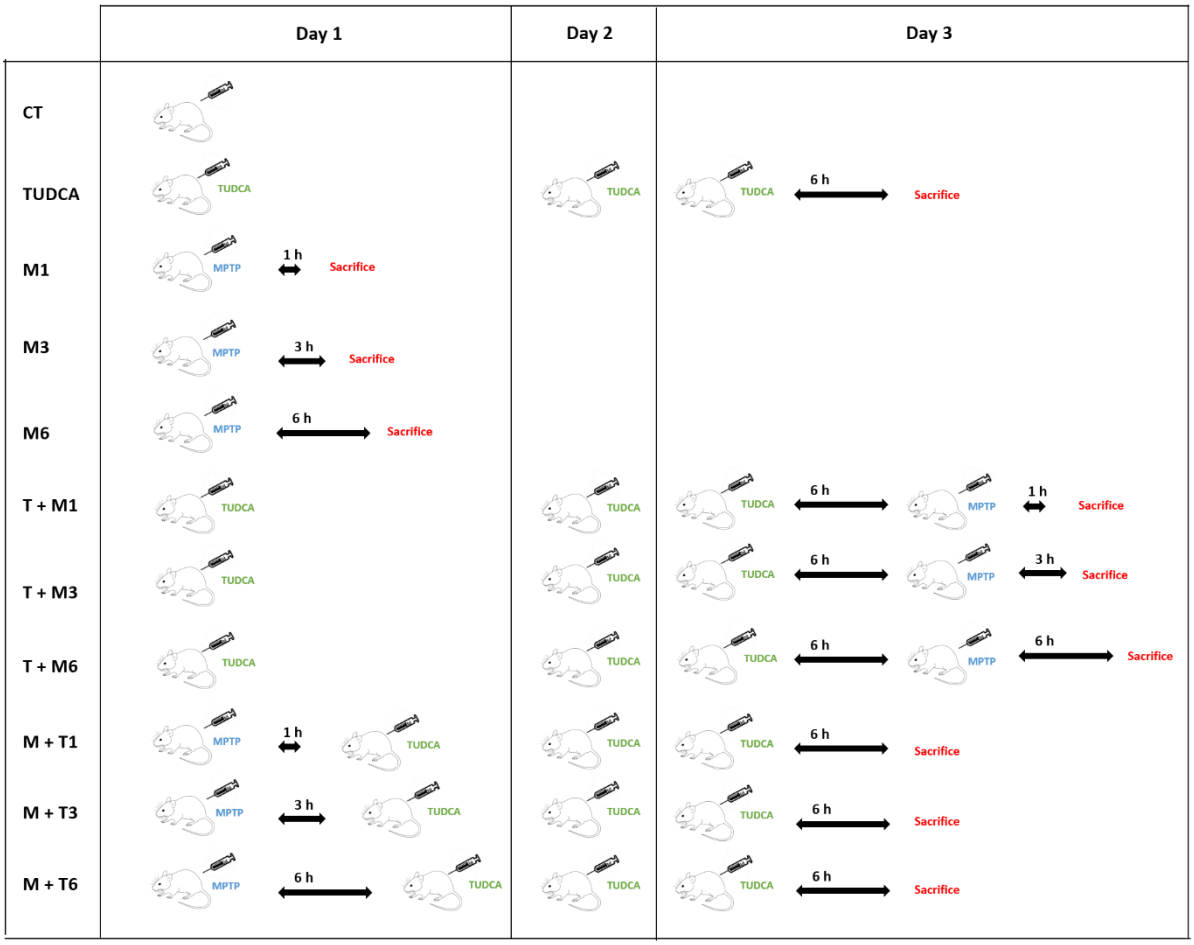

Figure II.1 - Schematic representation of C57BL/6 mice treatment course. Mice were i.p injected with TUDCA (50 mg/kg body weight) for three consecutive days. MPTP was administered at a single dose of 40 mg/kg body weight. Mice were divided into 8 main groups: CT – Control mice received saline; TUDCA – mice injected with TUDCA for three consecutive days sacrificed 6 hours after the last injection; M1 – mice treated with a single dose of MPTP at day one, sacrificed 1 hour after injection; M3 – mice treated with a single dose of MPTP at day one, sacrificed 3 hours after injection; M6 - mice treated with a single dose of MPTP at day one, sacrificed 6 hours after injection; T + M1 - mice treated with TUDCA for three consecutive days, injected with MPTP 6 hours after last treatment and sacrificed 1 hour after MPTP injection; T + M3 – mice treated with TUDCA for three consecutive days, injected with MPTP 6 hours after last treatment and sacrificed 3 hours after MPTP injection; T + M6 - mice treated with TUDCA for three consecutive days, injected with MPTP 6 hours after last treatment and sacrificed 6 hours after MPTP injection; M + T1 - TUDCA administration, for three consecutive days, occurred 1 hour after MPTP injection, and mice were sacrificed 6 hours after the last TUDCA injection, on day 3; M + T3 – TUDCA administration, for three consecutive days, occurred 3 hours after MPTP injection, and mice were sacrificed 6 hours after the last TUDCA injection, on day 3; M + T6 - TUDCA administration, for three consecutive days, occurred 6 hours after MPTP injection, and mice were sacrificed 6 hours after the last TUDCA injection, on day 3. i.p – intra-peritoneally; MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; TUDCA – Tauroursodeoxycholic acid.

2.2. Culture conditions and N2a treatment

Mouse neuronal cell line N2a was cultured and maintained in T75 flasks in DMEM and Opti-MEM® I (1:1), supplemented with 5% FBS, 1% L-glutamine and 1% P/S (penicillin and streptomycin), at 37ºC in a humidified atmosphere of 5% CO_2 in HERAcell 150 incubators (Thermo Scientific, Waltham, MA, USA). Cells were detached from the flaks with 1.5 ml of TrypleE express for 5 minutes at 37ºC when they reached about 80% confluence, counted, diluted in the previous medium and plated for different assays. For Western Blot cells were plated in 60 x 15 mm culture plates at a concentration of 1 x10^6 cells/plate and treated with TUDCA alone, MPP^+ plus TUDCA and MPP^+ alone, each at different time points, as illustrated in figure II.2 - A. Control cells were not treated. After treatment, cells were washed with PBS and lysed with lysis buffer (50 mM Tris-HCl pH 7.4, 180 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM DTT, proteases inhibitors). For immunocytochemistry cells were plated in a 12-well cell culture plate at a concentration of 1.9 x 10^5 and treated with TUDCA alone, MPP^+ alone, MPP^+ plus TUDCA or Tunicamycin and Tunicamycin with TUDCA, at different time points, as illustrated in figure II.2 – B. Treatment for immunocytochemistry is described in section II.2.4.

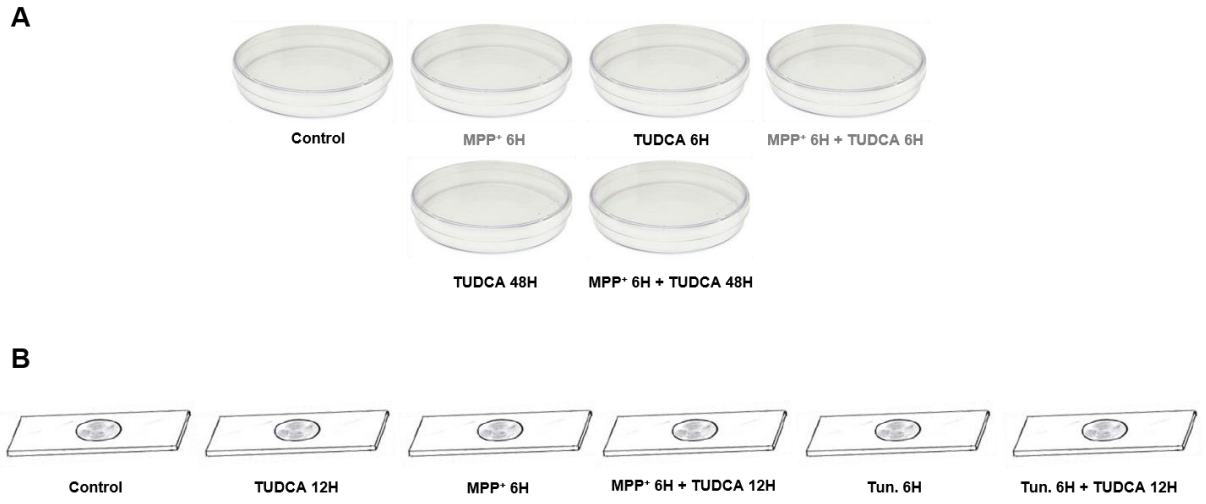

Figure II.2 – Different treatment conditions of N2a. Cells were plated as described above and after a 24h period of stabilization, culture medium was changed and N2a cells were treated. **A** – For protein extraction cells were treated with TUDCA alone (100 µM) at different timepoints or with MPP^+ (1mM) during 6 hours prior to TUDCA administration. Cells were also treated with MPP^+ or with 1mg/ml Tun alone or with Tun 6 hours prior to TUDCA administration. **B –** For immunocytochesmistry cells were treated with TUDCA during 12h, MPP^+ 6 hours, MPP^+ 6 hours before TUDCA administration, TUDCA during 12 hours and added MPP^+ 6 hours or with Tun 6 hours. Medium was always changed between treatments. Controls were always included and consisted of non treated cells. MPP^+ - 1-methyl-4-phenylpyridinium; TUDCA – Tauroursodeoxycholic acid; Tun – Tunicamycin.

2.3. Western Blot Analysis

Mice brains were collected as described under animals treatment section and cutted in slices containing the entire midbrain and striatum as previously described (Castro-Caldas et al., 2009). Tissue samples were homogenized in ice cold PBS using a Potter-Elvehjem homogenizer, centrifuged at 3000 rpm for 10 minutes at 4ºC and pellets were collected. Pellets were homogenized in lysis buffer 1x [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM Na_2EDTA, 1mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin] plus 200 mM Na_3VO_4, 1M NaF and Complete Mini Protease Inhibitors Cocktail. Lysates were sonicated, on ice, five times 5 seconds each in the Ultrasonic Processor UP100H (Hielscher-Ultrasound Technology, Teltow, Germany), centrifuged at 13000 rpm for 15 minutes at 4ºC and the supernatant was recovered and stored at -80ºC. N2a cells were also homogenized in lysis buffer, lysates were sonicated, on ice, three times 5 seconds each, centrifuged at 13000 rpm for 15 minutes at 4ºC and the supernatant was stored at -80ºC.

Total protein concentration was determined using the Bradford method using Bio-Rad's Protein Assay Reagent. Tissue extracts with 150 μg of total protein and cell extracts with 100 μg of total protein were added to denaturing buffer (1:1) [0.25 M Tris-HCl, 4% sodium dodecyl sulfate (SDS), 4% glycerol, 0.004% bromophenol blue, 1% β-mercaptoethanol, pH 6.8], boiled for 10 min and resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), in running buffer (25 mM Tris Base, 190 mM glycine, 0.1% SDS, pH 8,8) with fixed amperage of 35 mA per gel, for about 3,5h. Proteins were electrotransferred to an activated PVDF membrane (1 min in ethanol, 2 min H_2O, 5 min in transfer buffer), in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol), with a fixed amperage of 500 mA during 2h.

The 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 7.5), minimum 1h at room temperature (RT) and incubated overnight at 4ºC with specific primary antibodies (listed in table II.1). After being washed with TBS-T 3 times 15 minutes each, membranes were incubated 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 1h at RT. Membranes were again washed with TBS-T and incubated with Pierce™ ECL Westren Bloting Substrate (32106; ThermoScientific) or SuperSignal West Femto Maximum Sensitivity Substrate (34096; ThermoScientific) for detection of chemiluminescent immunocomplexes. Densitometric analyses were performed using ImageLab Software 5.1 Beta after scanning with ChemiDoc™, both from Bio-Rad Laboratories (Hercules, CA, USA). To reuse, membranes were stripped (1.5% glycine, 40% glacial acetic acid, 1% SDS, 10% Tween 20) for 10 minutes and washed several times with water and TBS-T. All membranes were then incubated with β-actin, used as a loading control.

2.4. Immunocytochemistry

N2a cells were treated as previously described. Cells were washed with PBS three times, five minutes each, and fixed with 4% (w/v) paraformaldehyde (PFA). After washing, cells were permeabilized with 1% Triton X-100 in PBS, 10 minutes, RT. Blocking was performed with goat serum, for 1h at RT. Cells were then incubated with anti-GSTπ primary antibody (1:50 in blocking solution (0.2% BSA, 0.05% Tween 20 in PBS)) overnight at 4ºC, in a humidified chamber. As secondary antibody goat anti-mouse Alexa Fluor® 488 (1:200 in blocking solution) was used and nuclei were stained with DAPI (1:100), both incubated for 1h RT. The cells were mounted in glass slides with Mowiol anti-fading mounting medium.

Green (for GSTπ) and blue (for nuclei) fluorescence and UV images of, at least, ten random microscope fields were acquired per sample, under 40x magnification, using a fluorescence microscope (model AxioScope.A1) with integrated camera AxioCam HR (Carl Zeiss, Inc – North America). The results were expressed as the percentage of green fluorescence per total number of cells, using the ImageJ software analysis (National Institutes of Health, USA).

2.5. Total RNA isolation and qRT-PCR analysis

Total RNA was extracted from mice striatum samples in accordance with manufacturers work instructions (Izol-RNA lysis reagent) as listed in section A of Annexes. RNA concentration was evaluated by spectrometry at UV light using the Nanodrop 1000 (Thermo Scientific, Rockford, USA). RNA samples were treated with DNase I recombinant RNase-free and 1 μg of treated RNA from each sample was submitted to reverse transcription with NZY Reverse Transcriptase, random primers and deoxynucleotides (NzyTech) to obtain cDNA. Quantitative real-time PCR (qRT-PCR) reactions were performed using SensiFAST™ SYBR® Hi-ROX Kit (Bioline USA Inc, Taunton, MA, USA) in an ABI 7300 sequence detection system (Applied Biosystems, Foster, CA, USA), using the following cycle conditions: 50ºC for 2 minutes; 95ºC for 2 minutes; followed by 40 cycles at a temperature of 95ºC for 5 seconds and at 60ºC for 30 seconds. For the melting curve a final step of 95ºC for 15 seconds, 60ºC for 30 seconds and 95ºC for 15 seconds was added. mRNA expression levels of the genes of interest were normalized to EEF expression levels. Primers used are listed in table II.2 and cycles of PCR are listed in table II.3. Data analysis is based on the ΔΔCt method with normalization of the raw data to housekeeping genes, as described in the manufacturer's manual. All PCR reactions were performed in duplicate from at least three independent experiments.

Table II.2 – Sequences of primers used for qRT.PCR analysis

Gene	Forward	Reverse
BiP/Grp78 /Hspa5	GCCAACTGTAACAATCAAGGTCT	TGACTTCAATCTGGGGAACTC
CHOP	CCACCACACCTGAAAGCAG	TCCTCATACCAGGCTTCCA
XBP1s	TCCGCAGCAGGTGCAG	CCAACTTGTCCAGAATGCCC
EEF	ACACGTAGATTCCGGCAAGT	AGGAGCCCTTTCCCATCTC

Table II.3 – Cycle treatments used for DNase sample treatment and cDNA synthesis

Treatment with DNase	20 minutes 37ºC
	10 minutes 75ºC
cDNA synthesis	5 minutes 65ºC
	10 minutes 25ºC
	50 minutes 50ºC
	5 minutes 85ºC

2.6. Statistical analysis

Data comparisons were conducted using one-way analysis of variance (ANOVA) followed by Tukey post hoc test. Analysis and graphic presentation are performed using GraphPad Prism software version 6 (GraphPad Software, Inc., San Diego, CA, USA). Results are presented as mean ± standard error of the mean (SEM).

3. RESULTS

1. Evaluation of autophagy markers in N2a cells and C57BL/6 mouse model

1.1. TUDCA alters autophagy markers expression through time as well as mTORC1 complex proteins, in N2a cells

TUDCA has been shown to have antioxidant properties as well as anti-apoptotic effects. In order to evaluate if the neuroprotective effect of TUDCA is related with induction of autophagy we decided to evaluate the levels of LC3 II and p62, classic autophagy markers, at different time points of TUDCA administration, namely in short period (6 hours) or longer time periods (48 hours).

The expression levels of LC3II/I and p62 were evaluated by Western blot using specific antibodies. N2a cells were treated with MPP^+ during 6 hours and followed by treatment with TUDCA during 6 or 48 hours. Levels of LC3II were higher at the shortest time point, decreasing with prolonged TUDCA administration and increased levels of LC3II observed after treatment with MPP^+ were reduced in the presence of TUDCA at both 6 hours and 48 hours, although values did not reach statistical significance (Figure III.1 – A). Accordingly, being p62 a substrate of autophagy, its values were particularly increased at 48 hours, with an accentuated decrease at 6 hours of treatment with TUDCA and levels of p62 were always similar to control when TUDCA was added, at both time periods (Figure III.1 – B). However, again no statistical significance was found. These preliminary results seem to suggest that TUDCA stimulates autophagy as a first response, but this response is diluted with longer exposures to this molecule.

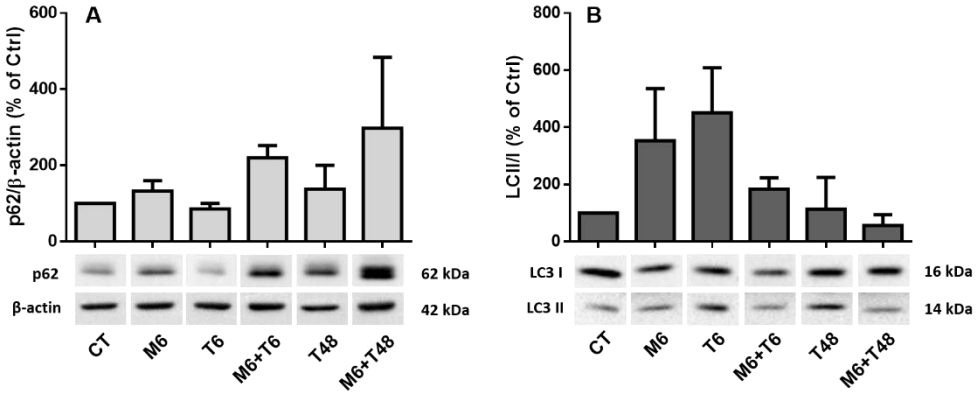

Figure III.1 – TUDCA modulates levels of LC3II and p62 in N2a cells. N2a cells were treated with MPP^+ and/or TUDCA as described in Methods. N2a cells extracts were analized by Western blot for LC3 (A) or p62 (B). β-actin was used as a loading control for p62 and values of LC3II were obtained through the ratio with LC3I. Intensity of the bands was quantified using image analysis software (Image Lab). Data are expressed as the mean values ± SEM, indicated as percentage of control. Results are representative of three independent experiments. CT – cells not treated; M6 – cells treated with MPP^+ for 6h; M6+T6 – cells treated with MPP^+ for 6h, followed by treatment with TUDCA for 6h; M6+T48 – cells treated with MPP^+ for 6h, followed by TUDCA for 48h; T6 – cells treated with TUDCA for 6h; T48 – cells treated with TUDCA for 48h.

Since levels of classic autophagy markers were altered in the presence of TUDCA we decided to search for modifications of mTORC1 complex proteins, in order to evaluate if the modulation observed in autophagy was done through mTORC1 signalling. Therefore, we evaluated the phosphorylation status of several proteins of the complex, as well as ULK1, since its activation/inhibition is a direct consequence of the correct gear of mTORC1.

Levels of mTOR, Raptor, AMPK and ULK1 phosphorylation were evaluated by Western blot assay using specific antibodies. The antibody used for mTOR recognises phosphorylation levels of the residue S2448, a reaction important for mTORC1 correct functioning. Phosphorylation of mTOR was increased with TUDCA administration during 6 hours, followed by a significant decrease of pmTOR levels with prolonged TUDCA administration, namely 48 hours ($p<0.01$). Also, levels of pmTOR significantly increased with previous administration of MPP^+ followed by TUDCA during 48 hours ($p<0.01$), however this effect was not observed when cells were treated with TUDCA for shorter time periods (Figure III.2 – A). The specific antibody used to determine phosphorylation levels of Raptor recognises residue S792, one of AMPK targets. Results obtained for p-Raptor showed an inverse pattern of p-mTOR, with levels of Raptor phosphorylation being lower in short time treatments with TUDCA, increasing proportionally with time of TUDCA exposure. Administration of MPP^+ before TUDCA treatment seems to increase levels of pRaptor at 6 hours, similar to what was observed at 48 hours (Figure III.2 – B). However, values for p-Raptor did not reach statistical significance. Levels of phosphorylated AMPK were determined using a specific antibody for T792. Phosphorylation of AMPK was significantly increased with administration of MPP^+, when compared to control ($p<0.01$) with subsequent TUDCA administration for 6 hours being able to significantly reduce this levels ($p<0.05$). Single administration of TUDCA during 6 or 48 hours significantly decreased levels of pAMPK when compared to single MPP^+ administration ($p<0.001$ and $p<0.01$, respectively) (Figure III.2 – C). The specific antibody used for determination of ULK1 phosphorylation recognises phosphorylation at residue S555, being this phosphorylation accomplished by activated AMPK. Activation levels, here shown by ULK1 phosphorylation rise with MPP^+ administration and seem to be partially restored with TUDCA administration after MPP^+, however no significant changes were observed. Also, phosphorylation levels of ULK1 were higher with longer periods of exposure of TUDCA, but no statistical significance was found (Figure III.2 – D).

Taken together, these preliminary results obtained in N2a cells suggest that TUDCA modulates autophagy and that mTORC1 plays an important role in this process, verified by the differences observed in protein expression of mTORC1 complex proteins.

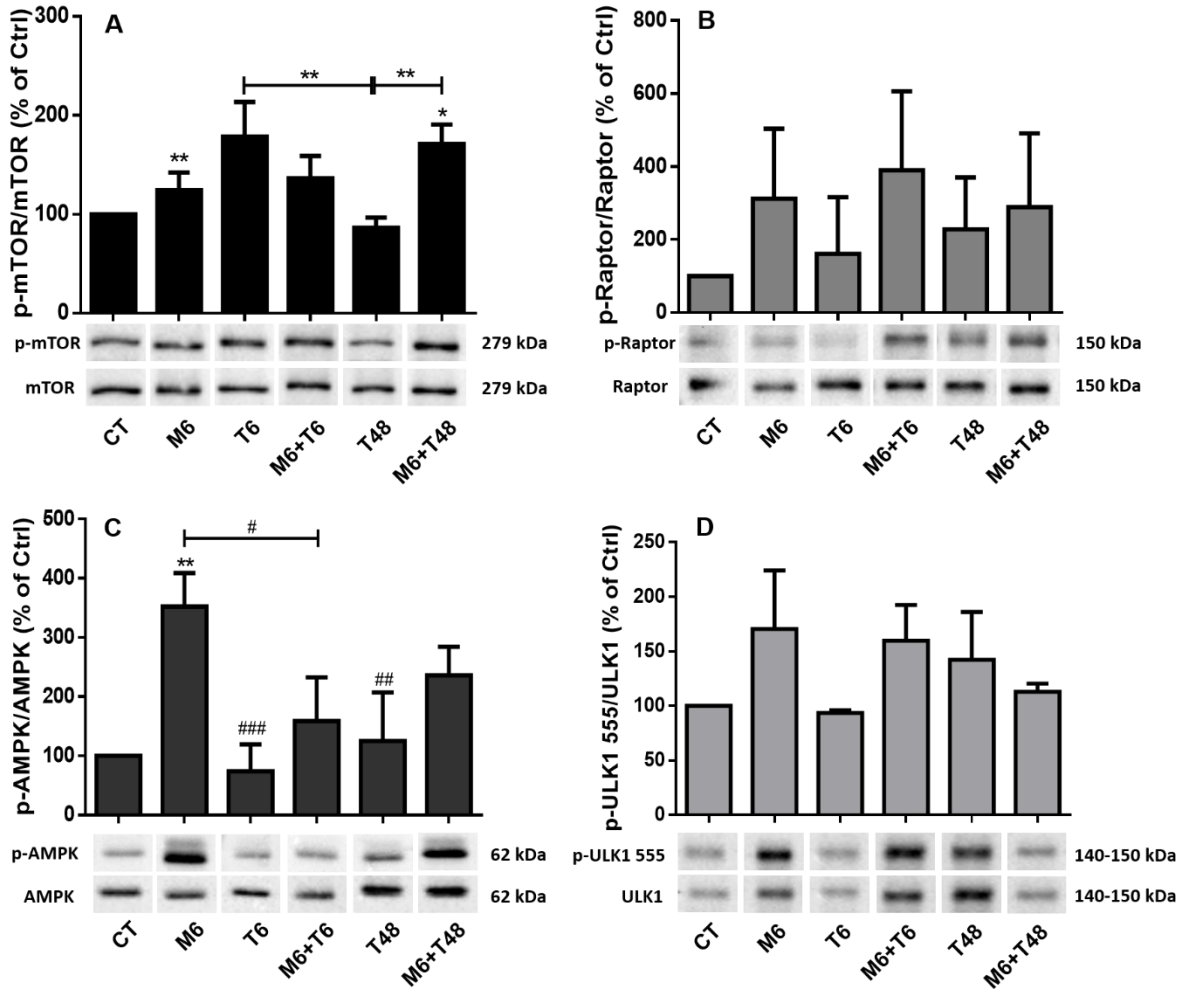

Figure III.2 – mTORC1 proteins phosphorylation levels in response to MPP^+ and TUDCA administration in N2a cells. N2a cells were cultured and treated with MPP^+ and/or TUDCA as previously described in Methods. N2a cells extracts were analysed by Western blot for p-mTOR (A), p-Raptor (B), p-AMPK (C) and p-ULK1 555 (D). Values of expression of the four proteins were obtained through the ratio with respective total protein levels. Intensity of the bands was quantified using image analysis software (Image Lab). Data are expressed as the mean values ± SEM, indicated as percentage of control. Results are representative of two or three independent experiments. CT – cells not treated; M6 – cells treated with MPP^+ for 6h; M6+T6 – cells treated with MPP^+ for 6h, followed by treatment with TUDCA for 6h; M6+T48 – cells treated with MPP^+ for 6h, followed by TUDCA for 48h; T6 – cells treated with TUDCA for 6h; T48 – cells treated with TUDCA for 48h. *p<0.05 vs. Control; **p<0.01 vs. Control; #p<0.05 vs. cells treated with MPP^+ in the absence of TUDCA; ##p<0.01 vs. cells treated with MPP^+ in the absence of TUDCA; ###p<0.01 vs. cells treated with MPP^+ in the absence of TUDCA.

1.2. TUDCA alters autophagy markers expression through time as well as mTORC1 complex proteins, in C57BL/6 mice brain

The results presented so far in N2a cells showed that TUDCA plays an important role in modulation of autophagy, at different time-points We sought to understand if this effect was also observed in animals, specifically in the *striatum*. We started to look at classic autophagy markers. The expression levels of LC3II/I and p62 were evaluated by Western blot using specific antibodies. Mice were treated with only TUDCA, only MPTP, TUDCA plus MPTP or MPTP plus TUDCA and total protein content was extracted from *striatum* samples.

LC3II levels did not vary much from the control, except for the increase observed in animals treated with TUDCA after MPTP exposure for 3 hours, when compared to all the other samples. We observed that with longer MPTP exposure the levels of LC3II decreased, both when TUDCA was administrated before or after MPTP (Figure III.3 – A). However, no statistical significance was achieved for LC3 levels. p62 levels were slightly increased with TUDCA administration and were not altered when mice were exposed to MPTP during 3 hours. Levels were significantly higher when mice were exposed to MPTP during 6 hours when compared to control mice (p<0.05). Pre-treatment with TUDCA before exposure to MPTP for 3 hours lead to a significant increase in p62 levels when compared to mice treated only with MPTP for the same time period (p<0.01). On the other hand, mice treated with MPTP for 3 hours previous to TUDCA administration (M+T 3H) showed a significant decrease in p62 when compared to mice treated with TUDCA before MPTP (T+M 3H) (p<0.05) (Figure III.3 – B).

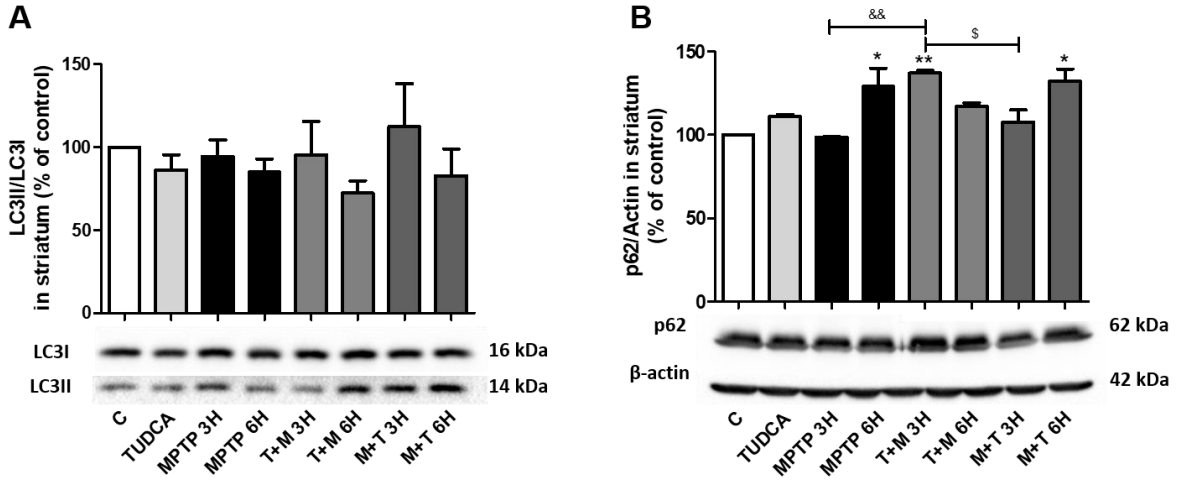

Figure III.3 – Expression levels of LC3II and p62 in the presence of MPTP and TUDCA in C57BL/6 mice striatum. C57BL/6 mice were treated with MPTP and/or TUDCA as indicated in Methods. Tissue extracts from mice striatum were subjected to SDS/PAGE and the corresponding blots were probed with antibodies for p62 (A) or LC3 (B). β-actin was used as a loading control for p62 and values of LC3II were obtained through the ratio with LC3I. Intensity of the bands was quantified using image analysis software (Image Lab). Data is expressed as the mean values ± SEM, indicated as percentage of control. The immunoblots presented are representative of three independent experiments. CT – mice received saline; TUDCA – mice treated with TUDCA during three consecutive days; MPTP 3H, 6H – mice treated with a single dose of MPTP at day one, sacrificed 3 or 6 hours, respectively, after MPTP injection; M+T 3H, 6H – mice received single dose administration of MPTP, for 3h or 6h, respectively,

followed by TUDCA injection during three consecutive days; T+M 3H, 6H – mice received TUDCA injections during three consecutive days, followed by MPTP administration for 3h or 6h, respectively. *p<0.05 vs. Control; **p<0.01 vs. Control; $$p$<0.05 vs. mice treated with TUDCA before MPTP for 3 hours; &&p<0.01 vs. Mice treated with MPTP for 3 hours.

In order to evaluate if the modulation observed in autophagy was done through mTORC1 signalling we decided once again to search for modifications of mTORC1 complex proteins. Therefore, we evaluated the same proteins previously accessed in N2a cells, proteins of the mTORC1 signalling pathway and phospho-ULK1 since its activation/inhibition is a direct consequence of mTORC1 correct functioning.

Levels of phosphorylated mTOR were not altered in the presence of TUDCA alone, with only a slight increase being observed. The same was observed for MPTP administration, at both time periods. However, administration of TUDCA before or after MPTP was able to decrease the levels of pmTOR. In particular, when TUDCA was administered after 6 hours of MPTP exposure, there was a significant decrease in p-mTOR levels when compared to control (p<0.01), TUDCA alone (p<0.001) and to MPTP administrated during 6 hours (p<0.001) (Figure III.4 – A). p-Raptor was particularly increased after MPTP administration during 3 hours, but this increase was attenuated at 6 hours of administration. Interestingly, administration of TUDCA before MPTP exposure for 3 hours was able to significantly decrease p-Raptor levels, when compared to samples of animals exposed only to MPTP for 3 hours (p<0.05). TUDCA was able to decrease p-Raptor levels when administrated before or after MPTP, at both time points. However, levels did not reach statistical significance (Figure III.4 – B). Accordingly with results obtained for p-mTOR, levels of p-AMPK slightly decreased in response to single dose administration of MPTP. When TUDCA was previously administrated we observed a slight increase in p-AMPK levels, but levels peaked when TUDCA was administrated after MPTP. TUDCA administration after treatment with MPTP for 3 hours significantly increased p-AMPK levels when compared to control (p<0.05) and to administration of TUDCA alone (p<0.05). Also, levels of pAMPK were significantly higher in this sample when compared to single administration of MPTP for 3 hours (p<0.01) and compared to treatment with TUDCA before treatment with MPTP for 3 hours (p<0.01) (Figure III.4 – C). Finally, levels of phosphorylated ULK1 were increased with the 3 hours of MPTP treatment and peaked when TUDCA was administered before MPTP at 3 hours. Levels of p-ULK were similar to control in all the other samples, with exception of treatment with MPTP for 6 hours, where we observed a slight decrease (Figure III.4 – D).

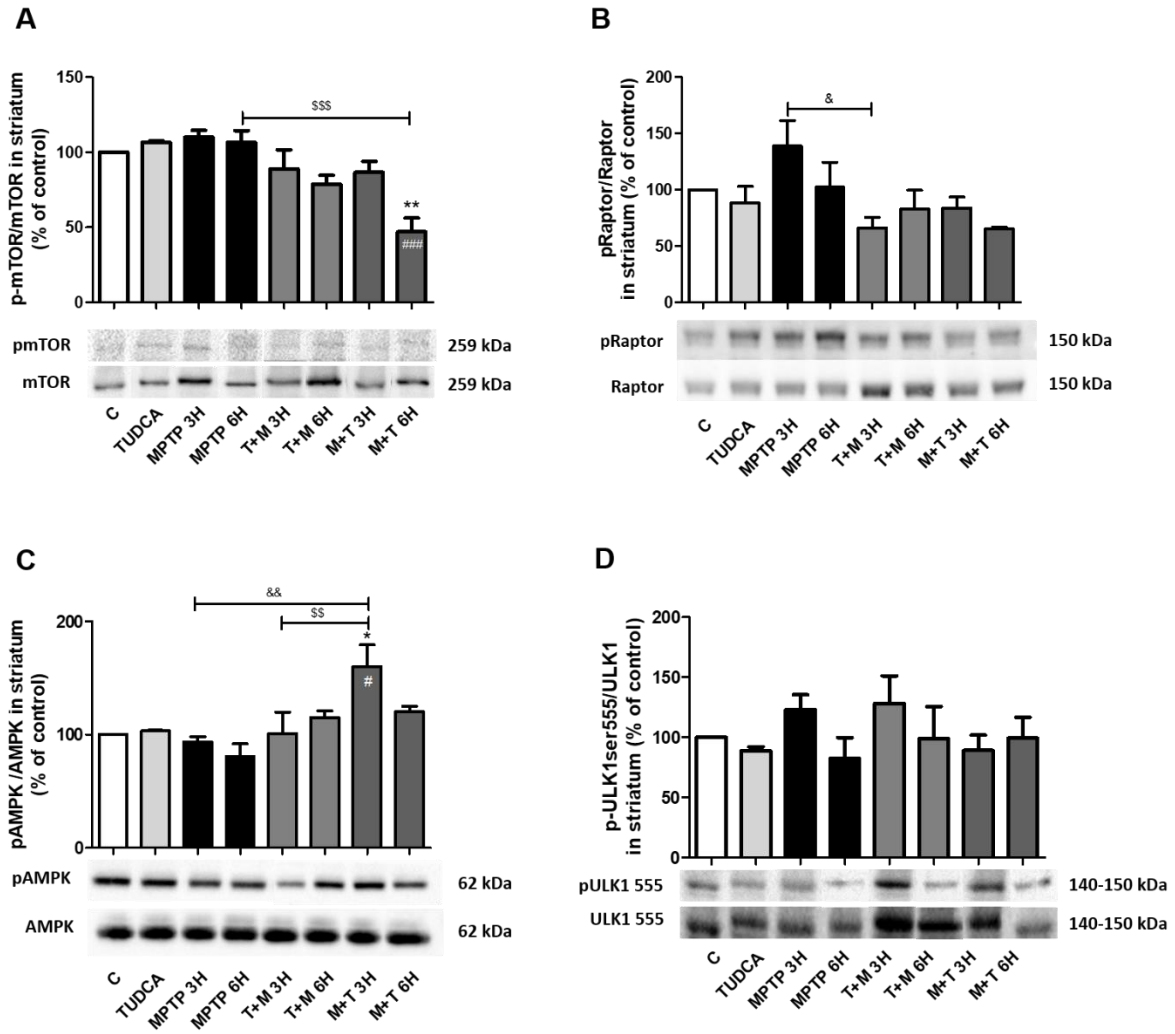

Figure III.4 - mTORC1 proteins phosphorylation levels in response to MPTP and TUDCA administration in C57BL/6 mice striatum. C57BL/6 mice were treated with MPTP and/or TUDCA as indicated in Methods. Tissue extracts from mice striatum were subjected to SDS/PAGE and the corresponding blots were probed with antibodies against p-mTOR (A), p-Raptor (B), p-AMPK (C) and p-ULK1 555 (D). Values of expression of the four proteins were obtained through the ratio with total protein levels. Intensity of the bands was quantified using image analysis software (Image Lab). Data is expressed as the mean values ± SEM, indicated as percentage of control. Results are representative of three independent experiments. . CT – mice received saline; TUDCA – mice treated with TUDCA during three consecutive days; MPTP 3H, 6H – mice treated with a single dose of MPTP at day one, sacrificed 3 or 6 hours, respectively, after MPTP injection; M+T 3H, 6H – mice received single dose administration of MPTP for 3h or 6h, respectively, followed by TUDCA injection during three consecutive days; T+M 3H, 6H – mice received TUDCA injections during three consecutive days, followed by MPTP administration for 3h or 6h, respectively. *p<0.05 vs. Control; **p<0.01 vs. Control; #p<0.05 vs.Mice treated only with TUDCA; ###p<0.001 vs. Mice treated only with TUDCA; $$p<0.01 vs.Mice treated with TUDCA before MPTP for 3 hours; $$$p<0.001 vs. Mice treated only with TUDCA for 3 hours; &p<0.05 vs. Mice treated only with MPTP for 3 hours; &&p<0.01 vs. Mice treated only with MPTP for 3 hours.

2. Evaluation of ER stress and UPR markers in N2a cells and C575BL/6 mouse model

2.1. Expression levels of mediators and downstream effectors of the UPR pathways are altered in N2a cells

After exploring the effects of MPP^+ and/or TUDCA in autophagy's modulation we decided to investigate whether there is a relation between ER stress and autophagy in our model, namely if autophagy might be up-regulated in response to ER stress and if this stress could be relieved with autophagy as a mediator.

As previously described in the introduction section, the precise mechanisms of interplay between oxidative stress and ER stress in the dopaminergic neurons have been sparsely described, although several studies show that ER stress may trigger the production of ROS and redox deviation in the ER (Zeeshan et al., 2016). However, if ER stress plays an important role in oxidative stress and anti-oxidant elicited neuronal response is currently unknown. To better understand this relation, treated N2a cells with MPP^+ in order to induce oxidative stress and/or TUDCA, a chemical chaperone, to understand if that oxidative stress response could be attenuated. We analysed the expression levels of two of the UPR branches, as well as two of their main downstream effectors.

Levels of UPR related proteins were evaluated by Western blot analyses, using specific antibodies against p-PERK, p-eIF2α, ATF4 and p-IRE1α. Our results demonstrated that levels of p-PERK are increased with MPP^+ administration and that the increased levels of this protein caused by MPP^+ administration are reverted when cells are exposed to TUDCA, both at 6 and 48 hours. Almost no differences were observed with single TUDCA administration, with a slight increase at 6 hours of exposure, when comparing to control (Figure III.5 – A). Surprisingly, phosphorylation levels of eIF2α were not altered in most of the conditions tested, with only an observable increase when cells were treated with MPP^+ and TUDCA during 6 hours (Figure III.5 – B). Despite the lack of differences observed in p-eIF2α, at the time points tested, ATF4 protein expression is highly increased with MPP^+ exposure and this increase seems to be reverted by exposure to TUDCA, both in short and longer time periods. Single TUDCA administration does not seem to alter ATF4 levels, with a slight increase when cells are exposed for 48 hours. (Figure III.5 – C). Last, phosphorylation levels of IRE1a were assessed, in order to evaluate another UPR branch. Levels of IRE1a phosphorylation were not altered in most of the conditions, only with a noticeable decrease of protein levels at 48 hours of TUDCA exposure. (Figure III.5 – D).

Although results in N2a cells experiments did not turned out to be conclusive, due to lack of statistical significance, previous results obtained in our lab showed that MPTP and TUDCA were able to induce differences in proteins involved in the UPR, namely JNK and Nrf2, so we decided to explore even further changes associated with ER stress using an *in vivo* PD model, the C57Bl/6 mice treated with MPTP.

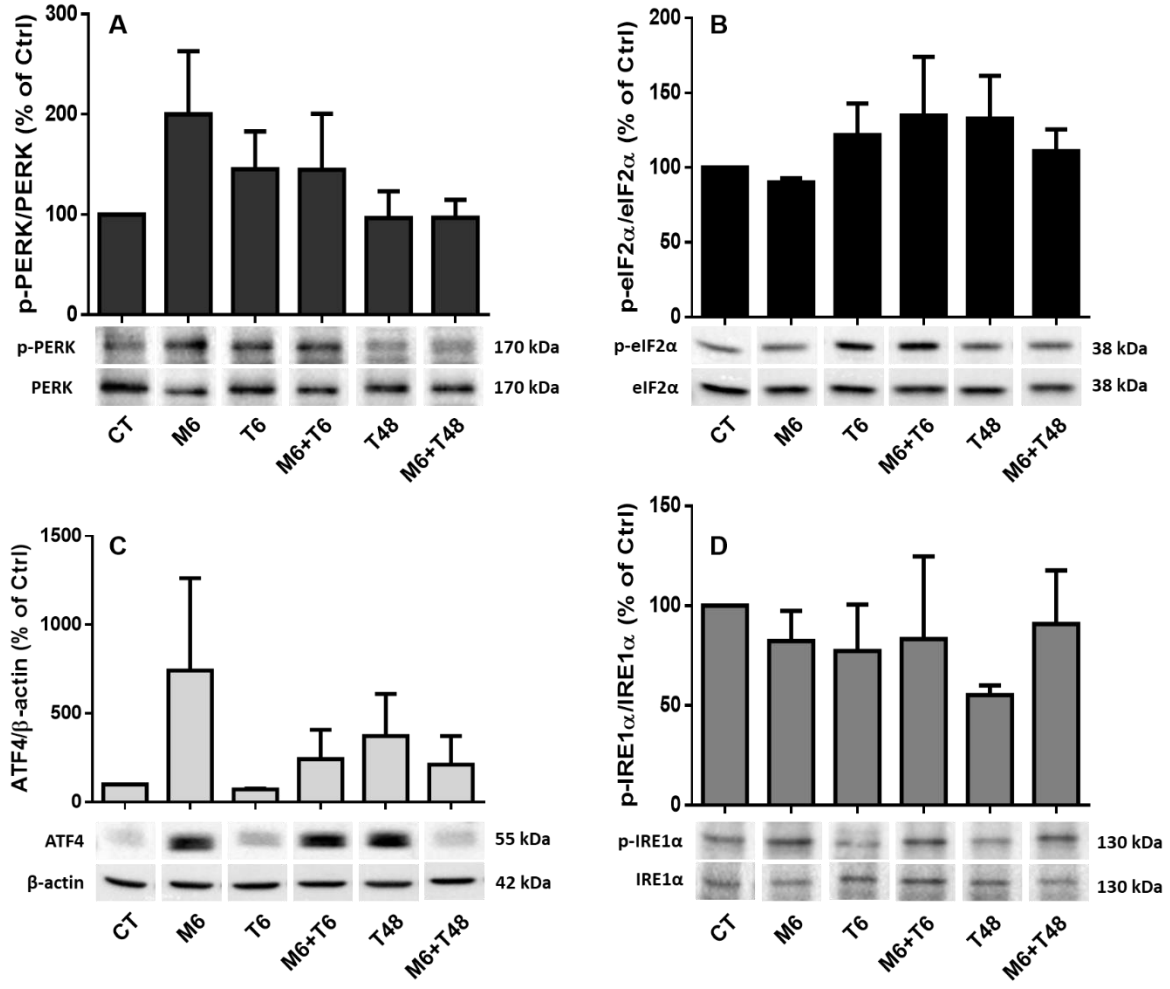

Figure III.5 – UPR related proteins expression levels in N2a cells after treatment with MPP^+ or TUDCA. N2a cells were cultured and treated with MPP^+ and/or TUDCA as previously described in Methods. N2a cells extract was subjected to SDS/PAGE and the corresponding blots were probed with antibodies for p-PERK (A), p-eIF2α (B), ATF4 (C) and p-IRE1a (D). Values of expression of the four proteins were obtained through the ratio with respective total protein levels. Intensity of the bands was quantified using image analysis software (Image Lab). Data is expressed as the mean values ± SEM, indicated as percentage of control. The immunoblots presented are representative of two or three independent experiments. CT – cells not treated; M6 – cells treated with MPP^+ for 6h; M6+T6 – cells treated with MPP+ for 6h, followed by treatment with TUDCA for 6h; M6+T48 – cells treated with MPP^+ for 6h, followed by TUDCA for 48h; T6 – cells treated with TUDCA for 6h; T48 – cells treated with TUDCA for 48h.

2.2. TUDCA regulates mRNA levels as well as proteins expression of ER stress related factors in C57BL/6 mice brain

The results presented so far demonstrated that the administration of MPP^+ in N2a cells seems to affect the levels of expression of proteins that are up-regulated in the presence of ER stress, providing evidence that the activation of the UPR probably plays a role in relieving the stress caused by this toxic metabolite. To further understand if this modulation of the UPR started upstream of the pathway or if it is a downstream process, total RNA was extracted from striatum samples of mice treated with TUDCA, MPTP, TUDCA plus MPTP or MPTP plus TUDCA and BiP, XBP1 and CHOP mRNA levels were quantified by qRT-PCR. All three genes were normalized for the expression of the housekeeping gene EEF, using the ΔΔCt method.

BiP mRNA levels were higher when mice were treated with MPTP during 1 hour but mRNA levels decreased when mice were treated with MPTP during 3 or 6 hours. Administration of TUDCA before MPTP treatment seemed to decrease levels of BiP mRNA at the three time points, with a less attenuated decrease observed at 3 hours of MPTP exposure. Similarly, BiP mRNA levels were also decreased when TUDCA was administered after MPTP exposure, at the three time-points (Figure III.6 – A). However, no statistical significance was achieved. CHOP mRNA levels were significantly decreased when TUDCA was administered after MPTP at 1 hour ($p<0.05$), 3 hours ($p<0.01$) and 6 hours ($p<0.001$) when compared to control and values of CHOP mRNA were even significantly lower than mice treated with MPTP for 1 hour ($p<0.05$), 3 hours ($p<0.05$) and 6 hours ($p<0.01$) (Figure III.6 – B). Last, XBP1 mRNA levels peaked when mice were treated with MPTP during 1 hour with a significantly decrease of mRNA levels when mice were previously treated with TUDCA ($p<0.01$) and when mice were injected with TUDCA after MPTP ($p<0.05$). Administration of TUDCA before or after MPTP at 3 or 6 hours slightly decreases XBP1 mRNA levels (Figure III.6 – C). However, no statistical significance was achieved in this time-points.

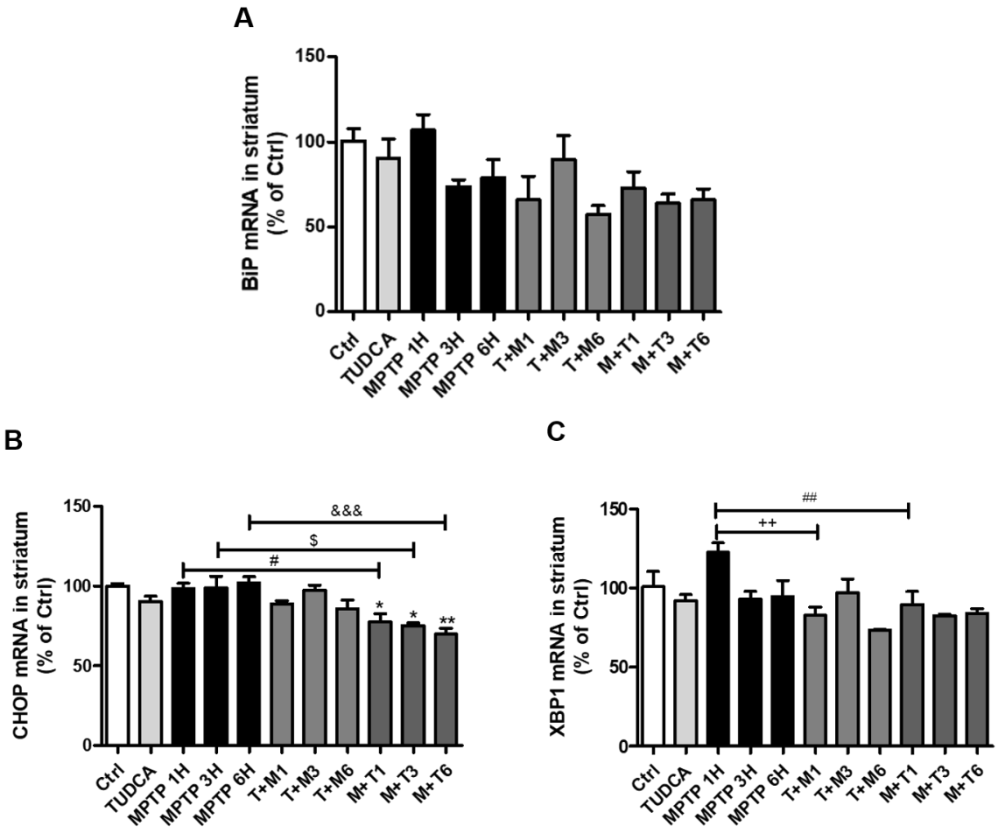

Figure III.6 - TUDCA modulates mRNA levels of ER stress related factors in C57BL/6 mice striatum. C57BL/6 mice were treated as indicated in Methods. Total RNA was extrated from striatum samples and levels of CHOP, XBP1 and BiP mRNA were determined by quantitative real-time PCR. All three genes were normalized for the expression of the housekeeping gene EEF, using the ΔΔCt method. Results are representative of 3 independent measures. Ctrl – mice received saline; TUDCA – mice treated with TUDCA during three consecutive days; MPTP 1H, 3H, 6H – mice treated with a single dose of MPTP at day one, sacrificed 1, 3 or 6 hours, respectively, after MPTP injection; M+T 1H, 3H, 6H – mice received single dose administration of MPTP for 1h, 3h or 6h, respectively, followed by TUDCA injection during three consecutive days; T+M 1H, 3H, 6H – mice received TUDCA injections during three consecutive days, followed by MPTP administration for 1h, 3h or 6h, respectively. *p<0.05 vs. Control; **p<0.01 vs. Control; ***p<0.001 vs. Control; #p<0.05 vs. MPTP 1 hours; ## p<0.01 vs. MPTP 1 hour; $$p$<0.05 vs. MPTP 3 hours; ++p<0.01 vs.MPTP 1 hour; &&&p<0.001 vs MPTP 6 hours.

After assessing the mRNA levels of this UPR related factors, given that the higher and significant changes were observed in the downstream effectors of the UPR we decided to look at the levels of protein expression of the three main mediators of the UPR, as well as some important downstream effectors, in order to understand if this pattern was replicated.

We decided to focus on the time periods of 3 and 6 hours, since 1 hour seemed a too short period of time to detect differences in protein expression. First, levels of protein expression were obtained by Western blot analysis using specific antibodies for p-PERK, p-IRE1α and ATF6. Phosphorylation levels of PERK increased at both time periods, with a higher increase at 3 hours of exposure. Administration of TUDCA before or after 3 hours MPTP exposure decreased the levels of activated PERK, when compared to single dose administration of MPTP. However, at 6 hours of MPTP

exposure TUDCA administration did not decrease the levels of p-PERK but actually increased this protein levels when administrated after MPTP (Figure III.7 – A). Although, no statistical significance was achieved. In contrast to PERK, phosphorylation levels of IRE1α were not increased with MPTP single administration during 3 and 6 hours, but in fact increased when TUDCA was administrated before treatment with MPTP during 3 hours. At 6 hours, TUDCA seems to decrease the levels of activated IRE1α when administrated after MPTP (Figure III.7 – B). However, no statistical significance was found. Last, levels of cleaved ATF6 were accessed and an accentuated peak at 3 hours of MPTP treatment, when TUDCA was previously administrated (Figure III.7 – C). Again, no statistical significance was found.

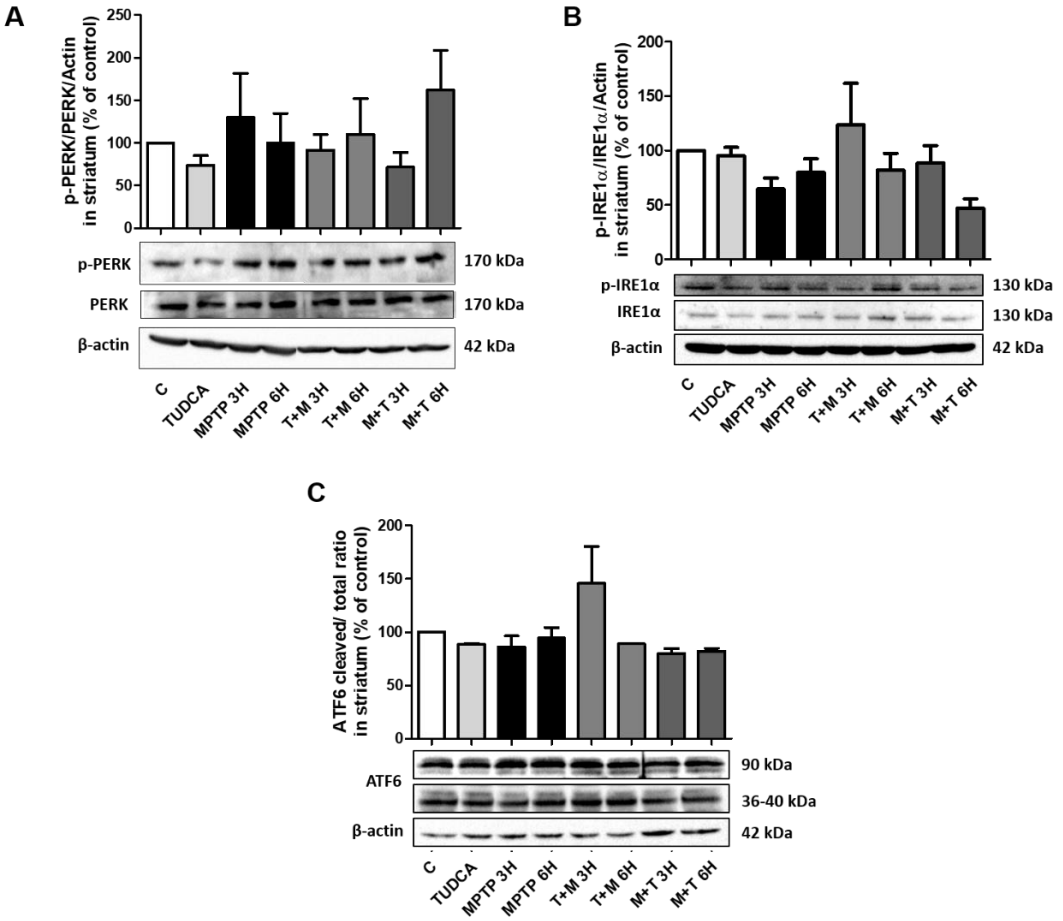

Figure III.7 - Protein expression levels of the three main initiators of UPR response. C57BL/6 mice were treated with MPTP and/or TUDCA as indicated in Methods. Tissue extracts from mice striatum were subjected to SDS/PAGE and the corresponding blots were probed with antibodies for p-PERK (A), p-IRE1a (B) or ATF6 (C). Values of the three proteins were obtained through the ratio with respective total protein levels. Intensity of the bands was quantified using image analysis software (Image Lab). Data is expressed as the mean values ± SEM, indicated as percentage of control. The immunoblots presented are representative of three independent experiments. . CT – mice received saline; TUDCA – mice treated with TUDCA during three consecutive days; MPTP 3H, 6H – mice treated with a single dose of MPTP at day one, sacrificed 3 or 6 hours, respectively, after MPTP injection; M+T 3H, 6H – mice received single dose administration of MPTP for 3h or 6h, respectively, followed by TUDCA injection during three consecutive days; T+M 3H, 6H – mice received TUDCA injections during three consecutive days, followed by MPTP administration for, 3h or 6h, respectively.

After assessing the alterations in the expression of these three transmembrane proteins, we decided to investigate if the downstream effectors of the UPR were affected in the same way. Levels of protein expression were obtained by Western blot analysis using specific antibodies against p-eIF2α and CHOP. Levels of phosphorylation of eIF2α were significantly higher in animals treated with MPTP during 3 hours, after TUDCA administration, when compared to control mice ($p<0.05$) and to mice treated with TUDCA only ($p<0.01$). When mice were treated first with MPTP and afterwards with TUDCA, levels of inactivated eIF2α significantly decreased at 3 hours when compared to mice treated with TUDCA before MPTP ($p<0.01$). Surprisingly, animals treated with MPTP during 6 hours after TUDCA administration showed lower levels of protein when compared to animals treated during 3 hours ($p<0.01$) (Figure III.8 – A). CHOP expression levels were significantly higher in animals treated with MPTP during 6 hours when compared to control and mice treated only with TUDCA ($p<0.01$). Animals treated with TUDCA prior to MPTP showed a significantly increase when compared to control at 3 hours ($p<0.001$) and 6 hours ($p<0.01$) as well as when compared to TUDCA ($p<0.001$ and $p<0.01$, respectively). We also observed a significant increase in CHOP levels in mice treated with TUDCA before MPTP for 3 hours when compared to mice treated only with MPTP 3 hours ($p<0.01$) and a significant decrease when the order of treatments was reversed, at 3 hours ($p<0.05$). Last, levels of CHOP expression significantly decreased in mice treated with TUDCA after MPTP 6 hours, both when compared to mice treated only with MPTP 6 hours ($p<0.01$) and mice treated in the reverse order (Figure III.8 – B).

Taken together these results show that the modulation done by MPTP and TUDCA is more proeminent in the UPR downstream effectors than upstream of the pathway.

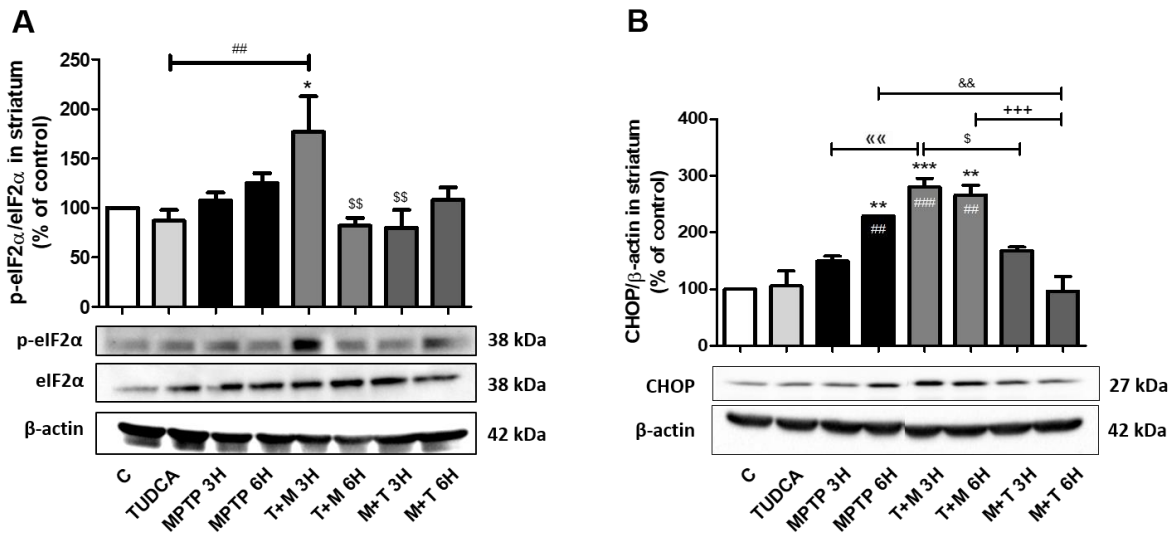

Figure III.8 – TUDCA and MPTP modulates the levels of UPR downstream effectors. C57BL/6 mice were treated with MPTP and/or TUDCA as indicated in Methods. Tissue extracts from mice striatum were subjected to SDS/PAGE and the corresponding blots were probed with antibodies against p-eIF2α (A) and CHOP (B). Values of peIF2α were obtained through the ratio with total protein levels and values of CHOP were normalized for β-actin. Intensity of the bands was quantified using image analysis software (Image Lab). Data are expressed as the mean values ± SEM, indicated as percentage of control. The immunoblots presented are representative of three independent experiments. C – mice received saline; TUDCA – mice treated with TUDCA during three consecutive days; MPTP 3H, 6H – mice treated with a single dose of MPTP at day one, sacrificed 3 or 6 hours, respectively, after MPTP injection; M+T 3H, 6H – mice received single dose administration of MPTP for 3h or 6h, respectively, followed by TUDCA injection during three consecutive days; T+M 3H, 6H – mice received TUDCA injections during three consecutive days, followed by MPTP administration for 1h, 3h or 6h, respectively. *p<0.05 vs. Control; **p<0.01 vs. Control; ***p<0.001 vs. Control; ##p<0.01 vs. Mice treated with TUDCA; ###p<0.001 vs. Mice treated with TUDCA; \$$p$<0.05 vs. Mice treated with TUDCA before MPTP administration for 3 hours; \$\$$p$<0.01 vs. . Mice treated with TUDCA before MPTP administration for 3 hours &&p<0.01 vs. Mice treated with MPTP for 6 hours; ««p<0.01 vs.Mice treated with MPTP for 3 hours; +++p<0.001 vs. Mice treated with TUDCA before MPTP administration for 3 hours.

3. GSTp may play an important role in TUDCA potential neuronal defense

3.1. TUDCA modulates GSTp expression levels in N2a cells

Previous studies in our group showed that pre-treatment with TUDCA attenuates the deleterious effects of MPTP especially by the blockage of ROS production and JNK activation, together with the activation of pro survival mediators (Castro-Caldas et al., 2012b). Also, our group showed as well that another mechanism underlying TUDCA neuroprotection is through modulation of mitophagy (Rosa et al., 2017). In order to understand if this protection observed and the antioxidant effects of TUDCA could be derived from the up regulation of antioxidant enzymes, we investigated if the levels of GSTp were altered in the presence of TUDCA and if TUDCA was able to increase the levels of this enzyme in the presence of oxidative stress.

With that purpose, we treated N2a cells with TUDCA, MPP^+ and tunicamycin, a classic ER stress inducer, alone and in combination with TUDCA and evaluated the expression levels of GSTp by Western blot. Preliminary results indicate that TUDCA alone is able to increase the levels of GSTp and that in the presence of MPP^+ or tunicamycin TUDCA was able to increase levels of the enzyme, especially in the presence of tunicamycin (Figure III.9). However, to further confirm this hypothesis we would need to increase the number of independent experiments, which was not possible at the moment. We also performed immunohistochemistry for GSTp in treated cells and interestingly we observed that MPP^+ and tunicamycin altered the cell morphology, namely by reducing ramifications (Figure III.10 -A and III.10 – B, respectively) and that TUDCA was able to partially revert this effect.

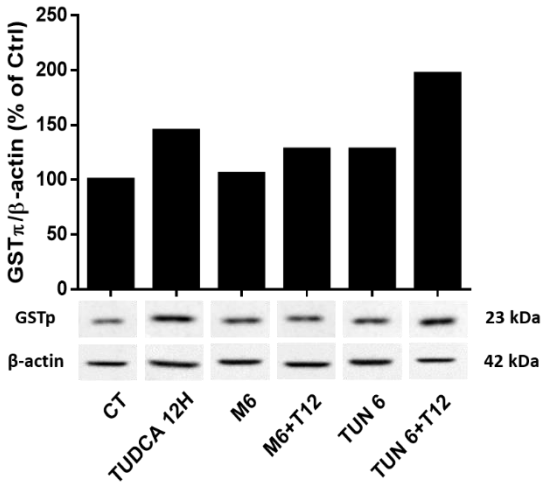

Figure III.9 – GSTp levels are altered in the presence of TUDCA in N2a cells. N2a cells were cultured and treated with MPP^+ and/or TUDCA as well as tunicamycin alone or with TUDCA as previously described in Methods. N2a cells extracts were analysed by Western blot using a specific antibody against GSTp and results were normalized for -βactin.. Intensity of the bands was quantified using image analysis software (Image Lab). Results are preliminary and representative of one independent experiment. CT-cells not treated; M6 – cells treated with MPP^+ during 6 hours; M6+T12 – cells treated with MPP^+ 6 hours before TUDCA during 12 hours;TUDCA 12H – cells treated with TUDCA during 12 hours; TUN 6 – cells treated with tunicamycin during 6 hours; TUN6+T12 – cells treated with tunicaycin 6 hours before TUDCA 12 hours.

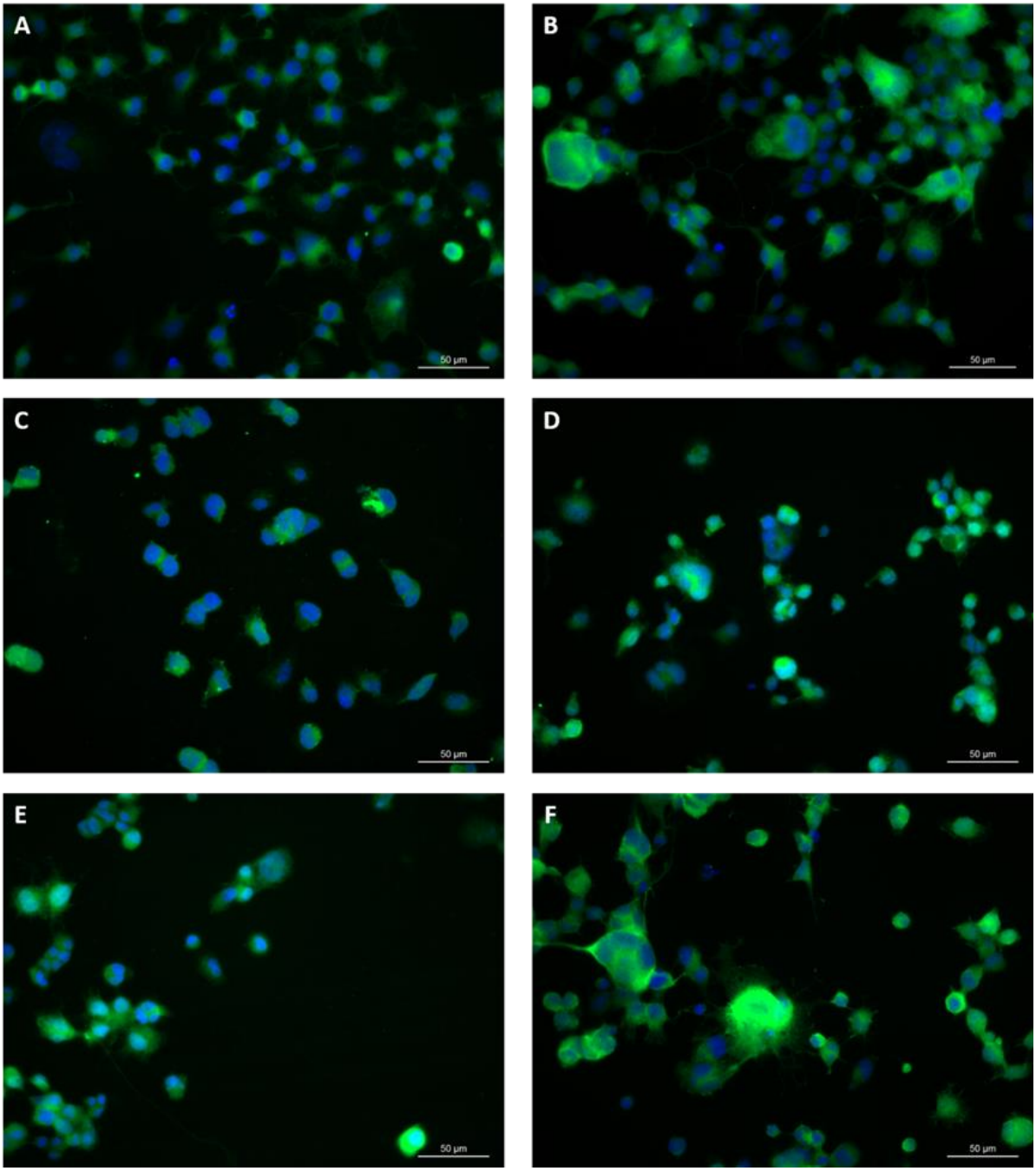

Figure III.10 – GSTp fluorescence in N2a cells treated with MPP^+, TUDCA and Tunicamycin. N2a cells were cultured and not treated (A), treated with TUDCA 12 hours (B), treated with MPP^+ for 6 hours (C) treated with MPP^+ for 6 hours followed by TUDCA 12 hours (D) or tretated wih tunicamycin 6 hours (E) or with tunicamycin 6 hours followed by TUDCA 12 hours (F) as described in Methods. Cells were immunoe stained for GSTp (green) and DAPI was used as a nuclear marker (blue). Microphotograps shown are representative of three independent experiments. Scale bar=50 µm.

3.2. CHOP mRNA levels are altered in GSTp KO C57BL/6 mice midbrain

Results previously obtained in our lab showed that MPTP-induced dopaminergic neuronal degeneration is an earlier event when comparing GSTp null (KO) vs wild-type (WT) mice, suggestive of a protective role for GSTp (Castro-Caldas et al., 2012a). Therefore, our future work will be focused on the study of this protective role, namely in trying to understand if it is related with regulation of ER stress and/or autophagy pathways. Differences in protein expression levels of several UPR related factors were already found in cortex of C57BL/6 GSTp KO mice, when comparing to WT mice (data published in Santos MA Master Thesis, 2015).

In order to investigate if this effect affected specific brain regions affected by PD, total RNA was extracted from midbrain samples of control WT mice and control KO mice and CHOP, BiP and XBP1 mRNA levels were quantified by qRT-PCR. All three genes were normalized for the expression of the housekeeping gene EEF, using the ΔΔCt method. Results showed a significant increase (p<0.05) in mRNA CHOP levels in KO mice, when compared to control (Figure III.11– A) and a slight increase in BiP mRNA levels also in KO mice, however no statistical significance was observed (Figure III.11 – B). Changes between WT and KO in XBP1 mRNA levels were not observed (Figure III.11 – C).

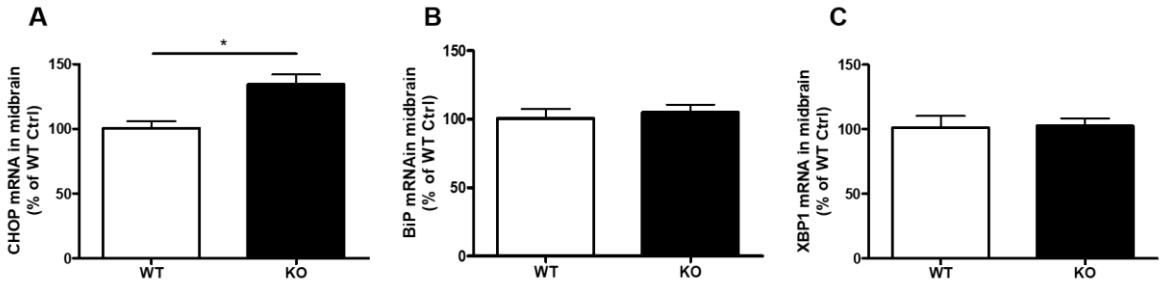

Figure III.11 – mRNA levels of CHOP, BiP and XBP1 in WT and GSTp KO mice in the midbrain. C57BL/6 KO mice were obtained as indicated in Methods. Total RNA was extrated from midbrain samples and levels of CHOP (A), BiP (B) and XBP1 (C) mRNA were determinated by quantitative real-time PCR. All three genes were normalized for the expression of the housekeeping gene EEF, using the ΔΔCt method. Results are representative of 4 independent measures. *p<0.05 vs. wildtype

IV. DISCUSSION

In this thesis, we aimed to understand the alterations caused by MPTP in two important pathways: the UPR and autophagy. Given that MPTP mimics the phenotype of PD the characterization of those pathways in this model will allow to better understand their involvement in PD and to find new possible therapeutic targets. Moreover, it is important to explore the relation between those pathways, understanding if the previously established connections between the UPR and autophagy in the context of other pathologies are similar in PD (section I – 3.2). In parallel, given the previously described potential of TUDCA as a neuroprotector and antioxidant, we evaluated the effects of this endogenous bile acid in both cellular pathways.

First, we wanted to assess if cells and animals treated with MPP^+/MPTP showed an increase in autophagy levels, since it was previously described that MPTP induced autophagy in monkeys' brains. (Su et al., 2015). Accordingly, our results showed that MPP^+ induced autophagy in N2a cells, observable by an increase in LC3II levels, possibly translating an increase in phagosome formation. Levels of p62 did not decrease in comparison to control, however this can be partially explained by the dual role of p62 in autophagy, functioning both as a substrate and as an upstream regulator of autophagy. Surprisingly and in contrast with the previous results, MPP^+ increased the phosphorylation levels of mTOR. On the other hand, levels of pAMPK considerably increased, which can be explained by the decrease in ATP levels caused by MPP^+ treatment. Since AMPK is phosphorylated when there is a high ratio of ADP/ATP it is reasonable to expect that the levels of p-AMPK are increased in the presence of this toxic, as in fact we observed. Levels of p-Raptor and p-ULK1 are also increased in the presence of MPP^+, supporting the idea that MPP^+ induces autophagy. Cells treated with TUDCA during 6 hours seem to have an increase in autophagy represented by the increase in LC3II levels. However, the expression levels of this protein decrease when cells are treated with TUDCA for longer time periods. Also, when cells are treated with TUDCA after MPP^+, the addition of TUDCA leads to a decrease in LC3II levels, especially at 48 hours. These results are better understood when we look at mTORC1 protein expression. TUDCA increased the levels of p-mTOR at 6 hours but decreased them at 48h, pointing to an increase in autophagic response at 48 hours. Levels of p-AMPK also did not increase in the presence of TUDCA alone at both time periods and TUDCA decreased the levels of pAMPK in the presence of MPP^+, when compared to cells treated only with MPP^+. This can be explained by the fact that TUDCA prevents ATP depletion, decreasing the ratio ADP/ATP (Xavier et al., 2014). Levels of p-Raptor and p-ULK1 show a similar pattern to p-AMPK, with a deviation at 48 hours of TUDCA exposure, when levels of p-ULK1 are high. These results suggest that the modulation of autophagy via mTORC1 complex occurs only for longer time periods of treatment with TUDCA (48 hours), in N2a cells. Nevertheless, autophagy is still activated since an increase of LC3II levels at that time period is observed (6 hours). Also, the decrease in autophagy levels when in the presence of MPP^+ and TUDCA could be related to the fact that when autophagy is over stimulated it can have a harmful effect to the cell and stimulate cell death by apoptosis (Cai et al., 2016).

Regarding our results in C57BL/6 mice, we observed that the levels of p62 peaked at 6 hours following MPTP exposure, both in the presence or absence of TUDCA. Like previously said, p62 has a dual role in autophagy (Rusten & Stenmark, 2010). Results obtained for p62 and LC3 in mice can be confusing, once, for example, both LC3 and p62 are increased in mice treated with TUDCA before MPTP for 3 hours. Taking into account that the remaining results seem to show that autophagy increases with longer MPTP treatment, we can deduce that levels of p62 are increased at longer MPTP time periods in order to stimulate autophagy and not as a signal that autophagy is compromised. High levels of p62 at 3 hours of TUDCA treatment may be explained by the close relation between p62 and Nrf2 (Jiang et al., 2015). The relation between p62 and Nrf2 was first described in 2007 by (Liu et al., 2007) but only recently this connection was fully understood. p62 is able to link to Keap1 and this interaction allows p62 to sequester Keap1 into the autophagosomes, which impairs the ubiquitylation of Nrf2, leading to activation of the Nrf2 signalling pathway (Jiang et al., 2015). In fact, our group have already shown that pre-treatment with TUDCA positively modulates Nrf2 protein levels in both *striatum* and *midbrain* of C57BL/6 mice (Moreira et al., 2017). Therefore, we think that one of the reasons why there is an increase in p62 might be the need to up-regulate Nrf2 expression in order to increase the amount of antioxidant proteins to relieve the cellular stress. In contrast to N2a cells, MPTP did not increase the levels of p-AMPK in the striatum of C57BL/6 mice. We also observed that the administration of TUDCA after MPTP insult is more efficient in inducing autophagy than the pre-treatment with TUDCA. This may be related to the capacity of TUDCA to act as a scavenger/chaperone protecting cells in response to a toxic stimulus.

Concerning the UPR modulation it has been shown that ATF6α and PERK/eIF2α/ATF4 pathways are activated in mice nigrostriatal dopaminergic neurons upon treatment with MPTP (Hashida et al., 2012). Our results regarding N2a cells showed that administration of MPP^+ increased levels of p-PERK and ATF4, however levels of p-eIF2α were not altered at the time periods analysed. Animals treated with MPTP showed an increase in levels of phosphorylated PERK and in the downstream effectors of PERK, namely p-eIF2α and CHOP, and this increase was more pronounced with the longer time of exposure to MPTP. Although our results regarding the PERK pathway are in accordance with what was previously described, we were not able to detect any differences in ATF6 protein expression in MPTP treated mice. However, mRNA levels of XBP1 are increased in animals injected with MPTP after 1 hour of exposure, what might indicate that modulation of the ATF6 pathway by MPTP occurs downstream in the pathway. Considering the IRE1α branch of the UPR, our results suggest that this pathway may not be involved in the ER-stress response to MPTP exposure, once levels of this protein are not increased compared to control. This result is in accordance with previously described work (Sado et al., 2009).

Administration of TUDCA after MPTP reduced the levels of CHOP mRNA and protein expression of eIF2α and CHOP, especially when animals were exposed to MPTP during longer time periods. Levels of ATF6 were not altered with TUDCA administration, indicating that the ATF6 pathway is probably not the main branch of the UPR involved in the protective response against MPTP. p-PERK expression levels increased when animals were exposed to MPTP during 6 hours before TUDCA administration. These results are very interesting suggesting that the neuroprotective properties of TUDCA are also

effective after the toxic insult, which is important for long term progressive diseases namely PD, to prevent further degeneration of remaining neurons.

Ideally neuroprotective agents should be administered before the first symptoms of the disease appear (Emborg, 2004). This is extremely important when it comes to PD, since clinical diagnosis only occurs when the degenerative process has already been triggered and about 60-80% of DA neurons have already die. Our results regarding previous administration of TUDCA in mice injected with MPTP (T+M) showed an increase in p-IRE1α, ATF6, p-eIF2α and CHOP levels. Previous studies showed that the eIF2α/ATF4 pathways is up-regulated in the presence of stress in order to initiate autophagy (B'Chir et al., 2013). Therefore, the observed increase at 3 hours of treatment (T+M 3H) may be in part explained by the triggering of autophagy. TUDCA seems to increase UPR related factors as a first response to MPTP insult, in order to further activate autophagy. In most cases, both mRNA levels and protein expression of UPR factors tend to decrease with longer MPTP exposure after TUDCA treatment, probably because autophagy was triggered as a mediator to relieve ER stress.

In this thesis, we also aimed to evaluate if TUDCA was altering the expression levels of GSTp. Our results, although preliminary, showed that TUDCA indeed increased the levels of GSTp. In the presence of an insult to the cells, in this case represented by MPP^+ and tunicamycin, the levels of GSTp are highly induced in the presence of TUDCA. The increased expression of GSTp is probably related with the activation of the Nrf2 signalling pathway by TUDCA, once the up-regulation of Nrf2 increases the expression and/or activity of cytoprotective and antioxidant enzymes (Carvalho et al., 2016; Moreira et al., 2017). Nrf2 controls the cellular oxidant level and antioxidant signalling by regulating the expression of three groups of ARE-dependent genes: drug metabolizing enzymes/transporters, oxidant signalling proteins and antioxidant enzymes/proteins, GSTp fitting in this last category (Moreira et al., 2017). In the presence of MPP^+ and tunicamycin, ER stress inducers, the activation of the UPR leads to an activation of Nfr2 that probably involves PERK. PERK phosphorylates Nrf2 on Thr-80 to activate Nrf2 and induce ARE genes, which increase GSH level, reduce ROS in ER, and promote survival (Ma, 2013).We propose that Nrf2 is responsible for the up-regulation of GSTp by TUDCA. Importantly, we have already demonstrated that GSTp is able to induce Keap1 S-glutathionylation, up-regulating the levels of Nrf2 (Carvalho et al., 2016). This shows that not only Nrf2 can up-regulate GSTp expression levels but also GSTp is able to increase Nrf2 expression levels.

One interesting result regarding N2a cells and GSTp expression is the altered morphology observed in the presence of MPP^+ and tunicamycin. When cells were exposed to these molecules there seems to be a reduction in the number of axon-like processes. However, TUDCA is able to revert this process when added after the toxic. Previous studies have already shown that some substances were able to restore this phenotype but no studies have been done with TUDCA (De Girolamo et al., 2001) Further studies need to be done in order to understand by which molecular mechanisms is TUDCA reverting this phenotype, and future implications in neuronal recovery.

Preliminary experiments analysing the levels of factors implicated in the UPR comparing control WT to control GSTp KO mice showed that the lack of GSTp by itself is able to increase levels of CHOP, although mRNA levels of other factors does not seem to be altered (Carvalho et al, unpublished data). This increase in CHOP levels may be an indicative that GSTp, being an antioxidant enzyme plays an

important role in the UPR, especially in the PERK branch. However, high levels of CHOP may also be an indicator of apoptosis once under prolonged ER stress, PERK signalling can trigger cell death. In fact, the artificial sustained activation of PERK, but not IRE1α, induces proliferation arrest and cell death in certain cell models, especially mediated through CHOP (Urra et al., 2013). Like previously mentioned, results obtained by the group showed differences in several UPR pathways in the cortex of GSTp KO mice (Santos MA Master thesis, 2015). Future work should focus on expanding this work to *midbrain* and *striatum*, once these are the main areas affected by PD and to understand if the modulation of pathways involving GSTp could be a new future target to PD treatment.

Although speculative, we may conclude that TUDCA modulates ER stress by stimulation of UPR pathways as an early response and by stimulation of autophagy when ER stress is persistent. Regarding TUDCA time of exposure, we conclude that, in early stages, it mediates autophagy in a mTORC1 independent manner, but that this complex is later activated in order to stimulate autophagy. Taken together, all the information about TUDCA as a neuroprotective and antioxidant agent that efficiently crosses the BBB with no associated toxicity and the work presented in this thesis, it is plausible to think in TUDCA as a potential therapeutic agent in PD.

In the future, it will be important to better understand and classify the interplay of UPR and autophagy in the pathogenesis of PD, and explore possible novel therapeutic targets to reduce the oxidative load in DA neurons that may be beneficial in slowing the progression of this neurodegenerative disease. Also, it will be important to observe if the lack of GSTp in KO mice will alter the response observed in the presence of MPTP, both in autophagy and UPR.

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ANNEXES

A - Protocol for Trizol extraction of total RNA from tissue samples

1. Lyse tissues by the addition of 750 µl of Trizol reagent for 50-100 mg of tissue;

2. Homogenize samples and centrifuge at 12000 x g for 10 minutes at 4ºC;

3. Transfer the supernatant for a new eppendorf and incubate the homogenized sample for 5 minutes;

4. Add 200 µl of chloroform for each 1 ml of Trizol used for homogenization;

5. Vortex and incubate the tubes at room temperature for 2-3 minutes;

6. Centrifuge at 12000 x *g* for 15 minutes;

7. Recover and transfer the aqueous phase to a fresh tube;

8. Precipitate the RNA with 500 µl of isopropanol for each 1 ml of Trizol used for homogenization;

9. Incubate the samples for 10 minutes at room temperature;

10. Centrifuge at 12000 x *g* for 10 minutes at 4ºC;

11. Remove the supernatant and wash the RNA pellet by adding 1 ml of etanol 75% for each 1 ml of Trizol used for homogenization;

12. Vortex and centrifuge at 7500 x *g* for 5 minutes at 4ºC;

13. Briefly air-dry the final RNA pellets and dissolve in DEPC treated H_2O;

14. Incubate at 55ºC for 10 minutes;

15. Store at -80ºC.