Inês Martins Ribeiro da Fonseca

Licenciada em Biologia Celular e Molecular

Unraveling the neuroprotective role of TUDCA in Parkinson´s disease

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

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

Co-orientador: Elsa Rodrigues, PhD, Faculdade de Farmácia, Universidade de Lisboa

Júri:

Presidente: Prof. Doutora Ilda Maria Barros dos Santos Gomes Sanches

Arguente: Prof. Doutora Helena Luísa de Araújo Vieira

Vogal: Prof. Doutora Margarida Casal Ribeiro Castro Caldas Braga

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Setembro 2015

Inês Martins Ribeiro da Fonseca

Licenciada em Biologia Celular e Molecular

Unraveling the neuroprotective role of TUDCA in Parkinson´s disease

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

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

Co-orientador: Elsa Rodrigues, PhD, Faculdade de Farmácia, Universidade de Lisboa

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Setembro 2015

Unraveling the neuroprotective role of TUDCA in Parkinson´s disease

Copyright © Inês Martins Ribeiro da Fonseca, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa.

A Faculdade de Ciências e Tecnologia e a Universidade Nova de Lisboa têm o direito, perpétuo e sem limites geográficos, de arquivar e publicar esta dissertação através de exemplares impressos reproduzidos em papel ou de forma digital, ou por qualquer outro meio conhecido ou que venha a ser inventado, e de a divulgar através de repositórios científicos e de admitir a sua cópia e distribuição com objetivos educacionais ou de investigação, não comerciais, desde que seja dado crédito ao autor e editor.

Part of the results discussed in this thesis were presented in the following publications/communications:

Fonseca I., Moreira S., Lemos L., Silva-Azevedo C., Nunes M.J., Rodrigues E., Gama M.J., Rodrigues C.M.P, Castro-Caldas M. Neuroprotective effects of TUDCA in models of Parkinson's disease: targeting autophagy/mitophagy. 7th iMed.UL post-graduate students meeting, 15th-16th July, 2015, Faculty of Pharmacy, Universidade de Lisboa. [Poster communication];

Fonseca I., Moreira S., Lemos L., Silva-Azevedo C., Nunes M.J., Rodrigues E., Gama M.J., Rodrigues C.M.P, Castro-Caldas M. Unraveling the TUDCA role on autophay/mitophagy in models of Parkinson's disease. XIV Meeting of the Portuguese Society for Neurosciences, 4th-5th June 2015, Póvoa de Varzim, Portugal. [Poster communication];

Fonseca I., Moreira S., Silvia-Azevedo C., Nunes M.J., Gordino G., Gama M.J., Rodrigues E., Rodrigues C.M.P, Castro-Caldas M., [Ferreira Mendes A.]. Mechanisms involved in TUDCA-dependent neuroprotection: Is mitophagy involved?. Annual Meeting of the Portuguese Society of Pharmacology 6th February 2015, Lisbon, Portugal. [Oral communication];

Fonseca I. Unravelling the neuroprotective role of TUDCA in Parkinson's disease: targeting mitophagy. Jornadas intercalares das dissertações anuais dos Mestrados dos Departamentos de Química e de Ciências da Vida. 5th February 2015,Faculty of Sciences and Technology, Universidade NOVA de Lisboa. [Oral communication].

À minha Família...

AGRADECIMENTOS

As minhas primeiras palavras de agradecimento são dirigidas á Professora Doutora Margarida Castro-Caldas. Muito obrigada por me ter concedido a oportunidade de realizar este trabalho, por me ter orientado ao longo deste último ano, pelo apoio constante, por todos os conhecimentos transmitidos, pela disponibilidade e prontidão no esclarecimento das minhas dúvidas, por todo o entusiasmo perante novos resultados, por toda a atenção quando tudo parecia mais complicado e acima de tudo por me ter feito crescer tanto a nível pessoal como profissional.

À Professora Doutora Elsa Rodrigues, coorientadora desta tese, agradeço a constante simpatia e carinho ao longo deste ano, a prontidão em esclarecer as minhas dúvidas, as sugestões feitas no decorrer do trabalho e principalmente o apoio constante que sempre me proporcionou.

À Professora Doutora Maria João Gama, pela disponibilidade demonstrada e pelo envolvimento na resolução dos problemas que foram surgindo no decorrer do trabalho.

À Professora Doutora Cecília Rodrigues, por toda a simpatia demonstrada e pelo apoio que me deu.

À Maria, ao Miguel Moutinho, ao Miguel Santos, à Carla, à Luísa e à Alexandra, obrigado por tudo o que me ensinaram, pelo apoio, pelo interesse demonstrado e pela companhia nos dias mais extensos.

À Sara, com quem partilhei a bancada, muitas horas no laboratório e também fora dele, por todos os momentos partilhados, pelos desabafos e principalmente, pela amizade que nos uniu. Que podia ter passado este último ano sem a sua companhia, podia. Mas não era definitivamente a mesma coisa. Por tudo isto, muito obrigado.

Aos restantes elementos do grupo "Cellular Function and Therapeutic Targeting" com quem partilhei o laboratório agradeço a simpatia e a disponibilidade demonstrada.

Ao Instituto de Investigação do Medicamento, Faculdade de Farmácia da Universidade de Lisboa, que foi uma verdadeira segunda casa durante o último ano.

Ao António e ao Leonardo, por me terem recuperado todo o trabalho que o Word do meu computador amavelmente fez questão de corromper. Muito obrigada.

Um especial obrigado os meus pais, por todo o apoio que sempre me deram independentemente da distância a que por vezes se encontravam, pela compreensão e preocupação, e por nunca me terem permitido desistir quando tudo me parecia impossível. Mas principalmente, pelo carinho que sempre me deram nos momentos mais difíceis de ultrapassar.

A uma pessoa muito especial, por toda a compreensão demonstrada, pelo apoio e pela constante boa disposição e otimismo. Muito obrigada.

Finalmente, à minha família e amigos, por todo o apoio que sempre me deram, por todos os incentivos e motivação.

Não menos importante, gostaria de agradecer à Fundação para a Ciência e Tecnologia – FCT, Portugal, por ter financiado o projeto científico PTDC/NEUNMC/02482012 em que se inseriu este trabalho.

ABSTRACT

Accumulating evidence suggests that mitochondrial dysfunction play a central role in Parkinson's disease (PD). Evidence is based on the identification of PD-associated mutations in genes that affect mitochondrial function, such as phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (*PINK1*) and *parkin*. This was further reinforced by the discovery that exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a mitochondrial complex I inhibitor, leads to clinical symptoms similar to sporadic PD. Thus, one therapeutic approach that has recently arisen in PD research is the selective clearance of damaged mitochondria by mitophagy.

The bile acid tauroursodeoxycholic acid (TUDCA) is an anti-apoptotic molecule shown to interfere with the mitochondrial pathway of cell death. Importantly, TUDCA was demonstrated to protect against MPTP-induced neurodegeneration in mice, but the mechanisms involved are still unknown.

Herein we investigate whether autophagy/mitophagy and mitochondrial biogenesis are part of TUDCA-mediated neuroprotection and discuss the molecular mechanisms involved, using two different models of PD, C57B/L6 mice and SH-SY5Y neuroblastoma cells treated with TUDCA and/or MPTP/MPP^+. Our preliminary results reveal that in mice brain, TUDCA induced microtubule-associated protein 1 light chain 3 (LC3) lipidation, and increased voltage-dependent anion channel (VDAC), full length PINK1 and parkin protein expression, thereby suggesting that autophagy/mitophagy and mitochondrial biogenesis are part of TUDCA-mediated neuroprotection. In SH-SY5Y cells, TUDCA prevents MPP^+-induced cell death and mitochondrial damage. Moreover, this bile acid was also shown to modulate parkin phosphorylation, and parkin expression levels in the presence of MPP^+. Importantly, modulation of parkin was accompanied by increased levels of autophagy.

Impaired mitochondrial turnover has been associated to PD, thus, mitochondrial protective agents represent an attractive direction for the development of new therapeutic drugs. Our results point to the pharmacological up-regulation of mitochondrial turnover by TUDCA as a novel neuroprotective mechanism of this molecule, and contribute to the validation of TUDCA clinical application in PD.

Keywords: Parkinson's disease, mitochondrial dysfunction, TUDCA, mitophagy, biogenesis

RESUMO

Vários estudos sugerem que a principal causa da Doença de Parkinson (DP) seja uma disfunção mitocondrial. Esta hipótese deriva da identificação de mutações em genes associados à doença que afetam o funcionamento mitocondrial, tais como *tensin homolog (PTEN)-induced putative kinase 1* (*PINK1*) e *parkina*. A descoberta de que a exposição a 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP), um inibidor do complexo I mitocondrial, induzia sintomas semelhantes aos observados em pacientes com DP, veio reforçar esta hipótese. Assim, a degradação seletiva de mitocôndrias danificadas por mitofagia é considerada uma possível via terapêutica na DP.

O ácido biliar taurousodeoxicólico (TUDCA) é uma molécula anti-apoptótica que interfere com a via de morte celular mitocondrial, tendo já sido demonstrado que previne neurodegenerescência induzida pelo MPTP em murganhos. No entanto, os mecanismos envolvidos são desconhecidos.

Neste estudo pretende-se investigar se a autofagia/mitofagia e biogénese mitocondrial fazem parte da neuroprotecção mediada pelo TUDCA e discutir quais os mecanismos moleculares envolvidos, utilizando dois modelos diferentes: murganhos C57B/L6 e células SH-SY5Y tratados com TUDCA e/ou MPTP/MPP^+. Os resultados preliminares obtidos revelam que, no cérebro de murganhos, o TUDCA induz a lipidação da proteína *microtubule-associated protein 1 light chain 3* (LC3), e aumenta a expressão proteica de *voltage-dependent anion channel* (VDAC), da forma não-clivada de PINK1 e da parkina, sugerindo que a autofagia/mitofagia e biogénese mitocondrial fazem parte da neuroprotecção mediada pelo TUDCA. Nas células SH-SY5Y, observou-se que o TUDCA previne a morte celular e a depleção mitocondrial induzida pelo MPP^+. Adicionalmente, este ácido biliar demonstrou ser capaz de modular a fosforilação da parkina, e os níveis de expressão da mesma na presença de MPP^+. A modulação da parkina revelou ser acompanhada por um aumento dos níveis de autofagia.

Um *turnover* mitocondrial deficiente tem sido associado à DP, pelo que agentes protetores mitocondriais representam uma direção atrativa para o desenvolvimento de novas terapias. Os resultados obtidos sugerem um aumento do *turnover* mitocondrial mediado pelo TUDCA como um novo mecanismo neuroprotetor do mesmo, contribuindo para a sua validação na DP.

Palavras-Chave: Doença de Parkinson, disfunção mitocondrial, TUDCA, mitofagia, biogénese.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AMPK	AMP-activated protein kinase
AR	Autosomal recessive
Atg	Autophagy-related protein
ATP	Adenosine triphosphate
i.p	Intra-peritoneal
LBs	Lewy Bodies
LC3	Microtubule-associated protein 1 light chain 3
LDH	Lactate dehydrogenase
MIM	Mitochondrial inner membrane
MOM	Mitochondrial outer membrane
MPP^+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mTOR	Mammalian target of rapamycin
PARIS	Parkin interacting substrate
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PD	Parkinson's disease
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator-1α
PINK1	Phosphatase and tensin homolog (PTEN)-induced putative kinase 1
qRT-PCR	quantitative Real-Time PCR
Raptor	Regulatory-associated protein of TOR
ROS	Reactive oxygen species
RT	Room temperature
SNpc	*Substantia nigra pars compacta*
TBS-T 0.1%	Tris-buffered saline with 0.1%Tween-20
TUDCA	Tauroursodeoxycholic acid
Ub	Ubiquitin
ULK1	UNC-51-like kinase 1
VDAC	Voltage-dependent anion channel

1. INTRODUCTION

1.1 Parkinson's disease

Parkinson's disease (PD), the second most common neurodegenerative disorder after Alzheimer's disease was first described in 1817 by James Parkinson. PD is a motor disorder, characterized by the progressive loss of dopaminergic neurons of the *substantia nigra pars compacta* (SNpc) and the degeneration of their dopaminergic axons in the striatum, with the presence of Lewy Bodies (LBs), which are intraneuronal proteinacious cytoplasmic inclusions (Dauer & Przedborski, 2003; Dexter & Jenner, 2013). Nevertheless, neurodegeneration and LBs formation are not confined to the nigrostriatal pathway. These processes extend to other brain regions, namely to noradrenergic, serotonergic and cholinergic systems, to the cerebral cortex, olfactory bulb and autonomic nervous system (Dauer & Przedborski, 2003). The disorder has a mean age of onset of approximately 55 years, and patients experience mainly motor but also nonmotor symptoms (Table 1.1), which strongly impair their quality of life (Chaudhuri, Healy, & Schapira, 2006; Jankovic, 2008; Thomas & Flint Beal, 2007). These symptoms only appear after depletion of approximately 50-70% of dopaminergic neurons of the nigrostriatal pathway (Barzilai & Melamed, 2003). PD therapies have not changed much in the past 30 years, and are all symptomatic, not preventing the progression of the disease (Dexter & Jenner, 2013).

Epidemiological studies revealed that approximately 90% of PD cases have a sporadic origin, which may be caused by unknown environmental factors, and the remaining 10% of the cases are inherited, being referred as familial forms of PD (Thomas & Flint Beal, 2007). Importantly, the identification of genes associated to familial PD contributed to the knowledge of the molecular pathogenesis of the disease (Thomas & Flint Beal, 2007).

Table 1.1 - Motor and nonmotor symptoms of PD

Motor Symptoms	Nonmotor Symptoms
- Tremor at rest, which decreases with voluntary movement	- Cognitive and affective abnormalities
- Rigidity	- Depression
- Bradykinesia (slowness of movement)	- Dementia
- Freezing (impaired ability to initiate movement)	- Sleep disorders, like insomnia, high motor activity while sleeping
- Postural instability; flexed posture	- Autonomic symptoms, as bladder disturbances
- Hypokinesia (reduction of the movement amplitude)	
- Akinesia (absence of unconscious movements, as reduced arm swing in walking); hypophonia (decreased voice volume); drooling, due to impaired swallowing; micrigraphia (decreased size of handwriting); hypomimia (loss of facial expressions)	

1.1.1 Familial Parkinson's disease

The first mutations responsible for PD were found in 1996, demonstrating that PD can be hereditary. Nowadays, about 28 distinct chromosomal regions have been related to PD, but only six of them lead to monogenic PD, meaning that a mutation in a single gene is enough to cause the disease. Out of the six chromosomal regions responsible for monogenic PD, two are accountable for autosomal dominant PD forms (*α-synuclein* and *LRRK2*) and the remaining four for autosomal recessive (AR)-PD (*PARK2*, *PINK1*, *DJ-1* and *ATP13A2*) (Klein & Westenberger, 2012). This section will focus in three of the genes responsible for monogenic PD, which have been associated with mitochondrial dysfunction.

α-synuclein

The α-*synuclein* gene (also referred as *SNCA* gene) encodes a 140 amino acid cytosolic protein, highly expressed in specific brain regions, which include dopaminergic neurons of the *substantia nigra*. Importantly, α-synuclein is a pre-synaptic protein, and is thought to play an active role in recycling, storage and compartmentalization of synaptic vesicles (Barzilai & Melamed, 2003; Thomas & Flint Beal, 2007). Mutations in α-*synuclein* have been demonstrated to cause autosomal-

dominant PD, and patients often present an early-onset of the disease (patients develop the disease before age of 50). Moreover, in patients with α-*synuclein* mutations the disease has a rapid progression, and presents α-synuclein aggregates, the main component of LBs. Only a small number of mutations have been reported for this gene, which includes three missense mutations, duplications and triplications (Klein & Westenberger, 2012).

Parkin

The *parkin* gene (*PARK2*) encodes for a 465 amino acid protein that functions as an E3 ubiquitin ligase, and is mainly localized in the cytosol. Parkin is composed of an N-terminal ubiquitin like domain, a central linker region and a C-terminal Really Interesting New Gene (RING) domain, and selectively targets proteins for degradation (Martin *et al*., 2011; Mata *et al.*, 2004). Additionally, this protein has been demonstrated to be involved in the mitochondrial quality control pathway (Martin *et al*., 2011). Mutations in the gene encoding for parkin are known to cause AR-PD, and are the most common cause of early outset of the disease (Klein & Westenberger, 2012; Thomas & Flint Beal, 2007). A wide variety of mutations in *PARK2* have been identified, which lead to loss of its E3 ubiquitin ligase activity. Parkin loss of function may lead to the accumulation of its substrates and possibly to PD pathogenesis (Dawson & Dawson, 2010; West & Maidment, 2004).

PINK1

The phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1) is a 581 amino acid serine/threonine protein kinase, that functions simultaneously with parkin to maintain mitochondrial homeostasis (Song *et al.*, 2013). Mutations in *PINK1* gene also cause AR-PD with early onset, being the first gene identified that suggested the involvement of impaired mitochondrial function in PD pathogenesis (Silvestri *et al.*, 2005). Most of the mutations identified in *PINK1* are nonsense or missense mutations affecting the serine/threonine kinase domain, suggesting that loss of kinase function may be an important part of PINK1 pathogenesis in PD (Klein & Westenberger, 2012; Song *et al*., 2013).

1.1.2 Sporadic Parkinson's disease

Although the cause of sporadic PD is unknown, evidence suggests that some environmental factors may be responsible for causing the progressive degeneration of dopaminergic neurons due to, for example, a chronic exposure to a toxin (Dauer & Przedborski, 2003). The best known example of a toxin that induces a PD-like syndrome is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (reviewed bellow in section 1.5). Pesticides, like rotenone and paraquat, are present in the environment and have also been suggested to play a role as PD risk factors, as well as cigarette smoking (Betarbet *et al*., 2000; Dauer & Przedborski, 2003; Manning-Bog *et al*., 2002).

However, the discovery of PD associated genes has suggested the interplay between environmental agents and gene expression. Substances present in the environment may be able to alter normal metabolic pathways, which in turn might originate toxic products responsible for the

disease (Dauer & Przedborski, 2003; Klein & Westenberger, 2012). Importantly, loss of function of parkin protein has been shown to occur in some cases of sporadic PD (Dawson & Dawson, 2010)

1.2 Neurodegeneration in PD

Dopaminergic neurons from the nigrostriatal pathway have their cell bodies in the SNpc of the ventral midbrain, and extend to the dorsal striatum (putamen and caudate nucleus) (Fig.1.1a/b (Chinta & Andersen, 2005; Dauer & Przedborski, 2003). The SNpc is essential for the control of voluntary movements, and is a dopamine (DA)-rich region containing neuromelanin with high iron content. Thus, degeneration of dopaminergic neurons causes SNpc depigmentation, a classic neuropathological feature of PD (Barzilai & Melamed, 2003; Dauer & Przedborski, 2003).

As mentioned above, in addition to the loss of dopaminergic neurons from the nigrostriatal pathway, another pathological hallmark of PD is the formation of LBs, even though these are not exclusive of the disease (Exner *et al*., 2012). LBs are intracytoplasmic protein aggregates, composed of several ubiquitinated proteins that are resistant to proteolysis (Fig. 1.1C). Even though α-synuclein is the major component of LBs, parkin and ubiquitin are also found in these structures (Barzilai & Melamed, 2003; Dauer & Przedborski, 2003). Until recently it was suggested that these protein aggregates contributed to neuronal degeneration in PD, but emerging evidence suggest that they may have a neuroprotective action sequestering potential harmful proteins (Keane *et al*., 2011; Kramer & Schulz-Schaeffer, 2007; Trojanowski *et al.*, 1998).

Even though the specific molecular mechanisms underlying the pathogenesis of the disease are not fully understood, growing evidence suggests that mitochondrial impairment plays a central role in both familial and sporadic PD (Martin *et al*., 2011).

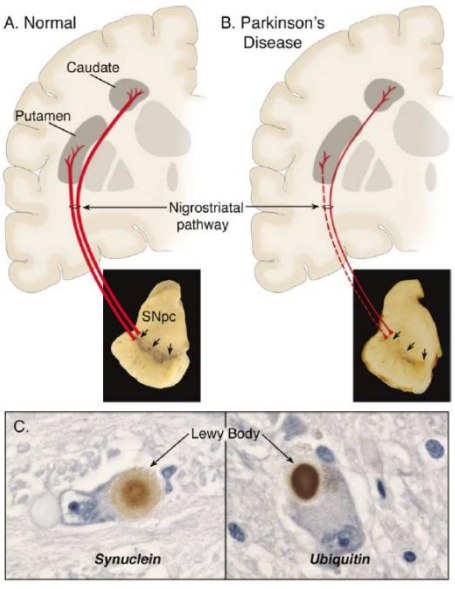


Figure 1.1 – Pathological hallmarks of Parkinson's disease. (a) Schematic representation of the nigrostriatal pathway. This is composed of dopaminergic neurons, which have their cell bodies in the SNpc (pointed with black arrows) and project the axons primarily to the putamen e then to the caudate. The SNpc has a characteristic pigmentation as dopaminergic neurons contain neuromelanin. (b) Shematic representation of nigrostriatal pathaway degeneration in PD. In PD there is an acentuated loss of dopaminergic neurons, which results in the degereration of the nigrostriatal pathway. Additionaly, due to loss of neuromelanin-containing neurons, it is observed a clear depigmentaion of the SNpc. (c) Immunohistochemical labeling of LBs in a SNpc dopaminergic neuron. The second hallmark of PD is the formation of protein agregates, known as LBs. These are composed of several diffetent proteins, such as parkin, ubiquitin and α-synuclein, being the last one mentiond the major component of these structures. In Dauer & Przedborski (2003).

1.2.1 Mitochondrial dysfunction

Mitochondria are double membrane organelles responsible for the generation of cellular energy in the form of adenosine triphosphate (ATP). The mitochondrial electron transport chain involved in the generation of ATP by oxidative phosphorylation is composed of four complexes plus an ATP-synthase, which are located in the mitochondrial inner membrane (MIM). During oxidative phosphorylation, formation of reactive oxygen species (ROS) can occur due to the reaction between electrons that leak from the electron transport chain and oxygen, which are then removed by mitochondrial antioxidants (Keane *et al*., 2011).

Discovery of two neurotoxins that induced a PD-like syndrome through the selective blockage of Complex I, namely rotenone and MPTP, suggested that mitochondrial dysfunction might be involved in PD pathogenesis (Betarbet *et al*., 2000; Zhu & Chu, 2010). *Post-mortem* observations of PD patients' brains revealed a decreased activity of Complex I in the SNpc, further reinforcing this idea (Mizuno *et al.*, 1989; Moore, West *et al*., 2005). Moreover, additional studies also revealed Complex I deficits in platelets and skeletal muscle of PD patients (Dexter & Jenner, 2013; Yoshino *et al.*, 1992).

Abnormalities in mitochondrial Complex I block the movement of the electrons along the electron transport chain, leading to a reduction of intracellular ATP levels and to the accumulation of electrons in the mitochondrial matrix. In the matrix, electrons react with oxygen generating high levels of ROS, which cause oxidative DNA damage, lipid peroxidation, protein oxidation, possibly also promoting excitotoxicity, and ultimately leading to cell death (Gandhi & Wood, 2005; Keane *et al.*, 2011).

The identification of mutations in mitochondrial associated genes, such as *PINK1* and *parkin*, responsible for familial forms of PD, also reinforces the idea that mitochondrial dysfunction plays a central role in PD pathogenesis (Morais *et al.*, 2009). Importantly, it was demonstrated that both in fruit flies and mouse models, loss of function of PINK1 resulted in decreased activity of mitochondrial Complex I, which resulted in mitochondria depolarization. Moreover, mutant or loss of function of PINK1 in cell cultures induced, in addition to mitochondrial dysfunction, impaired proteasomal function and α-synuclein accumulation (Liu *et al*., 2009; Morais *et al.*, 2009). Parkin loss of function in zebra fish embryos also lead to a reduction of Complex I activity and dopaminergic neurons, and *parkin* null mice demonstrated decreased oxidative phosphorylation, increased oxidative stress and higher nigrostriatal dysfunction (Flinn *et al*., 2009; Goldberg *et al*., 2003; Palacino *et al.*, 2004). More recently, a study from Imaizumi and collaborators (2012), using induced pluripotent stem cells-derived neurons from two PD patients with *parkin* mutations, suggested that mitochondrial dysfunction may lead to α-synuclein accumulation in these cells.

Importantly, Devi and co-workers (2008), demonstrated that, in human fetal dopaminergic primary neuronal cultures, α-synuclein may interact with mitochondrial Complex I, thus decreasing its activity. Simultaneously, *post-mortem* analysis of PD patients' brain tissues also revealed a significant accumulation of mitochondrial α-synuclein and decreased activity of Complex I, in both striatum and SNpc (Devi *et al.*, 2008).

Therefore, mitochondrial protective agents represent an attractive direction for the development of drug candidates that can modify the pathogenesis of neurodegeneration in PD.

1.3 Proteolytic systems for removal of intracellular components

Elimination of unwanted/unnecessary or damaged proteins/organelles is essential to maintain a balance between protein/organelle synthesis and degradation. The major cellular pathways for continuous degradation of intracellular components are ubiquitin-proteasome and autophagic-lysosomal pathway (Cuervo *et al.*, 2005; Reggiori & Klionsky, 2002).

Unwanted/unnecessary or damaged proteins can be selectively eliminated by the ubiquitin-proteasome pathway, where coordinated actions of different enzymes link chains of ubiquitin (Ub), a 76 amino acid protein, onto proteins marking them for proteasomal degradation (Lecker *et al.*, 2006). In this pathway Ub is activated by an Ub-activation enzyme (E1) in an ATP-dependent reaction, and further transferred to an Ub-carrier protein (E2). Then an Ub-protein ligase (E3) together with E2, catalyse the formation of the poly-Ub chain in the target protein, triggering its recognition by the 26S proteasome (a multicatalytic protease complex that degrades ubiquitinated proteins) (Fig. 1.2a). Importantly, the E3 protein ligase determines the selectivity of the pathway, as they are specific for different protein substrates. (Goldberg, 2003; Hershko *et al.*, 1984)

The degradation pathway of intracellular components in lysosomes is referred as autophagy. In this degradation pathway cargo is engulfed by a double membrane, originating an autophagosome, which fuses with a lysosomal compartment containing the required hydrolases for degradation (Fig. 1.2b) (Menzies *et al.*, 2015). Initiation of autophagy is signalled by the assembly of a kinase complex formed by Beclin 1, a class III phosphatidylinositol 3-kinase, vacuolar protein sorting 34 and other variable modulatory proteins (Kihara *et al.*, 2001; Liang *et al.*, 1999). Upon the assembly of this complex, precursor vesicles containing a transmembrane autophagy-related proteins (Atg) coalesce to origin a pre-autophagosomal structure known as phagophore (Mari *et al.*, 2010). Additionally, microtubule-associated protein 1 light chain 3 (LC3) processing is also essential for the autophagosome biogenesis. After being synthetized, LC3 is cleaved to form LC3-I, which is then conjugated with phosphatidylethanolamine, originating LC3-II (Kabeya *et al.*, 2003; Menzies *et al.*, 2015). Then, the phagophore elongates to sequester the cargo, and eventually seals to form a double membrane vesicle called autophagosome. The autophagosome fuses with a lysosome, and the acidification of the autolysosome lumen by the proton pump with simultaneous action of hydrolases provided by the lysosome, lead to the degradation of the cargo. Importantly, autophagy receptors confer specificity to this degradation pathway, through direct interaction with the cargo and with the autophagic machinery (Menzies *et al.*, 2015).

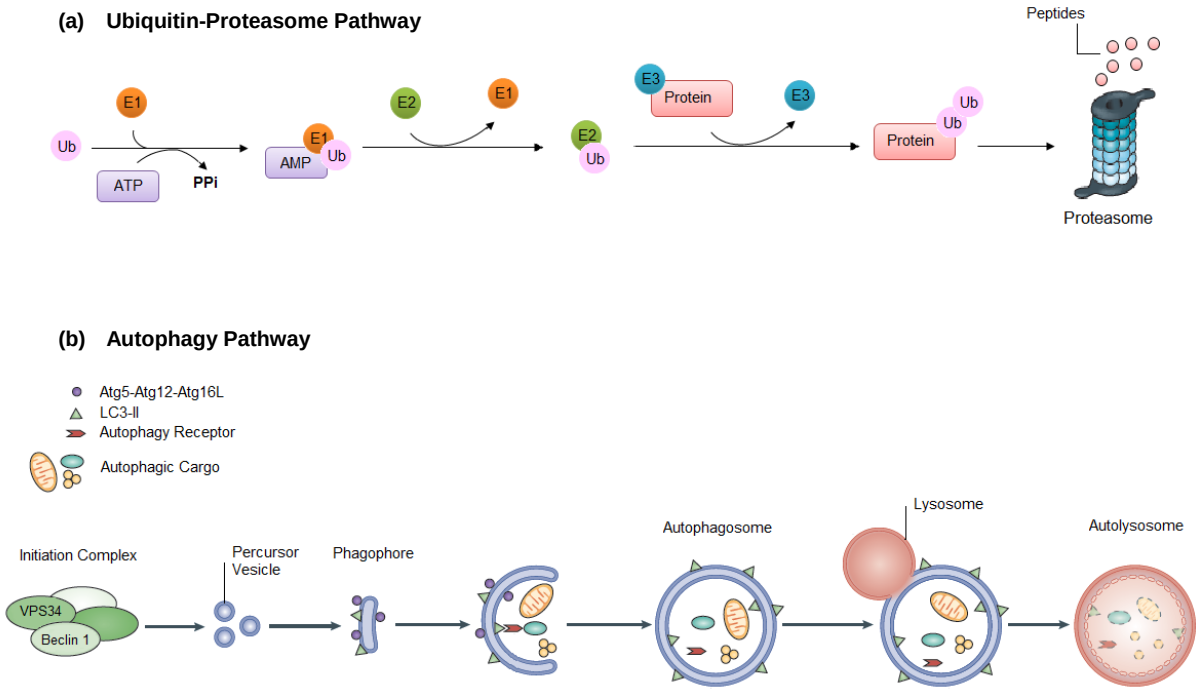


Figure 1.2 – Overview of the ubiquitin-proteasome pathway and autophay pathway. (a) Schematic representation of the ubiquitin-proteasome system. Briefly, ubiquitin is activated by an E1 protein in an ATP-dependent reaction, and further transferred to an E2 protein. Then an E3 together with an E2, catalyse the formation of a Ub chain in the target protein, targeting the protein for degradation by the 26S proteasome. (b) Schematic representation of the autophagy pathway. The first step of autophagy is the assembly of an intitiacion complex, formed by Beclin 1, vacuolar protein sorting 34 (VPS34) and other variable modulatory proteins, which is followed by the fusion of precursor vesicles to origin a pre-autophagosomal structure known as phagophore. Then the phagophore elongates to sequester the cargo and eventually seals, to form a double membrane vesicle called autophagosome. The autophagosome fuses with a lysosome, originationg an autolysosome, where cargo is degrated due to the acidic environment of the autolysosome lumen. Adapted from Menzies *et al*. (2015).

1.3.1 AMPK pathway of autophagy

Non-selective autophagy occurs upon nutrient deprivation, in order to provide cells with the necessary nutrients and energy to maintain essential cellular activities.

AMP-activated protein kinase (AMPK), a main cellular energy sensor, is a heterotrimeric serine/threonine kinase complex composed of a catalytic (α) subunit and two regulatory (β and γ) subunits, whose activation requires the phosphorylation of AMPK catalytic subunit. Upon energy stress conditions, intracellular AMP/ATP ratio levels increase, leading to AMPK phosphorylation through assembly of its regulatory γ subunit (Gwinn *et al.*, 2008). Activated AMPK phosphorylates downstream targets, thus allowing autophagy initiation (Egan *et al.*, 2011; Gwinn *et al*., 2008; Lee *et al*., 2010).

The UNC-51-like kinase 1 (ULK1) protein is a modulator of autophagy initiation, and its activity depends on the interaction with a focal adhesion kinase family-interacting protein of 200 kDa, mediated by Atg13 (Hara *et al*., 2008; Jung *et al*., 2009). Modulation of autophagy initiation by ULK1 involves the phosphorylation of Beclin 1 by this kinase, and consequent activation of the autophagic initiation complex. Moreover, ULK1 activity is regulated by the mammalian target of rapamycin (mTOR) complex 1 (mTORC) and AMPK (Russell *et al*., 2013).

mTOR is a serine/threonine kinase that forms two distinct multiprotein complexes, termed mTORC1 and mTORC2. mTORC1 is nutrient sensitive, negatively modulates autophagy, and comprises mTOR, mLST8 and the regulatory-associated protein of TOR (raptor). Importantly, raptor is an essential protein for mTOR-mediated phosphorylation of downstream targets, acting as a scaffold for substrate recruitment (Hara *et al*., 2002; Singh & Cuervo, 2011; Wang *et al.*, 2009). Under normal growth conditions, mTORC1 sequesters and phosphorylates ULK1 and Atg13, which inhibits ULK1 activity and consequently prevents autophagy induction (Fig. 1.3a) (Hosokawa *et al*., 2009; Jung *et al*., 2009).

As previously mentioned, upon energy stress conditions AMPK is activated, and phosphorylates ULK1 and raptor inhibiting mTOR activity, which results in autophagy initiation (Fig. 1.3b) (Egan *et al.*, 2011; Gwinn *et al*., 2008; Lee *et al*., 2010).

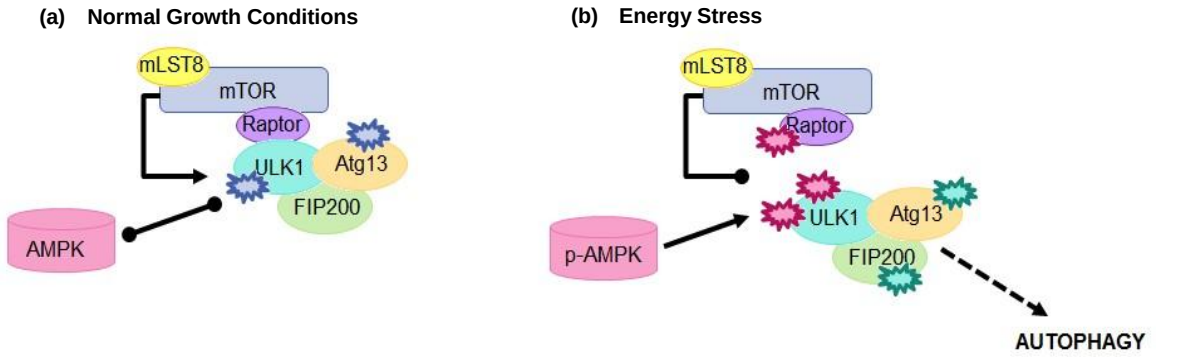


Figure 1.3 – Overview of the AMPK pathway of autophagy. (a) Schematic representation of autophagy inhibition by mTOR under normal growth contitions. Under normal growth conditions, AMPK is dephosphorylated, thus allowing mTORC1 to sequester and phosphorylate ULK1 and Atg13, which are present in the ULK1-Atg13-focal adhesion kinase family-interacting protein of 200 kDa (FIP200) complex, inhibiting ULK1 activity and consequently autophagy induction. (b) Schematic representation of autophagy induction by AMPK under energy stress. Upon energy stress AMPK is phosphorylated (activated), it phosphorylates ULK1 thus inhibiting mTOR activity, allowing ULK1-Atg13-FIP200 complex mobilization and autophagy initiation. Additionally, AMPK also inhibits mTOR activity through phosphorylation of raptor. Adapted from Singh & Cuervo (2011).

1.3.2 PINK1/parkin pathway of mitophagy

The specific degradation of damaged or superfluous mitochondria by autophagy is called mitophagy. Several studies have demonstrated that parkin together with PINK1 mediate impaired mitochondria degradation by mitophagy (Meissner *et al*., 2011).

PINK1 is mainly localized in mitochondria, as it contains an N-terminal mitochondrial targeting signal. Moreover, mitochondrial localized PINK1 has its C-terminus and kinase domain facing the cytosol (Silvestri *et al.*, 2005; Zhou *et al.*, 2008). In healthy mitochondria, PINK1 is translocated from the mitochondrial outer membrane (MOM) to the MIM through direct interaction with the translocases from the inner and outer mitochondrial membranes, where it is cleaved to a short fragment by a mitochondrial processing protease and by the mitochondrial protease presenilins-associated rhomboid-like protein (Greene *et al.*, 2012; Jin *et al.*, 2010; Meissner *et al*., 2011). Cleaved PINK1 is

reimported to the cytoplasm, where it is eliminated by proteasomal degradation (Fig. 1.4a) (Menzies *et al.*, 2015).

PINK1 translocation through the mitochondrial membrane and cleavage is dependent on the mitochondria membrane potential. Consequently, upon mitochondrial depolarization, native PINK1 stably associates with the translocator and accumulates in the outer mitochondrial membrane (Becker *et al.*, 2012; Lazarou *et al*., 2012; Narendra *et al.*, 2010*a*). Accumulated PINK1 selectively recruits parkin from the cytosol to depolarized mitochondria, and phosphorylates parkin in a conserved serine residue (Ser65) located within its N-terminal Ub-like domain, activating its E3 ligase activity (Kondapalli *et al*., 2012; Narendra *et al*., 2010*a*; Narendra *et al.*, 2008). Even though the mechanisms by which parkin induces mitophagy are not fully understood, growing evidence suggests that activated parkin ubiquitylates a number of mitochondrial proteins, such as the voltage-dependent anion-selective channel protein (VDAC) 1, which in turn may trigger the recruitment of the autophagy receptors to mitochondria, triggering the general autophagy machinery (Fig. 1.4b) (Geisler *et al.*, 2010; Narendra *et al.*, 2010*b*)..

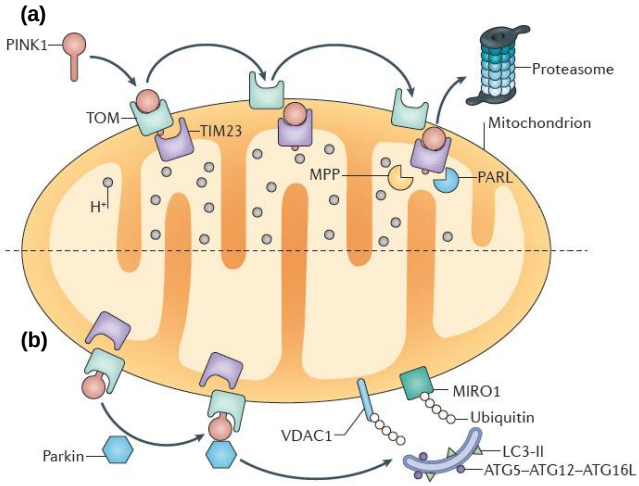


Figure 1.4 – Overview of the PINK1/parkin-mediated autophagy/mitophagy. (a) In healthy mitochondria, PINK1 is translocated to the mitochondrial inner membrane through direct interaction with the the translocases from the mitochondrial membranes (TOM20/TIM23), where is cleaved by the mitochondrial processing protease (MPP) and by the mitochondrial protease presenilins-associated rhomboid-like protein (PARL). Then, cleaved PINK1, is reimported to the cytoplasm, where is degradated by the 26S proteasome. (b) Upon mitochondrial depolarization, PINK1 accumulates in the mitochondrial outer membrane and recruits parkin to mitochondria through selective phosphorylation. Activated parkin ubiquitinates several mitochondrial proteins, which may trigger the recruitment of the autophagy receptors to mitochondria, as the p62/SQSTM1 that directly interact with the general autophagy machinery. In Menzies *et al*. (2015).

1.4 Parkin/PARIS pathway of mitochondrial biogenesis

Mitophagy and mitochondrial biogenesis are well coordinated events that contribute to the maintenance of mitochondria and cellular homeostasis. A recent study demonstrated the existence of

interplay between parkin and mitochondrial biogenesis, through the identification a novel parkin interacting substrate (PARIS or ZNF746) (Shin *et al.*, 2011).

PARIS is a transcriptional repressor of the peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α). The PGC-1α is a transcriptional coactivator of several genes, and a known master regulator of mitochondrial biogenesis (Shin *et al.*, 2011; Wu *et al.*, 1999). This transcriptional coactivator controls the expression of nuclear respiratory factors, which modulate the transcription of the main regulators of mitochondrial DNA transcription and replication, namely, the mitochondrial transcription factor A and transcription factor B proteins (Palikaras & Tavernarakis, 2014; Wu *et al.*, 1999), as well as nuclear mitochondrial genes, such as mitochondrial respiratory complexes, enzymes of heme biosynthesis, mitochondrial import machinery associated proteins and mitochondrial ribosomal proteins (Palikaras & Tavernarakis, 2014).

To maintain mitochondrial homeostasis parkin ubiquitinates PARIS, targeting it for proteasomal degradation. As PARIS is a negative regulator of the PGC-1α, degradation of this protein upregulates PGC-1α and PGC-1α-dependent nuclear respiratory factors gene expression, enabling mitochondrial biogenesis (Fig. 1.5) (Shin *et al.*, 2011).

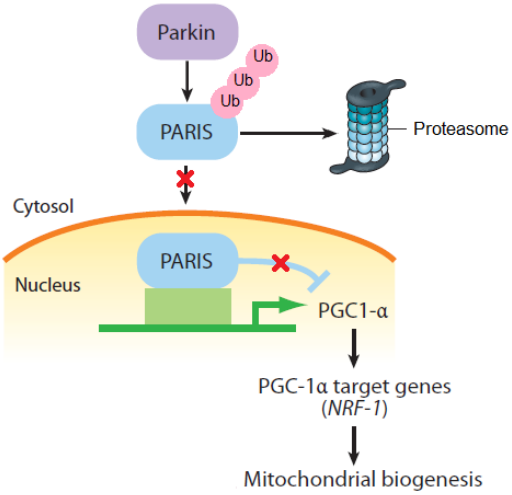


Figure 1.5 – Overview of Parkin-mediated mitochondrial biogenesis. PARIS is a transcriptional repressor of PGC-1α, a major regulator of mitochondrial biogenesis. After translocation to the nucleus, PARIS specifically downregulates PGC-1α expression by binding to its promotor, thus preventing mitochondrial biogenesis. Parkin modulates mitochondrial biogenesis by targeting PARIS for proteasomal degradation in the cytosol, thus allowing PGC-1α and PGC-1α-dependent nuclear respiratory factors (NRF) gene expression . Adapted from Martin *et al.* (2011).

1.5 The MPTP model of Parkinson's disease

In the late 70's young drug addicts developed an irreversible idiopathic parkinsonian syndrome after intravenous self-administration of 1-methyl-4-phenyl-propion-oxypiperidine, an analogue of heroin (Bové & Perier, 2012; Schober, 2004).The analysis of this synthetic drug revealed that MPTP, a

byproduct of MPPP synthesis, was the neurotoxin responsible for the Parkinsonian-like symptoms (Blum *et al*., 2001; Bové & Perier, 2012). In addition to the observed motor symptoms, like bradykinesia, rigidity, resting tremors and postural instability, *post-mortem* investigations confirmed lesion of the *substantia nigra* after exposure to MPTP (Blum *et al*., 2001). Different subsequent studies demonstrated that MPTP induced pathologic and biochemical features similar to idiopathic PD in monkeys and mice (Bové & Perier, 2012; Nicotra & Parvez, 2002). Although MPTP does not recapitulate all the hallmarks of PD, namely the formation of the characteristic LBs, nowadays it is a well-established parkinsonian toxin and the most widely used in PD animal models (Schober, 2004).

MPTP is a lipophilic molecule which, after systemic administration, easily crosses the blood-brain barrier due to its high lipophilicity (Fig. 1.6) (Bové & Perier, 2012; Schober, 2004). Subsequently, in the brain MPTP is metabolized into 1-methyl-4-phenyl-2,3-dihydropyridium by monoamine oxidase-B in the MIM of non-dopaminergic cells (essentially in astrocytes and serotonergic neurons) (Nicotra & Parvez, 2002; Schober, 2004; Watanabe *et al*., 2005). The recently formed intermediary is an unstable molecule, that rapidly deprotonates to generate 1-methyl-4-phenylpyridinium (MPP^+), the effective toxic metabolite of MPTP, which is in turn released into the extracellular space by an unknown mechanism (Bové & Perier, 2012; Smeyne & Jackson-Lewis, 2005). The polar molecule MPP^+ has a high affinity for the dopamine transporter, leading to a selective accumulation of MPP^+ in dopaminergic neurons (Blum *et al.*, 2001; Schober, 2004). In fact, it has been demonstrated that the pharmacological inhibition of the dopamine transporter (with mazindol) or its genetic ablation prevents MPTP neurotoxicity, suggesting that the uptake of MPP^+ is an important step in MPTP toxicity (Bové & Perier, 2012; Javitch *et al.*, 1985).

Once inside the dopaminergic neurons, MPP^+ can be accumulated in synaptic vesicles through interaction with the vesicular monoamine transporter, a proton-dependent transporter responsible for the uptake of neurotransmitters into synaptic vesicles (Blum *et al*., 2001; Schober, 2004; Smeyne & Jackson-Lewis, 2005). This mechanism is associated with a decrease of MPP^+ toxicity by preventing the interaction of the neurotoxin with mitochondria, since mice with 50% depletion of vesicular monoamine transporter are more susceptible to MPTP toxicity (Schober, 2004). Simultaneously, free cytosolic MPP^+ can accumulate in the mitochondrial matrix by means of passive transport due to the mitochondrial transmembrane gradient, where it inhibits the electron transport enzyme NADH-ubiquinone oxireductase (present in Complex I) (Bové & Perier, 2012; Keane *et al*., 2011; Przedborski *et al*., 2004; Smeyne & Jackson-Lewis, 2005). Complex I inhibition impairs mitochondrial respiration, resulting in the loss of mitochondrial membrane potential, cellular ATP depletion, alterations of calcium homeostasis and an increased production of ROS, and ultimately cell death (Du *et al*., 2001; Nicotra & Parvez, 2002; Przedborski *et al*., 2004).

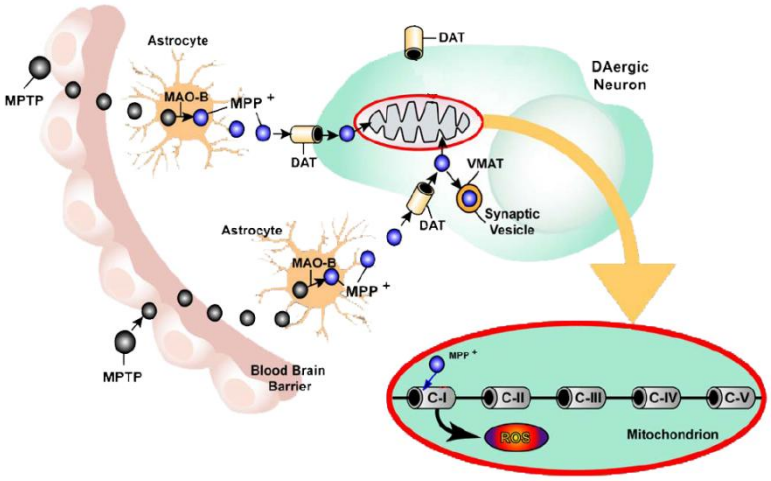


Figure 1.6 – Schematic representation of MPTP metabolism and MPP^+ intracellular pathways. After systemic administration, MPTP (represented as a grey vesicle) crosses the blood-brain-barrier. In the brain MPTP is taken up by the astrocytes, and converted to its toxic metabolit, the MPP^+ (represented as a blue vesicle), in mitochondria by the monoamine oxidase-B (MAO-B). Then the MPP^+ is released into the extracellular space, and specifically accumulates inside dopaminergic neurons via the dopamine transporter (DAT). Inside dopaminergic neurons, the neurotoxin can concentrate in mitochondria where inhibits the mitochondrial Complex I, or can be sequestered into synaptic vesicles via the vesicular monoamine transporter (VMAT). Adapted from Schober (2004)

1.6 Tauroursodeoxycholic acid as a neuroprotective agent

Bile acids represent a group of molecular species of acidic steroids, which are synthetized in the liver. At high concentrations hydrophobic bile acids are known to be cytotoxic, and have been implicated in many liver diseases and colon cancer. However, contrasting with the toxic action of hydrophobic bile acids, hydrophilic ones have been proved to be cytoprotective (Amaral, *et al.*, 2009).

Ursodeoxycholic acid, is a hydrophilic bile acid endogenously produced that has been used for centuries as a therapeutic agent of several maladies in Chinese medicine. This bile acid is a non-toxic anti-apoptotic molecule and is effective in the treatment of cholestatic liver disease, being the only molecule approved by the United States Food and Drug Administration for primary biliary cirrhosis (Parry *et al.*, 2010; Rodrigues *et al.*, 2002).

The ursodeoxycholic acid can be conjugated in the liver with taurine to form tauroursodeoxycholic acid (TUDCA) (Rodrigues *et al.*, 2002). TUDCA is a clinically safe hydrophilic bile acid capable of modulate cell death by interfering with classic apoptotic pathways, and which revealed to also be effective in the treatment of patients with primary cirrhosis (Parry *et al.*, 2010). Several reports have demonstrated that TUDCA is able to modulate cell death by interfering with mitochondria-mediated apoptosis, inhibiting mitochondrial depolarization and consequently limiting the production of ROS, preventing mitochondrial release of cytochrome *c* and Bax (a proapoptotic molecule) translocation from cytosol to mitochondria, and through inhibition of caspase activation (Rodrigues *et al.*, 2000*b*; Rodrigues *et al.*, 1998).

TUDCA can penetrate in the central nervous system, and growing evidence suggests that it plays an important protective role in both *in vitro* and *in vivo* models of several neurological disorders

(Rodrigues *et al*., 2002). TUDCA has been shown to be neuroprotective in Huntington's disease models, as it prevented 3-nitropropionic acid (a mitochondrial inhibitor of Complex II, which induces striatal lesions similar to those observed in Huntington's disease)-induced apoptosis in neuronal cell cultures. Moreover, systemic administration of TUDCA in a 3-nitropropionic acid mouse model and in a genetic mouse model of Huntington's disease, prevented striatal degeneration and ameliorated locomotor, cognitive and sensimotor deficits (Keene *et al*., 2001; Keene *et al*., 2002; Rodrigues *et al.*, 2000*a*). Additionally, it was shown that TUDCA has anti-apoptotic effects in experimental models of Alzheimer's disease. Notably, in addition to modulating upstream targets of apoptosis, TUDCA also modulates amyloid β-peptide-induced apoptosis by inhibiting mitochondrial permeabilization and subsequent cytochrome *c* release (Ramalho *et al.*, 2004, 2006; Rodrigues *et al.*, 2000*b*; Solá *et al.*, 2003). The neuroprotective action of TUDCA was also observed in *in vivo* models of stroke. In a rat model of cerebral ischemia, TUDCA was capable of reducing infarct volume, decreasing mitochondrial perturbations and to modulated apoptotic levels; and, in a rat model of intracerebral haemorrhage, TUDCA reduced brain injury through its anti-apoptotic action, and behavioural tests revealed an improved neurological function upon TUDCA administration (Rodrigues *et al.*, 2002, 2003). Importantly, TUDCA was shown to protect a genetic models of *Caenorhabditis elegans* from mitochondrial dysfunction, helped the survival and function of nigral transplant in a rat model of PD, and was effective against MPTP neurotoxicity, preventing MPTP-induced dopaminergic cell death in a mouse model of PD (Castro-Caldas *et al.*, 2012; Ved *et al.*, 2005). Finally, TUDCA was recently approved to be used in a pilot study for treatment of patients with amyotrophic lateral sclerosis, and data suggest that TUDCA may be effective in treatment of this disease (Elia *et al.*, 2015).

1.7 Aims

Herein we investigated whether autophagy/mitophagy and mitochondrial biogenesis, are part of the neuroprotective action of TUDCA against MPTP-induced neurodegeneration, using C57BL/6 mouse model. Then, to further characterize the molecular mechanisms involved in TUDCA-induced neuroprotection we used the human neuroblastoma cell line SH-SY5Y treated with MPP^+ in the presence or absence of TUDCA.

2. MATERIAL AND METHODS

2.1 Reagents

2.1.1 Supplements and chemicals

F12 Nutrient Mixture medium, Minimum Essential Medium, fetal bovine serum, streptomycin, penicillin, L-glutamine, non-essential amino acids and TryplE Express were acquired from GIBCO® (Life Technologies, Paisley, UK). Paraformaldehyde was obtained from Alfa Aesar® (Karlsruhe, Germany). MPTP, MPP^+, TUDCA, bovine serum albumin (fraction V) (BSA), Hoechst dye 33258, Mowiol mounting medium, Complete Mini Protease Inhibitor Cocktail and Amido black were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bio-Rad protein assay reagent was purchased from Bio-Rad Laboratories (Hercules, CA, USA), and Immobilon Polyvinylidene fluoride membrane from Milipore (Bedford, MA, USA). ECL Western blotting detection reagents were acquired from GEHealthcare (Buckinghamshire, UK). SuperSignal® West Femto Maximum Sensivity Substrate was purchased from Thermo Scientific (Rockford, USA). Mini protease inhibitors cocktail and Cytotoxicity detection kit (LHD) were obtained from Roche Diagnosis (Penzberg, Germany). SensiFAST™ SYBR® Hi-ROX kit was purchased from Bioline (London, UK). SuperScript® II Reverse Transcriptase was acquired from Invitrogen (Grand Island, NY, USA). Isol-RNA Lysis Reagent was obtained from 5 PRIME (Gaithersburg, US). Random hexamer primers and dNTPs were purchased from Promega (Madison, Wisconsin, USA). Cyto-ID® Autophagy detection kit (ENZ-51031-0050) was obtained from Enzo Life Sciences (Farmingdale, NY, USA). Mitotracker® Red CMXRos was from Molecular Probes® (Life Technologies, Eugene, Oregon, USA). Other chemicals and reagents were of the highest analytical grade and were purchased from local commercial sources.

2.1.2 Antibodies

Table 2.1 Primary antibodies

Antibody	Host	Preparation	Dilution	Brand
Anti-β-Actin	Mouse	5% non-fat dry milk in tris-buffered saline with 0.1%Tween-20 (TBS-T 0.1%)	1:40000	Santa Cruz Biotechnology® (Santa Cruz, CA, USA)
Anti-AMPKα	Rabbit	5% BSA in TBS-T 0.1%	1:1000	Cell Signaling Technology® (Beverly, MA, USA)
Anti-LC3I + LC3II (PA1-16931)	Rabbit	5% non-fat dry milk in TBS-T 0.1%	1:2000	Thermo Fisher Scientific (Rockford, IL, USA)
Anti-P-AMPKα	Rabbit	3% BSA in TBS-T 0.1%	1:1000	Cell Signaling Technology® (Beverly, MA, USA)
Anti-PARIS	Rabbit	3% BSA in TBS-T 0.1%	1:1000	Abcam® (Cambridge, UK)
Anti-Parkin (phosphor S65)	Rabbit	3% BSA in TBS-T 0,1%	1:300	Abcam® (Cambridge, UK)
Anti-Parkin (Prk8)	Mouse	3% BSA in TBS-T 0.1%	1:1000	Cell Signaling Technology® (Beverly, MA, USA)
Anti-PINK1	Rabbit	3% BSA in TBS-T 0.1%	1:1000	Novus Biologicals (Littleton, CO, USA)
Anti-VDAC	Rabbit	5% BSA in TBS-T 0.1%	1:1000	Cell Signaling Technology® (Beverly, MA, USA)

Table 2.2 Secondary antibodies

Antibody	Host	Preparation	Dilution	Brand
Horseradish peroxidase-conjugated anti-mouse IgG	Goat	3% non-fat dry milk in TBS-T 0.1%	1:5000	Invitrogen/Molecular Probes® (Eugene, OR, USA)
Horseradish peroxidase-conjugated anti-rabbit IgG	Goat	3% non-fat dry milk in TBS-T 0.1%	1:5000	Invitrogen/Molecular Probes® (Eugene, OR, USA)

2.2 Methods

2.2.1 Animals and treatments

All animal experiments were carried out in accordance with the institutional, Portuguese and European guidelines (*Diário da República, 2.ª série N.º121* of 27 June 2011; and 2010/63/EU European Council Directive) and methods were approved by the Direcção Geral de Alimentação e Veterinária (DGAV, reference 021943) and the Ethical Committee for Animal Experimentation of the Faculty of Pharmacy, University of Lisbon.

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

Mice were divided in five groups:

I) TUDCA-treated mice: TUDCA was dissolved in phosphate buffer saline (PBS) pH 7.4 and was injected intra-peritoneally (i.p) (50mg/kg body weight) for three consecutive days. Mice were sacrificed on day 3, 6 h after the last TUDCA injection;

II) MPTP-treated mice: MPTP was administrated i.p. at a single dose (40 mg/kg body weight), and mice were sacrificed 3 or 6 h after MPTP injection;

III) Mice treated with TUDCA prior to MPTP administration: TUDCA was administrated for three consecutive days and on day 3, 6 h after the last TUDCA injection, MPTP was injected. Mice were sacrificed 3 or 6 h after the MPTP treatment;

IV) Mice treated with MPTP prior to TUDCA administration: MPTP was injected on day 1 and, 3 or 6 h after MPTP treatment, TUDCA was administrated for three consecutive days. Mice were sacrificed 6 h after the last TUDCA injection;

V) Control mice: control mice received saline alone and were sacrificed at the same time as TUDCA-treated mice.

The schematic chronogram of TUDCA and MPTP administration is illustrated in Figure. 2.1 and studies were carried using groups of three mice per condition.

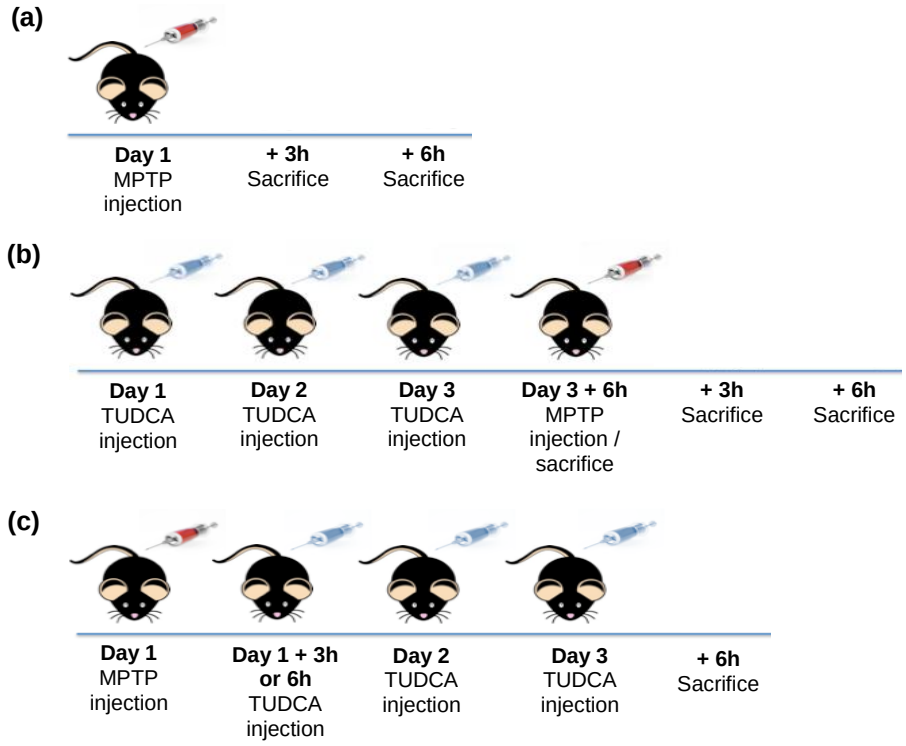


Figure 2.1 - Schematic representation of animal treatment. MPTP-treated C57BL/6 male mice were i.p. injected with MPTP at a single dose of 40 mg/kg body weight and sacrificed 3 or 6 hours after injection (a). TUDCA administration began on day 1 (50 mg/kg body weight) and was i.p. injected for three consecutive days. On day 3, 6 hours after the last injection of TUDCA, MPTP was administrated i.p at a single dose and TUDCA-treated mice were sacrificed. TUDCA+MPTP-treated mice were sacrificed 3 or 6 hours after MPTP injection (b). MPTP was injected at a single dose on day 1 and after 6 hours TUDCA was i.p administrated. TUDCA was injected for three consecutive days and mice were sacrificed 6 hours after the las injection (c).

2.2.2 Cell culture and treatments

The human neuroblastoma cell line SH-SY5Y was obtained from American Type Culture Collection (ATCC). Cells were cultured in Minimum Essential Medium:F12 Nutrient Mixture supplemented with 2mM L-glutamine, 1% of non-essential amino acids, 1% penicillin/streptomycin and 15% of fetal bovine serum, and maintained at 37°C, with 5% CO_2, in a humidified atmosphere, in a Heracell™ 150i CO_2 incubator (Thermo Scientific, Rockford, USA).

SH-SY5Y cells were plated at a density of 2 x 10^4 cells/ ml for cell death evaluation assays, at 1.5 x 10^4 cells/ ml for mRNA and proteins isolation, at 5 x 10^4 cells/ ml for specific labelling of autophagic vacuoles, and at 2 x 10^5 cells/ ml to stain metabolically active mitochondria. After seeded, neuroblastoma cells were treated as follows: (1) TUDCA plus MPP^+-treated cells were incubated in medium supplemented with TUDCA (100 µM) for 12 h, and further incubated with MPP^+ (1mM) for 48 or 72 h. (2) MPP^+-treated cells were incubated with the neurotoxin for 48 or 72h. (3) Control cells include cells treated with vehicle, and cells treated with TUDCA alone. Time points indicate time after MPP^+ addition to the medium. The schematic chronogram of neuroblastoma cells treatment with TUDCA and/or MPP^+ is illustrated in Figure. 2.2.

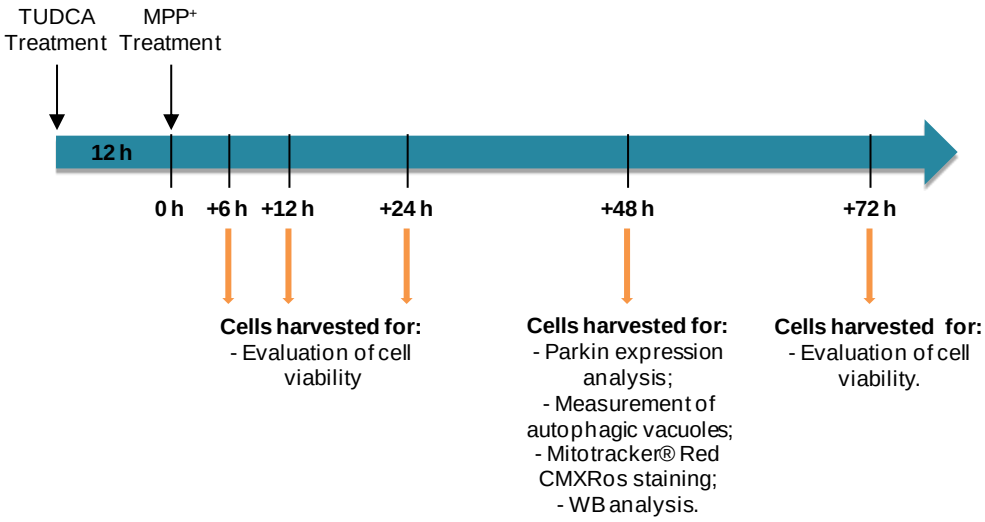


Figure 2.2 - Schematic representation of SH-SY5Y cells treatment. Once seeded, cells were treated with TUDCA (100 µM) and/ or MPP^+ (1mM) 12 h after TUDCA administration. Cells were collected in different time points after MPP^+ administration: 6,12,24 and 72h after for evaluation of cell viability, and 48 h after for parkin expression analysis, autophagic vacuoles measurement, Mitotracker® Red CMXRos staining and Western Blot analysis. Control cells were collected at the same time as MPP^+-treated cells, and include cells treated with vehicle and cells treated with TUDCA.

2.2.3 Evaluation of cell viability

Cell death was evaluated through assessment of lactate dehydrogenase (LDH) release using the Cytotoxicity detection kit. Briefly, SH-SY5Y cells were plated in a 96-well plate and after 10 hours were incubated with MPP^+ at different time points (6 h, 12 h, 24 h and 72h), or with TUDCA and/or MPP^+ for 72 h, as described. Culture medium was replaced before TUDCA addition. After treatments, the plate was centrifuged at 250 *g* for 10 min using Centrifuge 5810R (Eppendorf, Hamburg, Germany). Then 50µl of cell-free culture supernatant was collected and transferred to a new microplate. Finally 50µl/ well of reaction mixture (prepared immediately before use according to the manufacturer's instructions) was added to the cell-free culture supernatant, followed by an 20 min incubation in dark. Absorbance was measured at 492nm using a microplate reader Model 680 (Bio-Rad Laboratories, Hercules, CA, USA). Additionally, before adding the reaction mixture to the cell-free supernatant, the absorbance was measured and used as blank. As a positive control of LDH release, cells treated with vehicle were lysed with the lysis buffer provided in the kit. Results are expressed as percentage of control.

2.2.4 RNA extraction

SH-SY5Y cells were plated in 60x15 mm plates, and treated as previously described with TUDCA and/or MPP^+ for 48h. Culture medium was replaced immediately before MPP^+ addition. Total RNA was isolated using Isol-RNA Lysis Reagent following manufacturer's instructions. Briefly, cells were washed with PBS, 500µl of Isol-RNA Lysis Reagent was added, and plates were stored at -80°C

until further use. The plates were then thawn, samples were collected, homogenized and incubated at RT, for 5 min. Then 0.2 ml of chloroform per ml of Isol-RNA Lysis Reagent was added, samples were vortexed 15 s and further incubated at RT for 3 min. After being centrifuged 12000 x *g* for 15 min, at 4°C, the aqueous phase (containing RNA) was collected and the interphase and organic phase discarded. Then, 0.5 ml of isopropanol per ml of Isol-RNA Lysis Reagent was added, mixed by inversion and incubated 10 min at RT. Again, after being centrifuged at 12000 x *g* for 10 min, at 4°C, the supernatant was discarded, and 0.8 ml of 70% ethanol per ml of Isol-RNA Lysis Reagent was added and samples centrifuged at 7500 x *g* for 5 min, at 4°C. Finally, the supernatant was again discarded and the pellet was dissolved in RNase-free water. Samples were quantified using NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Rockford, IL, USA). RNA integrity was confirmed in 1% agarose gel.

2.2.5 Expression analysis

To analyse parkin (*PARK2)* expression, 1.5 µg of RNA was reversed transcribed using SuperScript II reverse-transcriptase and random hexamer primers, in UnoCycler Thermal Cycler (VWR, Pennsylvania, USA). First strand DNA from 1.5 µg of RNA was used as a template in quantitative Real-Time polymerase chain reaction (qRT-PCR) using SensiFAST™ SYBR® Hi-ROX kit, performed on a 7300 Real-Time PCR System (Applied Biosystems, CA, USA). The cycling program was set as follows: 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 45°C for 2 s and 62°C for 30 s. The sequences used as primers were: Park 2 fwd 5' CCCACCTCTGACAAGGAAACA 3' and rev 5' TCGTGAACAAACTGCCGATCA; β-actin fwd 5' CTGGAACGGTGAAGGTGACA 3' and rev 5' AAGGGACTTCCTGTAACAATCCA 3'. Results presented are from at least two independent experiments. Parkin mRNA levels were normalized to the level of β-actin and expressed as fold change from controls, using the ΔΔCt method.

2.2.6 Measurement of autophagic vacuoles

To specifically label autophagic vacuoles Cyto-ID® Autophagy detection kit (ENZ-51031-0050) was used. The procedures of the assay were conducted as described in the manufacturer's protocol for microplate reader. Cells were seeded in a 96-well plate, and 36 h after culture medium was replaced and cells were treated with TUDCA and/or MPP^+ for 48 h, as described. After rising the cells with 1X Assay Buffer, cells were incubated with Cyto-ID® Green dye for 30 min, at 37°C in dark. Fluorescence was measured by Glomax® Multidetection System (Promega, Madison, Wisconsin, USA) in assay buffer, at excitation and fluorescence emission wavelengths 463nm and 534nm, respectively. The measured fluorescence was normalized with the total protein content in the correspondent well. As a positive control, cells were treated with two autophagy inhibitors, rapamycin and hydroxychloroquine, provided in the kit. Results are expressed as percentage of control.

2.2.7 Labelling of mitochondria

SH-SY5Y cells were plated in 35x10 mm plates and treated with TUDCA and/or MPP^+ for 48 h as described above. Culture medium was replaced immediately before MPP^+ addition. Metabolically active mitochondria were stained with Mitotracker® Red CMXRos (50nM) for 30 min at 37°C in dark. Cells were then fixed with 4% paraformaldehyde in PBS for 10 min, incubated with Hoechst dye 33258 (1:500) in PBS for 5 min and plates were mounted with anti-fading Mowiol mounting medium. Red fluorescence and UV images of at least eight random microscopic fields were acquired per sample under model AxioScope A1 (Carl Zeiss,Inc, North America) with integrated camera (AxioCCamHRm), using ZEN Lite 2012 software. Fluorescence intensity was quantified by ImageJ software and normalized to the total number of cells. Results are expressed as percentage of control.

2.2.8 Western blot analysis

After being anesthetized with sodium pentobarbital (50 mg/kg i.p.), mice were decapitated and brains were quickly removed and placed in ice-cold freshly made PBS. The entire midbrain region containing SNpc, and the whole striatum were dissected as described in Castro-Caldas *et al.* (2009). Samples were stored at -80ºC until further use. Protein extracts were prepared from striatum and midbrain-enriched brain fragments. Briefly, fragments were homogenised in PBS using a tissue grinder and centrifuged at 3000 rpm for 10 min, at 4ºC. The supernatant was discarded and the pellet suspended in lysis buffer (20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM Na2EDTA, 1mM EGTA, 1% Triton, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM Na_3VO4, 1µg/ml leupeptin) supplemented with Complete Mini Protease Inhibitor Cocktail, 200mM Na_3VO_4 and 1M NaF, and further incubated 30 min on ice. After six times sonication for 5s each, samples were centrifuged at 13000 rpm for 15 min, at 4ºC, and the supernatants collected and stored at -80°C.

SH-SY5Y cells were plated in 60x15 mm plates and treated with TUDCA and/or MPP^+ for 48h. Cells were then lysed in ice-cold lysis buffer (50mM Tris-HCl pH 7,4, 180 mM NaCl, 1mM EDTA and 1% Triton-X 100) plus Complete Mini protease inhibitors cocktail, 200mM Na_3VO_4, 1M NaF, 1M DTT, and incubated on ice for 30 minutes. After sonication three times, for 5 sec each, on ice, samples were centrifuged at 13,000x*g* for 10 min, at 4°C, and the supernatants were collected and stored at -80°C.

Protein concentration was determined using the Bradford method according to the manufacturer instructions. Brain tissue extracts (100 µg) and SH-SY5Y protein extracts (25 µg) were added (5:1) to denaturing buffer (0.25 mMTris-HCL, pH 6.8, 4% SDS, 40% glycerol, 0.2% bromophenol blue, 1% β-mercaptoethanol), boiled for 5 min, resolved on 12,5% or 10% sodium dodecyl sulphate-polyacrilamide gel electrophoresis with a fixed amperage of 35 mA per gel (20 mA per gel for mini-gel), and electrotransfered to polyvinylidene fluoride membrane with a fixed amperage of 500 mA (200mA for mini gel). The membrane was then stained with Amido black 1X for 4 min, washed with distaining solution (25% isopropanol and 10% acetic acid) for 5 min, and dried at RT. After re-hydration with ethanol 1 min, wash with water 2 min and equilibrate for 5 min in TBS-T 0.1%, the membrane was blocked with 5% non-fat dry milk in TBS-T 0.1% for 1 h at RT and then incubated with

the specific primary antibodies, overnight at 4°C, with shacking. After washing with TBS-T 0.1%, the membranes were incubated with the specific secondary antibody for 1 h, at RT. After washing the membranes with TBS-T 0.1%, the immunocomplexes were visualized by chemiluminescent detection with ECL Western blotting detection reagent or SuperSignal® West Femto Maximum Sensivity Substrate in a ChemiDoc™ MP imaging system from Bio-Rad Laboratories (Hercules, CA, USA). β-actin expression was used as a loading control. The relative intensities of protein bands were analysed using the Image Lab™ analysis software from Bio-Rad Laboratories (Hercules, CA, USA).

2.3 Statistical analysis

All results are expressed as mean ± SEM. Data were analysed by one-way ANOVA and differences between groups were determined by post hoc Bonferroni's test (GraphPad, Prism 5.0, San Diego, CA, USA). Mean differences were considered statistically significant at a *p* value below 0.05. Importantly, statistical analysis of preliminary results with less than three independent experiments were not performed.

3. RESULTS

3.1 Evaluation of TUDCA effect on autophagy/ mitophagy and mitochondrial biogenesis associated proteins in C57BL/6 male mice brain

3.1.1 Effect of TUDCA on full-length PINK1, Parkin and VDAC protein expression

We started to investigate the possible role of TUDCA on mitophagy activation using C57BL/6 mice model. This strain was found to be the most sensitive to systemic administration of MPTP and the more selective in terms of targeting nigrostriatal dopaminergic neurons, being nowadays a established useful animal model of PD (Schober, 2004). For this we assessed whether TUDCA modulates PINK1 and parkin protein expression levels in the presence or absence of MPTP, in mice midbrain and striatum. Results from Figure 3.1a show that at the conditions and time points evaluated, neither TUDCA nor MPTP alone altered full-length PINK1 protein levels in the striatum. However, an increase in the expression levels of full-length PINK1 was observed in TUDCA plus MPTP-treated mice, 3 and 6 h after the insult, and in MPTP plus TUDCA-treated mice, 6 h after the neurotoxin administration, although the values did not reach statistical significance. In midbrain samples, the response was more exacerbated than in striatum, and full-length PINK1 expression was increased in mice treated with TUDCA or MPTP. Importantly, full-length PINK1 levels increase more strikingly in mice treated with TUDCA prior to MPTP, 6 h upon the insult (Fig. 3.1b).

Concerning parkin expression, results show an increase in protein levels in mice treated with TUDCA prior to MPTP administration, 3 h after the insult, in both striatum and midbrain (Fig. 3.1c/d).

Simultaneously, VDAC protein expression was analysed in these tissues extracts. In accordance to the previous observations, expression levels of VDAC increased more strikingly in the midbrain than in striatum (Fig. 3.1e/f). A significant increase in VDAC levels was observed in TUDCA plus MPTP-treated mice, 3 h after the insult, when comparing with control ($p<0.05$), and with the corresponding 3 h MPTP-treated mice ($p<0.05$), in striatum. Mice treated with TUDCA prior to MPTP administration, 3 or 6h, demonstrated higher VDAC expression levels in the midbrain when compared with control and the correspondent MPTP-treated mice. Interestingly, TUDCA administration alone increased VDAC protein expression in both brain regions.

Taken together, these results show that pre-treatment with TUDCA increases the levels of full-length PINK1, together with parkin and VDAC expression, even though with different dynamics and degrees in both striatum and midbrain. These findings suggest that TUDCA might modulate mitochondrial dynamics through PINK1/parkin-dependent mitophagy, and parkin-dependent

mitochondrial biogenesis in this PD model. However, to confirm results here presented from midbrain, corroborative experiments are ongoing.

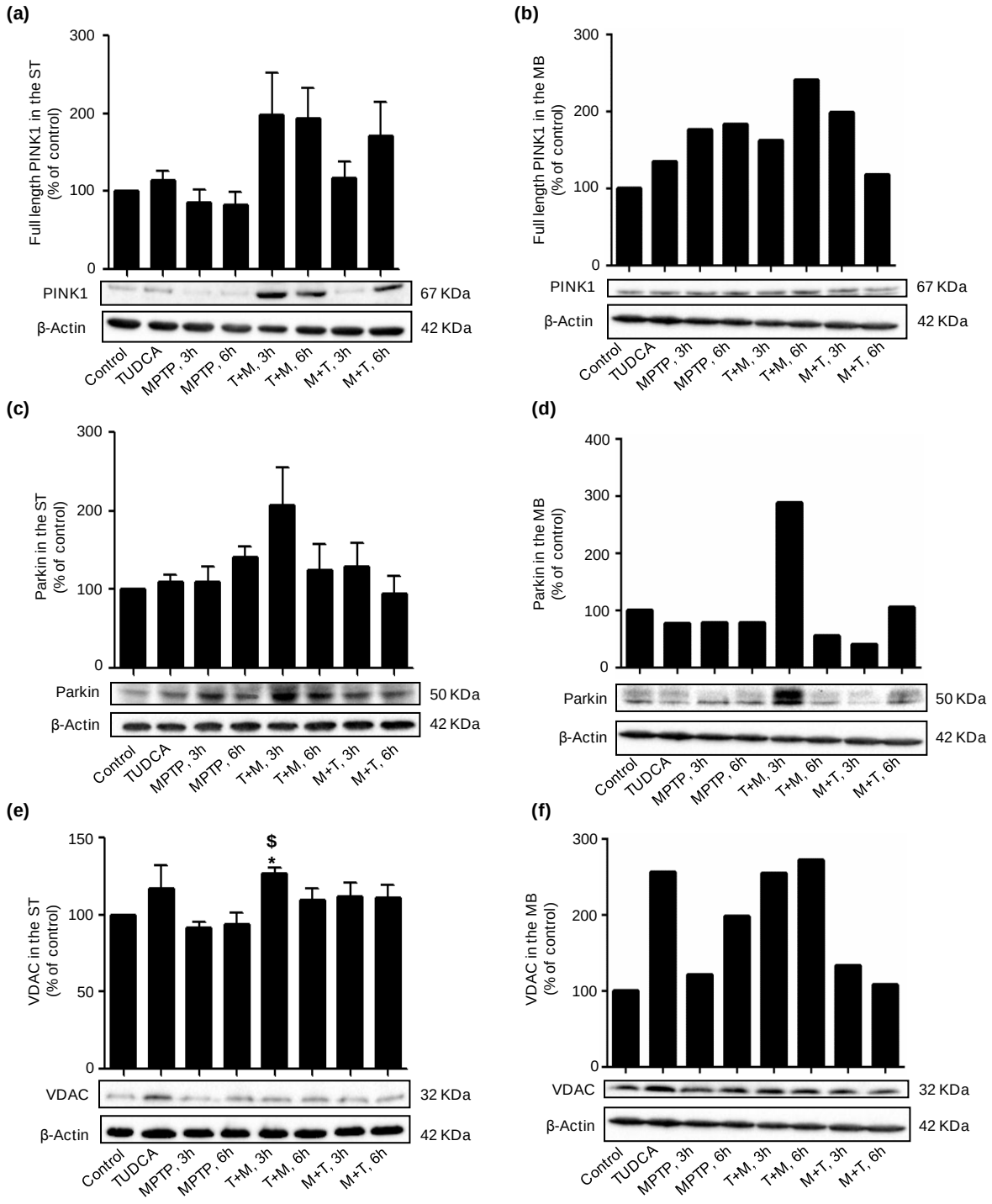


Figure 3.1- TUDCA induces full-lenght PINK1, parkin and VDAC protein expression. C57BL/6 male mice were treated with saline (control), TUDCA, MPTP, TUDCA+MPTP (T+M) or MPTP+TUDCA (M+T) as described in Materials and Methods. Tissue extracts from striatum (ST) (a,c,e) and midbrain (MB) (b,d,f) were subjected to Western blot analysis using anti-PINK1, anti-parkin and anti-VDAC antibodies. β-actin as used as loading control. The immunoblots shown are representative of the results obtained in diferent experiments, and are shown under the plotted quantification of protein levels. Protein levels of control mice were set as 100% and alterations in protein levels were calculated and plotted as a percentage of this value. Results are indicated as percentage of the respective control. Data shown from striatum are mean values ±SEM of at least three independent experiments (*p<0.05 vs control and \$$p$<0.05 vs MPTP).

3.1.2 Effect of TUDCA on LC3 Lipidation

To further elucidate whether TUDCA is involved in autophagy/mitophagy modulation, we investigated the ability of TUDCA to regulate LC3 lipidation in the presence or absence of MPTP. The levels of LC3 lipidation were determined using the ratio of the relative intensity of immunoblot bands, LC3-II, the lower molecular weight band, and LC3-I. Notably, lipidation of LC3 seems to increase in striatum from mice treated with MPTP for 6 h, and this is exacerbated when animals were pre-treated with TUDCA, where an evident peak is observed (Fig. 3.2a). On the other hand, in the midbrain, LC3 lipidation in mice treated with TUDCA in the presence or absence of MPTP was higher than in control and in MPTP-treated mice, but with no apparent peak in any time point evaluated (Fig. 3.2b).

Although preliminary, effects of TUDCA on LC3 lipidation suggest that this bile acid induces autophagy in this model of PD. To confirm these results, corroborative experiments are ongoing.

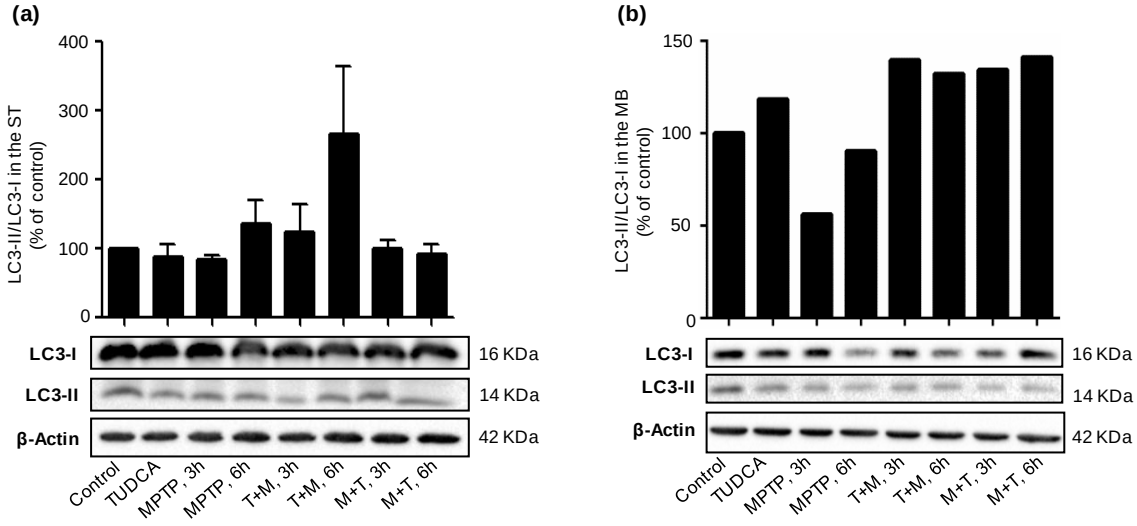


Figure 3.2 - TUDCA modulates LC3 lipidation. C57BL/6 male mice were treated with saline (control), TUDCA, MPTP, TUDCA+MPTP (T+M) or MPTP+TUDCA (M+T) as described in Material and Methods. Tissue extracts from striatum (ST) (a) and midbrain (MB) (b) were subjected to Western blot analysis using anti-LC3 and β-actin (as loading control) antibodies. LC3 lipidation was calculated using the ratio LC3-II/LC3-I. Representative immunoblots from each protein are shown under the correspondent graph,which are indicated in percentage of the respective control. Data shown from striatum are mean values ±SEM of at least three independent experiments.

3.1.3 Effect of TUDCA on AMPK phosphorylation

As MPTP neurotoxicity leads to cellular ATP depletion, to understand whether the alterations previously observed on LC3 lipidation could be due to AMPK activation, we determined the phosphorylation levels of AMPK (p-AMPK) and native AMPK, by Western blot analysis. Results from Figure 3.3a show that, in striatum, the levels p-AMPK are higher in mice treated with TUDCA and/or MPTP than those found in control. Interestingly, the results suggest higher levels of activated AMPK in

striatum of mice treated with TUDCA before or after MPTP. Even though in the striatum results suggest an increased TUDCA-dependent AMPK activation, in the midbrain the same trend was not observed (Fig. 3.3b).

The lack of statistical significance in these results, together with the discrepancies observed between striatum and midbrain samples imply that these assays have to be repeated with another batch of samples. However, in the striatum, results strongly point to an induction of AMPK activation when animals are treated with TUDCA in any condition evaluated, which is in accordance with the previous results regarding autophagy.

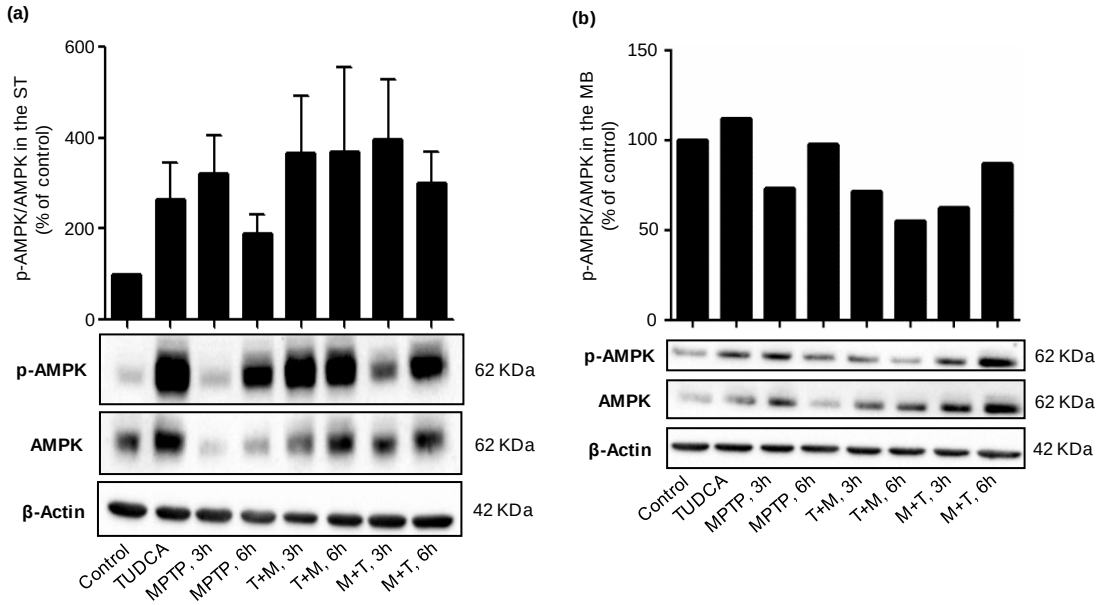


Figure 3.3 – TUDCA-dependent AMPK phosphorylation. C57BL/6 male mice were treated with saline (control), TUDCA, MPTP, TUDCA+MPTP (T+M) or MPTP+TUDCA (M+T) as described in Materials and Methods. Tissue extracts from striatum (ST) (a) and midbrain (MB) (b) were subjected to Western blot analysis using anti-pAMPKα, anti-AMPKα and β-actin antibodies. Representative immunoblots from each protein are shown under the correspondent graph, which are indicated in percentage of control. Data shown are mean values ±SEM of at least three independent experiments.

3.1.4 Effect of TUDCA on PARIS expression

To evaluate if TUDCA protective mechanisms depend on the modulation of mitochondrial biogenesis via degradation of PARIS by parkin, the expression levels of PARIS was evaluated by Western blot. PARIS expression was analysed only in midbrain, since by the time we had access to the anti-PARIS antibody, we had no time to analyse the striatum samples. Moreover, results shown are from one single experiment. However, our preliminary results suggest that TUDCA or MPTP induce a decrease in PARIS protein expression of about 56% and 82% of control, respectively (Fig. 3.4). Simultaneously, in TUDCA plus MPTP-treated mice the amount of PARIS protein attained the lowest expression levels, especially 6h after MPTP administration, were it reached a value of 6 % of control.

Even though these results are clearly preliminary, the decrease in PARIS levels is in accordance to the increased parkin and VDAC expression previously observed. Further work will be necessary to determine the accuracy of these data.

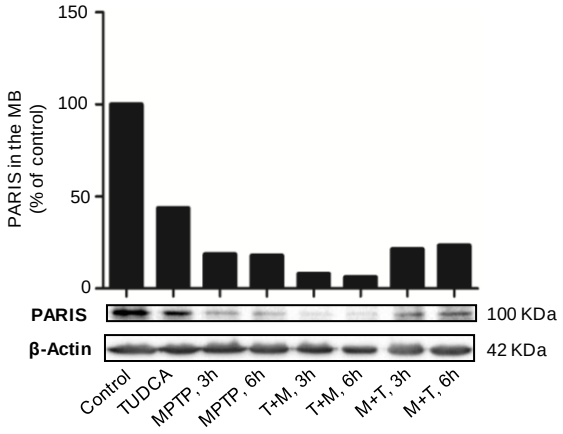


Figure 3.4 - TUDCA modulates PARIS protein expression. C57BL/6 male mice were treated with saline (control), TUDCA, MPTP, TUDCA+MPTP (T+M) or MPTP+TUDCA (M+T) as described in Materials and Methods. Tissue extracts from midbrain (MB) were subjected to Western blot analysis using anti-PARIS antibody. β-actin as used as loading control. Data shown are from one independent experiment. Results are expressed in percentage of control.

3.2 Molecular mechanisms involved in TUDCA protective action against MPP^+-induced cell death

3.2.1 Protective effect of TUDCA against MPP^+-induced cell death, in SH-SY5Y cells

To further discuss the molecular mechanisms that may be involved in TUDCA-mediated neuroprotection we used a human neuroblastoma cell line SHSY5Y, once it has been widely used to investigate disease mechanisms at a cellular level, and is suited for PD investigation as, when in the undifferentiated state, it presents the phenotype of immature catecholaminergic neurons (Constantinescu *et al*., 2007; Dayem *et al*., 2014; Krishna *et al*., 2014). Moreover, SHSY5Y cells present stem cells properties and can be maintained in a undifferentiated state (Dayem *et al*., 2014).

First we investigated MPP^+ (the active metabolite of MPTP)-induced cell death at different time points. Cell viability was assessed by the determination of the amount of LDH release to the cell culture supernatant. LDH is a stable cytoplasmic enzyme that is released to the cell culture supernatant when plasma membrane is damaged due to cell death. Results from Figure 3.5a show that MPP^+ only induced significantly differences in LDH release from control at 72 h ($p<0.05$).

To further investigate whether TUDCA exerts a protective action against MPP^+-induced cell death, SH-SY5Y cells were treated with TUDCA and/or MPP^+ as described in Materials and Methods. Results from Figure 3.5b show that MPP^+ significantly induced LDH release to 128% of control ($p<0.05$). Interestingly, pre-treatment with TUDCA significantly prevented MPP^+-induced LDH release ($p<0.01$), reaching levels similar to control.

These results indicate that MPP^+ significantly induces death of SH-SY5Y cells, which is completely abrogated when cells are pre-treated with TUDCA, showing a clear protective role of this bile acid.

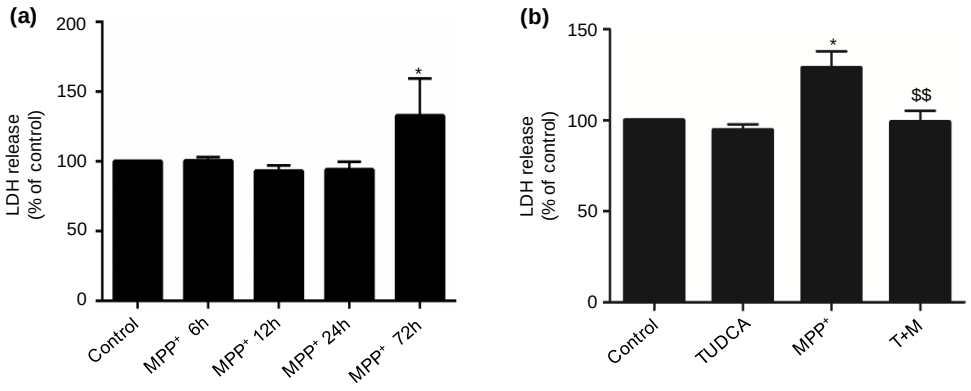


Figure 3.5 - TUDCA protects SH-SY5Y cells from MPP$^+$-induced cell death. (a) SH-SY5Y cells were treated with MPP$^+$ (1mM) at different time points namely, 6 h, 12 h, 24 h and 72 h. (b) SH-SY5Y cells were treated with TUDCA (100µM), MPP$^+$ (1mM) or TUDCA plus MPP$^+$ (T+M) for 72 h as described in Material and Methods. Cell death was acessed by the amount of LDH released to the cell culture supernatant. Data shown are mean values ± SEM of at least three independent experiments, expressed as percentage of the respective control (*p<0.05 vs control and \$\$$p$<0.01 vs MPP$^+$).

3.2.2 Effect of TUDCA on parkin expression and phosphorylation in SH-SY5Y cells

Our previous results showed that, in the animal model of PD, parkin protein expression was highly increased by TUDCA. Therefore, to further investigate TUDCA action on parkin protein expression, Western blot analysis was also performed in SH-SY5Y cells. Our preliminary results (Fig. 3.6a/b) indicate that parkin protein expression levels are increased in MPP$^+$-treated cells. Interestingly, and in accordance to what we found in animals, pre-treatment with TUDCA further increased parkin protein expression.

To understand whether TUDCA is able to modulate parkin expression at mRNA level, SH-SY5Y cells were treated with TUDCA (100 µM) and/ or MPP$^+$ (1mM) 12 h after TUDCA administration, and parkin mRNA levels were evaluated by qRT-PCR. Our results demonstrate that MPP$^+$ significantly increased parkin mRNA levels by 2 fold, when compared to control (Fig. 3.6c). Importantly, pre-treatment with TUDCA further increased parkin mRNA levels.

Together, these results strengthen the observations obtained in the *in vivo* experiments, and further confirm the ability of TUDCA to induce parkin expression in different models. As previously mentioned, parkin E3 ligase activity requires activation. Accumulated full-length PINK1 on the outer mitochondrial membrane is able to phosphorylate parkin at the Ser65 residue, thus activating it. Consequently, to explore whether TUDCA modulates parkin activation, parkin phosphorylation status was evaluated by Western blot analysis, using a specific antibody that recognizes phosphorylated Parkin at Ser 65 (Fig. 3.6a/d). Although preliminary, our results show that phosphorylated parkin (p-parkin) is increased relatively to control in all the conditions tested, being strongly elevated in TUDCA treated cells (Fig. 3.6d).

These results suggest that parkin is phosphorylated on Ser65 upon TUDCA or/and MPP^+ treatments; however, further assays are needed to clarify the conditions when an increased phosphorylation occurs.

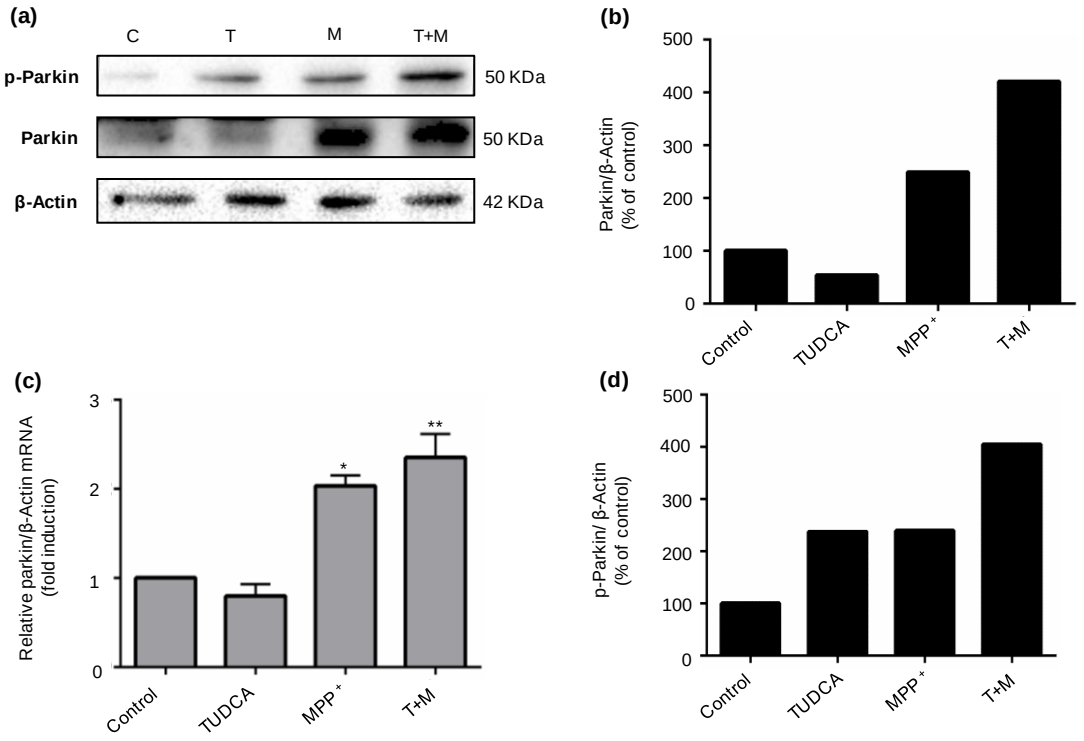


Figure 3.6 - TUDCA modulates parkin protein expression and phosphorylation in human neuroblastoma cells. SH-SY5Y cells were treated with TUDCA (T), MPP^+ (M) or TUDCA+MPP^+ (T+M) for 48hours as described in Material and Methods. (a) Representative immunoblot of phosphorylated parkin (p-Parkin) and total parkin protein expression. (b) Quantification of the relative levels of total parkin. β-Actin was used as loading control. Data represent mean values of two independent experiments, indicated as percentage of control. (c) Real-time PCR analysis of parkin mRNA levels. Values were normalized to the internal standard β-actin and expressed as relative fold change relative to control cells Data represent means ± SEM of at least three independent experiments (*p<0.05 and **p<0.01 vs control). (d). Quantification of the relative levels of phosphorylated parkin (p-parkin). β-Actin was used as loading control. Data represent mean values of two independent experiments, indicated as percentage of control.

3.2.3 Effect of TUDCA in modulation of autophagy/mitophagy in SH-SY5Y cells

Taking in consideration the results obtained with SH-SY5Y cells, and to further confirm the ability of TUDCA to induce autophagy, we used the Cyto-ID® Autophagy Detection Kit for monitoring autophagic activity in cells treated with TUDCA and/or MPP^+. This kit contains a green fluorescent dye that specifically labels autophagic vacuoles produced during autophagy. Results show that TUDCA significantly increased Cyto-ID fluorescence (p<0.05) (Fig. 3.7). Even though MPP^+ increased Cyto-ID fluorescence pre-treatment of neuroblastoma cells with TUDCA further and significantly increased dye fluorescence, compared with control and MPP^+-treated cells (p<0.001 and p<0.01).

Our results show that the treatment with the different compounds increased autophagy vacuoles, particularly when SH-SY5Y cells were treated with TUDCA in the presence or absence of MPP^+.

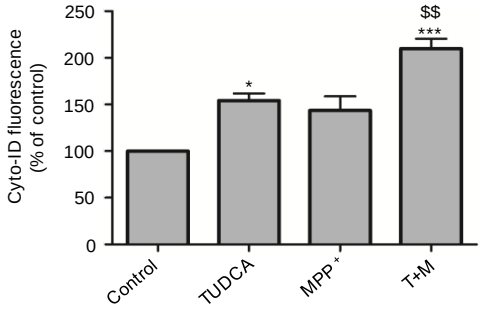
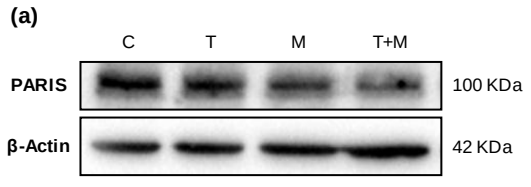


Figure 3.7 - TUDCA upregulates autophagy/mitophagy in SH-SY5Y MPP^+-treated cells. SH-SY5Y cells were treated with TUDCA (T), MPP^+ (M) or TUDCA+MPP^+ (T+M) for 48hours as described in Material and Methods.. Autophagic vacuoles were specifically labeled using Cyto-ID® Autophagy detection kit and fluorescence was measured. Fluorescence was normalized with the total protein content of each sample. Data shown are mean values ± SEM of at least three independent experiments, indicated as percentage of the respective control (*p<0.05 and ***p<0.001 vs control and $$$p$<0.01 vs MPP^+).

3.2.4 Effect of TUDCA in PARIS protein expression in SH-SY5Y cells

Afterwards, we decided to evaluate PARIS protein levels in SH-SY5Y cells treated with TUDCA and/or MPP^+. In agreement to what was observed *in vivo*, our preliminary results indicate that PARIS levels decrease in all the conditions tested (Fig. 3.9), with the lowest expression observed in cells treated with TUDCA and MPP^+. These results are in accordance to our previous results regarding Parkin expression, as well as mitochondrial network preservation by TUDCA.



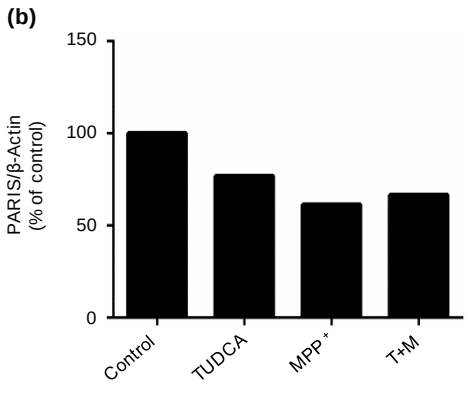


Figure 3.8 – TUDCA modulation of PARIS protein expression in neuroblastoma cells. SH-SY5Y cells were treated with TUDCA (T), MPP^+ (M) or TUDCA+MPP^+ (T+M) for 48hours as described in Material and Methods. (a) Western blot analysis of PARIS protein levels. (b) Quantification of the relative levels of PARIS protein. β-actin was used as loading control. Data represent mean values two independent experiments, indicated as percentage of control.

3.2.5 Effect of TUDCA on mitochondrial network in SH-SY5Y cells

The influence of TUDCA on mitochondrial network in SH-SY5Y cells was evaluated using Mitotracker® Red CMXRos, a red fluorescent probe that accumulates in active mitochondria. Results illustrated in Figure 3.8 show that treatment with MPP^+ significantly decreased Mitotracker fluorescence by 43% when compared to control ($p<0.01$). As can be observed in the microscopy images, MPP^+ induced mitochondrial network fragmentation, making it more diffuse, leading to the formation of localizes mitochondrial accumulation (Fig. 3.8a, white arrows). Importantly, even though some mitochondrial accumulation could still be found when cells were pre-treated with TUDCA, pre-treatment completely prevented the disruption of mitochondrial network, and abrogated the accentuated decrease of fluorescence observed in MPP^+-treated cells.

Together, these results show that TUDCA preserves mitochondrial network morphology, even in the presence of a toxic insult such as MPP^+.

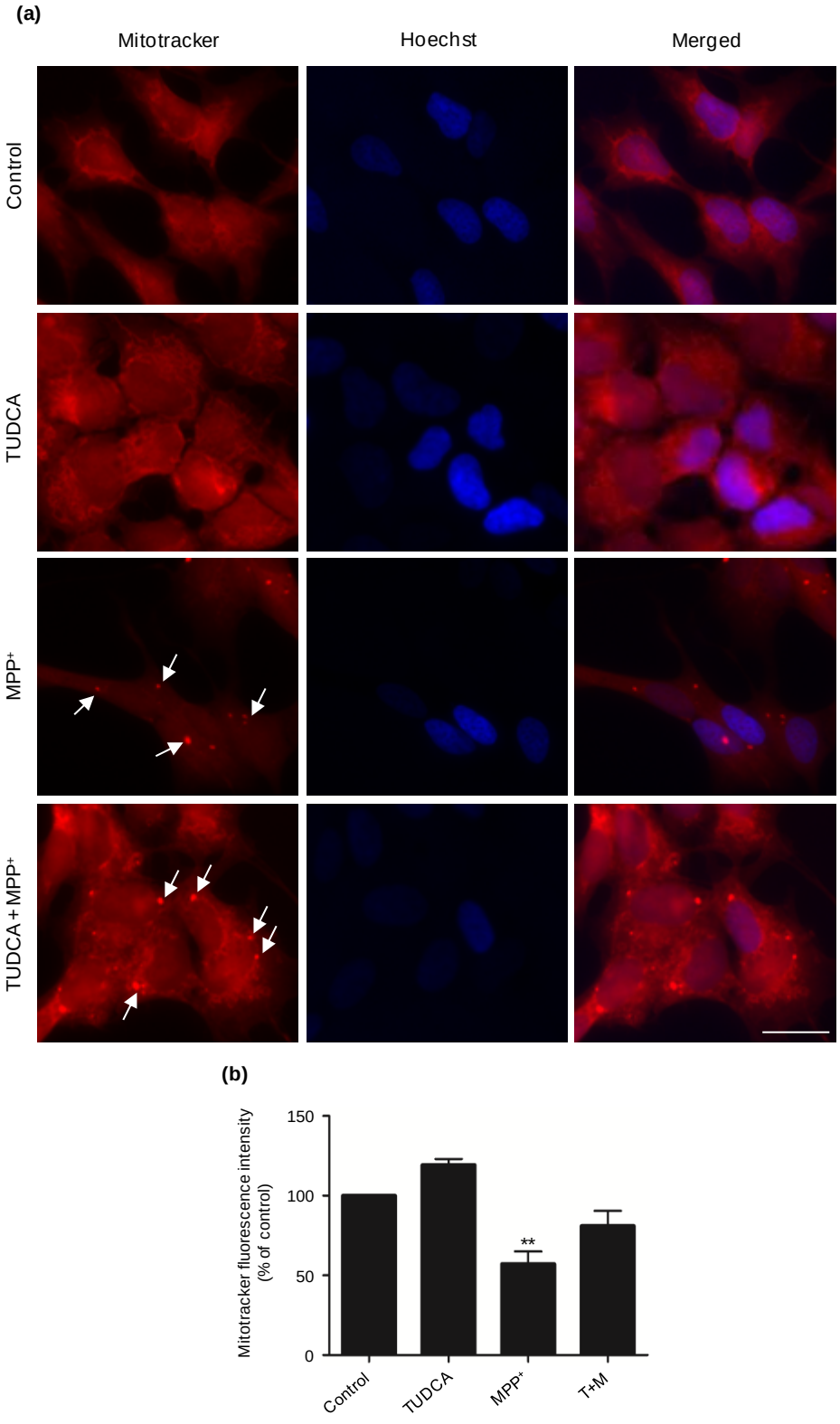


Figure 3.9- TUDCA prevents mitochondrial depletion in SH-SY5Y MPP$^+$-treated cells. SH-SY5Y cells were treated with TUDCA, MPP$^+$ or TUDCA plus MPP$^+$ (T+M) for 48hours as described in Material and Methods. (a) Metabolically active mitochondria were specifically stained with Mitotracker® Red CMXRos (50nM) and nuclei stained with Hoechst dye 33258.Whitte arrows point to mitochondrial accumulations. *Scale bar*= 20µm. (b) Relative fluorescence per cell was measured using ImajeJ software. Data shown are mean values ± SEM of at least three independent experiments, indicated as percentage of the respective control (**p<0.01 vs control).

4. DISCUSSION

Mitochondrial complex I dysfunction and oxidative stress are early events in the progression of PD (Keane *et al*., 2011). The identification of rare mutations in mitochondrial-associated genes, such as *PINK1* and *Parkin*, associated with familial cases of PD, as well as the neuropathological features of sporadic PD caused by the complex I inhibitor MPTP, further reinforce this idea (Zhu & Chu, 2010).

TUDCA is an endogenous bile acid, that has been described to have a neuroprotective role against MPTP-induced dopaminergic cell death, but the mechanisms involved are still unknown. In this context, we investigated part of the mechanisms underlying TUDCA neuroprotective action, using two different models, namely, MPTP-treated C57BL/6 mice and human neuroblastoma SH-SY5Y cells treated with MPP^+.

Here we demonstrate that pre-treatment with TUDCA increases autophagy/mitophagy-associated proteins expression in the brain of C57BL/6 mice, namely full-length PINK1, parkin and LC3-II, suggesting that modulation of PINK1/parkin-mediated mitophagy might be part of TUDCA neuroprotective mechanisms. These data are supported by an *in vivo* study conducted by Fang and collaborators (2013), that also demonstrate that TUDCA is able modulate autophagy in podocyte (kidney differentiated cells) under diabetic conditions. Moreover, the increase in VDAC protein levels induced by TUDCA may play an important part in its' neuroprotective role. In fact, Geisler and coworkers (2010) identified VDAC1 as a target of parkin E3 ligase on PINK1/parkin-mediated mitophagy. Thus, to further reinforce our hypothesis, it would be interesting to investigate the role of TUDCA on VDAC ubiquitination by parkin. Moreover, our results are supported by unpublished work from Castro-Caldas and colleagues, which demonstrated that in cells treated with Carbonyl cyanide *m*-chlorophenyl hydrazon, a mitochondrial uncoupler, pre-treatment with TUDCA induced both parkin expression and parkin translocation to mitochondria.

Compounds with antioxidant proprieties, other than TUDCA, have been shown to have a neuroprotective role in PD models. For instance, resveratrol is a polyphenol present in red grapes, red wine and peanuts, that has been demonstrated to have antioxidant proprieties, protecting cells from oxidative stress by preventing mitochondrial dysfunction through modulation of AMPK and AMPK downstream targets phosphorylation (Shin *et al.*, 2009). Importantly, it was also demonstrated that resveratrol is a protective agent against rotenone-induced apoptosis, by inducing autophagy through AMPK activation (Wu *et al.*, 2011). However, the role of TUDCA on AMPK activation, to our knowledge, has never been shown. In this context, we investigated whether TUDCA could be mediating autophagy/ mitophagy through AMPK activation. In fact, we found that the levels of phosphorylated AMPK were apparently increased by TUDCA in striatum, but not in midbrain samples.

Additionally, to further evaluate AMPK activation, we analysed the well-known downstream targets of this protein in the context of autophagy, namely phosphorylated ULK1 and phosphorylated raptor. However, these results were not elucidative due to very low expression of these proteins in mice brain (data not shown).

To complement and strengthen the results obtained in mice, we used a cell model with SH-SY5Y treated with MPP^+. Importantly, MPP^+ was found to significantly induce SH-SY5Y cell death 72h after treatment, which is in accordance with previous work of Itano & Nomura (1995).

Our preliminary data demonstrate that in human neuroblastoma cells TUDCA also up-regulates parkin protein levels in the presence of the neurotoxin, and additionally showed that TUDCA has the ability to modulate parkin mRNA levels in the presence of a mitochondrial insult like MPP^+.

Moreover, we also observed that TUDCA significantly up-regulated autophagy in SH-SY5Y cells, in the presence or absence of MPP^+, which is in accordance with the results obtained in mice brain. Importantly, TUDCA was shown to modulate parkin phosphorylation in Ser65 residue, in the presence or absence of MPP^+. Together these results suggest that increased levels of autophagy may be due to the activation of the PINK1/parkin pathway of mitophagy. This is supported by a study conducted by Shiba-Fukushima and co-workers (2012), that demonstrated that parkin phosphorylation on Ser65 by PINK1 plays an important role in mitophagy. As expected MPP^+ also increased autophagy levels, which is in agreement with what was previously demonstrated by Zhu and colleagues (2007), that showed MPP^+ increasing autophagy via extracellular signal-regulated protein kinase activation.

Coordination between clearance of damaged mitochondria by autophagy/ mitophagy and mitochondrial biogenesis is very important in the maintenance of mitochondrial and cellular homeostasis (Palikaras & Tavernarakis, 2014). Kuroda and coworkers (2006) found that parkin overexpression in human neuroblastoma cells enhanced mitochondrial biogenesis, providing evidence of a novel pathway regulated by this protein. The identification of PARIS protein, a negative regulator of PGC-1α, as a new substrate of parkin further reinforced this idea (J. H. Shin *et al*., 2011). Importantly, these authors also demonstrated the existence of interplay between this new subtract of parkin and PD. They found that in the cortex of AR-PD patients lacking functional parkin, levels of PARIS were higher than found in controls, and the same protein accumulation was found in SN of patients with sporadic PD. Although we presented preliminary data, our studies suggest that parkin up-regulation by TUDCA induced PARIS down-regulation in mice midbrain and in human neuroblastoma cells, associating the modulation of mitochondrial biogenesis as mechanism underlying TUDCA protection. However, it is unknown whether PINK1 is involved in parkin-dependent degradation of PARIS, or if an overexpression of parkin protein is sufficient to trigger the activation of this pathway (Castillo-Quan, 2011). Thus, it would be interesting to investigate the ability of parkin to induce PARIS proteasomal degradation, in cells with stable knockdown of PINK1.

Lanosterol, a cholesterol precursor, is known to protect dopaminergic neurons against MPTP-induced cell death (Lim et al., 2012). It was previously demonstrated that lanosterol induces mitochondrial uncoupling and subsequent clearance of defective mitochondria by autophagy/ mitophagy (Lim *et al.*, 2012). In this context, herein, we show in vivo that, TUDCA increased full-length

PINK1, VDAC and LC3 protein expression, and decreases PARIS protein expression. In agreement, in neuroblastoma cells, TUDCA significantly increases autophagy levels and also decreases PARIS protein expression. These data suggests that, like lanosterol, TUDCA may induce mitochondrial uncoupling of damaged mitochondria, up-regulating mitophagy and, in parallel, modulating mitochondrial biogenesis. Although this hypothesis needs further validation, we have already performed preliminary experiments using a voltage-sensitive fluorescent dye (data not shown), which revealed an increased mitochondrial depolarization upon TUDCA treatment.

In cells, mitochondria are organized in a dynamic network, which is maintained by processes of fission and fusion of mitochondria. These two processes can also be controlled by PINK1 and parkin proteins (Gaweda-Walerych & Zekanowski, 2013). Even though we did not explore this pathway we decided to analyse whether pre-treatment of human neuroblastoma cells with TUDCA could prevent the disruption of mitochondrial network induced by MPP^+. We observed that treatment with MPP^+ completely disrupted mitochondrial network and induced mitochondrial accumulations (Cartelli *et al.*, 2010; Scarffe *et al*., 2014). Importantly, pre-treatment with TUDCA clearly prevented the disruption of the mitochondrial network, demonstrating that this bile acid is able to reduce MPP^+ toxicity.

In summary, this work provides for the first time strong evidence that one of the mechanisms underlying TUDCA neuroprotection against MPTP/MPP^+ neurotoxicity is the modulation of autophagy/mitophagy via PINK1/parkin-mediated pathway, with concomitant alterations in mitochondrial biogenesis. In fact, we have shown that TUDCA specifically modulates proteins associated with these two metabolic pathways, namely, full-length PINK1 expression, LC3 lipidation, parkin gene and protein expression and PARIS protein degradation.

Impaired mitochondrial turnover has been associated to PD, thus, mitochondrial protective agents represent an attractive direction for the development of new therapeutic drugs. Our results point to the pharmacological up-regulation of mitochondrial turnover by TUDCA as a novel neuroprotective mechanism of this molecule, and to contribute to the validation of TUDCA clinical application in PD.

Future work

Due to technical problems that we could not overcome in the course of this thesis, part of the results presented here are still preliminary, and are presented as single values or averages of two independent experiments. Indeed, we had intended to analyse parkin activation by PINK1 by Western blot analysis in both models, in order to obtain corroborating data supporting the hypothesis that PINK1/parkin pathway of autophagy is involved in TUDCA neuroprotective action. Afterwards, we also plan to confirm the importance of parkin in TUDCA neuroprotective action, by loss-of-function experiments using human neuroblastoma cells transfected with parkin small interfering RNA. The transfected cells will be treated with TUDCA and/or MPP^+ and cell viability will be assessed by measuring the amount of LDH released to the medium.

We are also interested to see if, besides inducing parkin expression, TUDCA can contribute to the stabilization parkin protein, extending its half-life. This experiment will be carried out using SH-

SY5Y cells treated with TUDCA and/or MPP+, in the presence of cycloheximide, to impair protein *de novo* synthesis. Analysis of the total protein expression levels will be performed by Western blots, with extracts prepared from cells treated for different time points.

Additionally, we also intent to explore the hypothesis proposed formerly, namely, that TUDCA acts on mitochondria in a similar way as lanosterol. In this matter, we are going to proceed with more experiments using a voltage-sensitive fluorescent dye (JC-1 dye) to monitor mitochondrial membrane potential upon treatment with TUDCA and/or MPP^+.

5. REFERENCES

Amaral, J. D., Viana, R. J. S., Ramalho, R. M., Steer, C. J., & Rodrigues, C. M. P. (2009). Bile acids: regulation of apoptosis by ursodeoxycholic acid. Journal of Lipid Research 50(9): 1721–1734.

Barzilai, A., & Melamed, E. (2003). Molecular mechanisms of selective dopaminergic neuronal death in Parkinson's disease. Trends in Molecular Medicine 9(3): 126–132.

Becker, D., Richter, J., Tocilescu, M. a., Przedborski, S., & Voos, W. (2012). Pink1 kinase and its membrane potential-dependent cleavage product both localize to outer mitochondrial membrane by unique targeting mode. Journal of Biological Chemistry 287(27): 22969–22987.

Betarbet, R., Sherer, T. B., MacKenzie, G., Garcia-Osuna, M., Panov, a V, & Greenamyre, J. T. (2000). Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nature Neuroscience 3(12): 1301–1306.

Blum, D., Torch, S., Lambeng, N., Nissou, M. F., Benabid, A. L., Sadoul, R., & Verna, J. M. (2001). Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: Contribution to the apoptotic theory in Parkinson's disease. Progress in Neurobiology 65(2): 135–172.

Bové, J., & Perier, C. (2012). Neurotoxin-based models of Parkinson's disease. Neuroscience 211: 51–76.

Cartelli, D., Ronchi, C., Maggioni, M. G., Rodighiero, S., Giavini, E., & Cappelletti, G. (2010). Microtubule dysfunction precedes transport impairment and mitochondria damage in MPP+-induced neurodegeneration. Journal of Neurochemistry 115(1): 247–258.

Castillo-Quan, J. I. (2011). Parkin' control: regulation of PGC-1α through PARIS in Parkinson's disease. Disease Models & Mechanisms 4(4): 427–429.

Castro-Caldas, M., Carvalho, A. N., Peixeiro, I., Rodrigues, E., Lechner, M. C., & Gama, M. J. (2009). GSTpi expression in MPTP-induced dopaminergic neurodegeneration of C57BL/6 mouse midbrain and striatum. Journal of Molecular Neuroscience 38(2): 114–127.

Castro-Caldas, M., Carvalho, A. N., Rodrigues, E., Henderson, C. J., Wolf, C. R., Rodrigues, C. M. P., & Gama, M. J. (2012). Tauroursodeoxycholic acid prevents MPTP-induced dopaminergic cell death in a mouse model of Parkinson's disease. Molecular Neurobiology 46(2): 475–86.

Chaudhuri, K. R., Healy, D. G., & Schapira, a H. (2006). Non-motor symptoms of Parkinson's disease: diagnosis and management. Lancet Neurol 5(3): 235–245.

Chinta, S. J., & Andersen, J. K. (2005). Dopaminergic neurons. The International Journal of Biochemistry & Cell Biology 37(5): 942–946.

Constantinescu, R., Constantinescu, a T., Reichmann, H., & Janetzky, B. (2007). Neuronal differentiation and long-term culture of the human neuroblastoma line SH-SY5Y. Journal of Neural Transmission. Supplementum, (72), 17–28.

Cuervo, A. M., Bergamini, E., Brunk, U. T., Ffrench, M., & Terman, A. (2005). Autophagy and Aging: The Importance of Maintaining “Clean” Cells. Landes Bioscience 1(3): 131–140.

Dauer, W., & Przedborski, S. (2003). Parkinson’s Disease. Neuron 39(6): 889–909.

Dawson, T. M., & Dawson, V. L. (2010). The role of parkin in familial and sporadic Parkinson’s disease. Movement Disorders, 25(SUPPL. 1): 32–39.

Dayem, A. A., Kim, B., Gurunathan, S., Choi, H. Y., Yang, G., Saha, S. K., … Cho, S. G. (2014). Biologically synthesized silver nanoparticles induce neuronal differentiation of SH-SY5Y cells via modulation of reactive oxygen species, phosphatases, and kinase signaling pathways. Biotechnology Journal 9(7): 934–943.

Devi, L., Raghavendran, V., Prabhu, B. M., Avadhani, N. G., & Anandatheerthavarada, H. K. (2008). Mitochondrial import and accumulation of alpha-synuclein impair complex I in human dopaminergic neuronal cultures and Parkinson disease brain. Journal of Biological Chemistry 283(14): 9089–9100.

Dexter, D. T., & Jenner, P. (2013). Parkinson disease: from pathology to molecular disease mechanisms. Free Radical Biology & Medicine 62: 132–44.

Du, Y., Ma, Z., Lin, S., Dodel, R. C., Gao, F., Bales, K. R., … Paul, S. M. (2001). Minocycline prevents nigrostriatal dopaminergic neurodegeneration in the MPTP model of Parkinson’s disease. Proceedings of the National Academy of Sciences of the United States of America 98(25): 14669–14674.

Egan, D. F., Shackelford, D. B., Mihaylova, M. M., Gelino, S. R., Rebecca, A., Mair, W., … Shaw, R. J. (2011). Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. Science 331(6016): 456–461.

Elia, a. E., Lalli, S., Monsurrò, M. R., Sagnelli, A., Taiello, a. C., Reggiori, B., … Albanese, A. (2015). Tauroursodeoxycholic acid in the treatment of patients with amyotrophic lateral sclerosis. European Journal of Neurology 0: 1–8

Exner, N., Lutz, A. K., Haass, C., & Winklhofer, K. F. (2012). Mitochondrial dysfunction in Parkinson’s disease: molecular mechanisms and pathophysiological consequences. The EMBO Journal 31(14): 3038–3062.

Fang, L., Zhou, Y., Cao, H., Wen, P., Jiang, L., He, W., … Yang, J. (2013). Autophagy Attenuates Diabetic Glomerular Damage through Protection of Hyperglycemia-Induced Podocyte Injury. PLoS ONE 8(4): e60546

Flinn, L., Mortiboys, H., Volkmann, K., Kster, R. W., Ingham, P. W., & Bandmann, O. (2009). Complex i deficiency and dopaminergic neuronal cell loss in parkin-deficient zebrafish (Danio rerio). Brain 132(6): 1613–1623.

Gandhi, S., & Wood, N. W. (2005). Molecular pathogenesis of Parkinson’s disease. Human Molecular Genetics 14(18): 2749–2755.

Gaweda-Walerych, K., & Zekanowski, C. (2013). Integrated pathways of parkin control over mitochondrial maintenance - Relevance to Parkinson’s disease pathogenesis. Acta Neurobiologiae Experimentalis 73(2): 199–224.

Geisler, S., Holmström, K. M., Skujat, D., Fiesel, F. C., Rothfuss, O. C., Kahle, P. J., & Springer, W. (2010). PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. Nature Cell Biology 12(2): 119–131.

Goldberg, A. L. (2003). Protein degradation and protection against misfolded or damaged proteins. Nature 426(6968): 895–899.

Goldberg, M. S., Fleming, S. M., Palacino, J. J., Cepeda, C., Lam, H. a., Bhatnagar, A., … Shen, J. (2003). Parkin-deficient Mice Exhibit Nigrostriatal Deficits but not Loss of Dopaminergic Neurons. Journal of Biological Chemistry 278(44): 43628–43635.

Greene, A. W., Grenier, K., Aguileta, M. a, Muise, S., Farazifard, R., Haque, M. E., … Fon, E. a. (2012). Mitochondrial processing peptidase regulates PINK1 processing, import and Parkin recruitment. EMBO Reports 13(4): 378–385.

Gwinn, D. M., Shackelford, D. B., Egan, D. F., Mihaylova, M. M., Mery, A., Vasquez, D. S., … Shaw, R. J. (2008). AMPK Phosphorylation of Raptor Mediates a Metabolic Checkpoint. Molecular Cell 30(2): 214–226.

Hara, K., Maruki, Y., Long, X., Yoshino, K. I., Oshiro, N., Hidayat, S., … Yonezawa, K. (2002). Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell 110(2): 177–189.

Hara, T., Takamura, A., Kishi, C., Iemura, S. I., Natsume, T., Guan, J. L., & Mizushima, N. (2008). FIP200, a ULK-interacting protein, is required for autophagosome formation in mammalian cells. Journal of Cell Biology: 181(3) 497–510.

Hershko, a, Leshinsky, E., Ganoth, D., & Heller, H. (1984). ATP-dependent degradation of ubiquitin-protein conjugates. Proceedings of the National Academy of Sciences of the United States of America 81(6): 1619–1623.

Hosokawa, N., Hara, T., Kaizuka, T., Kishi, C., Takamura, A., Miura, Y., … Mizushima, N. (2009). Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. Molecular Biology of the Cell 20(7): 1981–1991.

Imaizumi, Y., Okada, Y., Akamatsu, W., Koike, M., Kuzumaki, N., Hayakawa, H., … Okano, H. (2012). Mitochondrial dysfunction associated with increased oxidative stress and α-synuclein accumulation in PARK2 iPSC-derived neurons and postmortem brain tissue. Molecular Brain 5: 35.

Itano, Y., & Nomura, Y. (1995). 1-methyl-4-phenyl-pyridinium ion (MPP+) causes DNA fragmentation and increases the Bcl-2 expression in human neuroblastoma, SH-SY5Y cells, through different mechanisms. Brain Research 704(2): 240–245.

Jankovic, J. (2008). Parkinson's disease: clinical features and diagnosis. Journal of Neurology, Neurosurgery, and Psychiatry 79(4): 368–376.

Javitch, J. a, D'Amato, R. J., Strittmatter, S. M., & Snyder, S. H. (1985). Parkinsonism-inducing neurotoxin, N-methyl-4-phenyl-1,2,3,6 -tetrahydropyridine: uptake of the metabolite N-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity. Proceedings of the National Academy of Sciences of the United States of America 82(7): 2173–2177.

Jin, S. M., Lazarou, M., Wang, C., Kane, L. a., Narendra, D. P., & Youle, R. J. (2010). Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL. Journal of Cell Biology 191(5): 933–942.

Jung, C. H., Jun, C. B., Ro, S.-H., Kim, Y.-M., Otto, N. M., Cao, J., … Kim, D.-H. (2009). ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. Molecular Biology of the Cell 20(7): 1992–2003.

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., … Yoshimori, T. (2003). LC3, a mammalian homolog of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO Journal, 22(17): 4577.

Keane, P. C., Kurzawa, M., Blain, P. G., & Morris, C. M. (2011). Mitochondrial dysfunction in Parkinson's disease. Parkinson's Disease, 2011: 1-18.

Keene, C. D., Rodrigues, C. M., Eich, T., Linehan-Stieers, C., Abt, a, Kren, B. T., … Low, W. C. (2001). A bile acid protects against motor and cognitive deficits and reduces striatal degeneration in the 3-nitropropionic acid model of Huntington's disease. Experimental Neurology 171(2): 351–360.

Keene, C. D., Rodrigues, C. M. P., Eich, T., Chhabra, M. S., Steer, C. J., & Low, W. C. (2002). Tauroursodeoxycholic acid, a bile acid, is neuroprotective in a transgenic animal model of Huntington's disease. Proceedings of the National Academy of Sciences of the United States of America 99(16): 10671–10676.

Kihara, a., Kabeya, Y., Ohsumi, Y., & Yoshimori, T. (2001). Beclin-phosphatidylinositol 3-kinase complex functions at the trans-Golgi network. EMBO Reports 2(4): 330–335.

Klein, C., & Westenberger, A. (2012). Genetics of Parkinson's disease. Cold Spring Harbor Perspectives in Medicine 2(1): a008888.

Kondapalli, C., Kazlauskaite, a., Zhang, N., Woodroof, H. I., Campbell, D. G., Gourlay, R., … Muqit, M. M. K. (2012). PINK1 is activated by mitochondrial membrane potential depolarization and stimulates Parkin E3 ligase activity by phosphorylating Serine 65. Open Biology, 2(5): 120080–120080.

Kramer, M. L., & Schulz-Schaeffer, W. J. (2007). Presynaptic alpha-synuclein aggregates, not Lewy bodies, cause neurodegeneration in dementia with Lewy bodies. The Journal of Neuroscience : The Official Journal of the Society for Neuroscience 27(6): 1405–1410.

Krishna, A., Biryukov, M., Trefois, C., Antony, P. M., Hussong, R., Lin, J., … May, P. (2014). Systems genomics evaluation of the SH-SY5Y neuroblastoma cell line as a model for Parkinson's disease. BMC Genomics 15: 1–21.

Kuroda, Y., Mitsui, T., Kunishige, M., Shono, M., Akaike, M., Azuma, H., & Matsumoto, T. (2006). Parkin enhances mitochondrial biogenesis in proliferating cells. Human Molecular Genetics 15(6): 883–895.

Lazarou, M., Jin, S. M., Kane, L. a., & Youle, R. J. (2012). Role of PINK1 Binding to the TOM Complex and Alternate Intracellular Membranes in Recruitment and Activation of the E3 Ligase Parkin. Developmental Cell 22(2): 320–333.

Lecker, S. H., Goldberg, A. L., & Mitch, W. E. (2006). Protein degradation by the ubiquitin-proteasome pathway in normal and disease states. Journal of the American Society of Nephrology : JASN 17(7): 1807–1819.

Lee, J. W., Park, S., Takahashi, Y., & Wang, H. G. (2010). The association of AMPK with ULK1 regulates autophagy. PLoS ONE 5(11): 1–9.

Liang, X. H., Jackson, S., Seaman, M., Brown, K., Kempkes, B., Hibshoosh, H., & Levine, B. (1999). Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature, 402(6762): 672–676.

Lim, L., Jackson-Lewis, V., Wong, L. C., Shui, G. H., Goh, a X. H., Kesavapany, S., … Wenk, M. R. (2012). Lanosterol induces mitochondrial uncoupling and protects dopaminergic neurons from cell death in a model for Parkinson's disease. Cell Death and Differentiation, 19(3): 416–427.

Liu, W., Vives-Bauza, C., Acín-Peréz-, R., Yamamoto, A., Tan, Y., Li, Y., … Li, C. (2009). PINK1 defect causes mitochondrial dysfunction, proteasomal deficit and alpha-synuclein aggregation in cell culture models of Parkinson's disease. PloS One 4(2): e4597.

Manning-Bog, A. B., McCormack, A. L., Li, J., Uversky, V. N., Fink, A. L., & Di Monte, D. a. (2002). The herbicide paraquat causes up-regulation and aggregation of ??-synuclein in mice: Paraquat and ??-synuclein. Journal of Biological Chemistry 277(3): 1641–1644.

Mari, M., Griffith, J., Rieter, E., Krishnappa, L., Klionsky, D. J., & Reggiori, F. (2010). An Atg9-containing compartment that functions in the early steps of autophagosome biogenesis. Journal of Cell Biology 190(6): 1005–1022.

Martin, I., Dawson, V. L., & Dawson, T. M. (2011). Recent advances in the genetics of Parkinson's disease. Annual Review of Genomics and Human Genetics, 12: 301–325.

Mata, I. F., Lockhart, P. J., & Farrer, M. J. (2004). Parkin genetics: one model for Parkinson's disease. Human Molecular Genetics, 13(1): R127–R133.

Meissner, C., Lorenz, H., Weihofen, A., Selkoe, D. J., & Lemberg, M. K. (2011). The mitochondrial intramembrane protease PARL cleaves human Pink1 to regulate Pink1 trafficking. Journal of Neurochemistry 117(5): 856–867.

Menzies, F. M., Fleming, A., & Rubinsztein, D. C. (2015). Compromised autophagy and neurodegenerative diseases. Nature Reviews Neuroscience, 16(6): 345–357.

Mizuno, Y., Ohta, S., Tanaka, M., Takamiya, S., Suzuki, K., Sato, T., … Kagawa, Y. (1989). Deficiencies in complex I subunits of the respiratory chain in Parkinson's disease. Biochemical and Biophysical Research Communications, 163(3): 1450–1455.

Moore, D. J., West, A. B., Dawson, V. L., & Dawson, T. M. (2005). Molecular pathophysiology of Parkinson's disease. Annual Review of Neuroscience 28: 57–87.

Morais, V. a., Verstreken, P., Roethig, A., Smet, J., Snellinx, A., Vanbrabant, M., … De Strooper, B. (2009). Parkinson's disease mutations in PINK1 result in decreased Complex I activity and deficient synaptic function. EMBO Molecular Medicine, 1(2): 99–111.

Narendra, D. P., Jin, S. M., Tanaka, A., Suen, D. F., Gautier, C. a., Shen, J., … Youle, R. J. (2010*a*). PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biology 8(1): e1000298.

Narendra, D. P., Kane, L. a., Hauser, D. N., Fearnley, I. M., & Youle, R. J. (2010*b*). p62/SQSTM1 is required for Parkin-induced mitochondrial clustering but not mitophagy; VDAC1 is dispensable for both. Autophagy 6(8): 1090–1106.

Narendra, D., Tanaka, A., Suen, D. F., & Youle, R. J. (2008). Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. Journal of Cell Biology 183(5): 795–803.

Nicotra, a., & Parvez, S. H. (2002). Apoptotic molecules and MPTP-induced cell death. Neurotoxicology and Teratology 24(5): 599–605.

Palacino, J. J., Sagi, D., Goldberg, M. S., Krauss, S., Motz, C., Wacker, M., … Shen, J. (2004). Mitochondrial dysfunction and oxidative damage in parkin-deficient mice. The Journal of Biological Chemistry 279(18): 18614–18622.

Palikaras, K., & Tavernarakis, N. (2014). Mitochondrial homeostasis: The interplay between mitophagy and mitochondrial biogenesis. Experimental Gerontology 56: 182–188.

Parry, G. J., Rodrigues, C. M. P., Aranha, M. M., Hilbert, S. J., Davey, C., Kelkar, P., … Steer, C. J. (2010). Safety, tolerability, and cerebrospinal fluid penetration of ursodeoxycholic Acid in patients with amyotrophic lateral sclerosis. Clinical Neuropharmacology 33(1): 17–21.

Przedborski, S., Tieu, K., Perier, C., & Vila, M. (2004). MPTP as a mitochondrial neurotoxic model of Parkinson's disease. Journal of Bioenergetics and Biomembranes 36(4 SPEC.ISS.): 375–379.

Ramalho, R. M., Borralho, P. M., Castro, R. E., Solá, S., Steer, C. J., & Rodrigues, C. M. P. (2006). Tauroursodeoxycholic acid modulates p53-mediated apoptosis in Alzheimer's disease mutant neuroblastoma cells. Journal of Neurochemistry 98(5): 1610–1618.

Ramalho, R. M., Ribeiro, P. S., Solá, S., Castro, R. E., Steer, C. J., & Rodrigues, C. M. P. (2004). Inhibition of the E2F-1/p53/Bax pathway by tauroursodeoxycholic acid in amyloid β-peptide-induced apoptosis of PC12 cells. Journal of Neurochemistry 90(3): 567–575.

Reggiori, F., & Klionsky, D. J. (2002). Autophagy in the Eukaryotic Cell. Eukaryotic Cell 1(1): 10–21.

Rodrigues, C. M. P., Fan, G., Ma, X., Kren, B. T., & Steer, C. J. (1998). A novel role for ursodeoxycholic acid in inhibiting apoptosis by modulating mitochondrial membrane perturbation. Journal of Clinical Investigation 101(12): 2790–2799.

Rodrigues, C. M. P., Linehan-Stieers, C., Keene, C. D., Ma, X., Kren, B. T., Low, W. C., & Steer, C. J. (2000*a*). Tauroursodeoxycholic acid partially prevents apoptosis induced by 3-nitropropionic acid: Evidence for a mitochondrial pathway independent of the permeability transition. Journal of Neurochemistry 75(6): 2368–2379.

Rodrigues, C. M. P., Sola, S., Nan, Z., Castro, R. E., Ribeiro, P. S., Low, W. C., & Steer, C. J. (2003). Tauroursodeoxycholic acid reduces apoptosis and protects against neurological injury after acute hemorrhagic stroke in rats. Proceedings of the National Academy of Sciences of the United States of America 100(10): 6087–6092.

Rodrigues, C. M. P., Spellman, S. R., Solá, S., Grande, A. W., Linehan-Stieers, C., Low, W. C., & Steer, C. J. (2002). Neuroprotection by a bile acid in an acute stroke model in the rat. Journal of Cerebral Blood Flow and Metabolism : Official Journal of the International Society of Cerebral Blood Flow and Metabolism 22(4): 463–471.

Rodrigues, C. M., Solá, S., Silva, R., & Brites, D. (2000*b*). Bilirubin and amyloid-beta peptide induce cytochrome c release through mitochondrial membrane permeabilization. Molecular Medicine (Cambridge, Mass.) 6(11): 936–946.

Russell, R. C., Tian, Y., Yuan, H., Park, H. W., Chang, Y.-Y., Kim, J., … Guan, K.-L. (2013). ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase. Nature Cell Biology 15(7): 741–50.

Scarffe, L. a, Stevens, D. a, Dawson, V. L., & Dawson, T. M. (2014). Parkin and PINK1: much more than mitophagy. Trends in Neurosciences 37(6): 315–24.

Schober, A. (2004). Classic toxin-induced animal models of Parkinson's disease: 6-OHDA and MPTP. Cell and Tissue Research 318(1): 215–224.

Shiba-Fukushima, K., Imai, Y., Yoshida, S., Ishihama, Y., Kanao, T., Sato, S., & Hattori, N. (2012). PINK1-mediated phosphorylation of the Parkin ubiquitin-like domain primes mitochondrial translocation of Parkin and regulates mitophagy. Scientific Reports 2: 1002.

Shin, J. H., Ko, H. S., Kang, H., Lee, Y., Lee, Y. Il, Pletinkova, O., … Dawson, T. M. (2011). PARIS (ZNF746) repression of PGC-1α contributes to neurodegeneration in parkinson's disease. Cell 144(5): 689–702.

Shin, S. M., Cho, I. J., & Kim, S. G. (2009). Resveratrol Protects Mitochondria against Oxidative Stress through AMP-Activated Protein Kinase-Mediated Glycogen Synthase Kinase-3 ␤ Inhibition Downstream of Poly (ADP- ribose) polymerase-LKB1 Pathway. Molecular Pharmacology, 76(4), 884–895. http://doi.org/10.1124/mol.109.058479.reactive

Silvestri, L., Caputo, V., Bellacchio, E., Atorino, L., Dallapiccola, B., Valente, E. M., & Casari, G. (2005). Mitochondrial import and enzymatic activity of PINK1 mutants associated to recessive parkinsonism. Human Molecular Genetics 14(22): 3477–3492.

Singh, R., & Cuervo, A. M. (2011). Autophagy in the cellular energetic balance. Cell Metabolism 13(5): 495–504.

Smeyne, R. J., & Jackson-Lewis, V. (2005). The MPTP model of Parkinson's disease. Molecular Brain Research 134(1): 57–66.

Solá, S., Castro, R. E., Laires, P. a, Steer, C. J., & Rodrigues, C. M. (2003). Tauroursodeoxycholic Acid Prevents Amyloid-β Peptide–Induced Neuronal Death Via a Phosphatidylinositol 3-Kinase–Dependent Signaling Pathway. Molecular Medicine 9(9-12): 226–234.

Song, S., Jang, S., Park, J., Bang, S., Choi, S., Kwon, K. Y., … Chung, J. (2013). Characterization of PINK1 (PTEN-induced putative kinase 1) mutations associated with parkinson disease in mammalian cells and drosophila. Journal of Biological Chemistry 288(8): 5660–5672.

Thomas, B., & Flint Beal, M. (2007). Parkinson's disease. Human Molecular Genetics 16(R2): 183–194.

Trojanowski, J. Q., Goedert, M., Iwatsubo, T., & Lee, V. M. (1998). Fatal attractions: abnormal protein aggregation and neuron death in Parkinson's disease and Lewy body dementia. Cell Death and Differentiation 5(10): 832–837.

Ved, R., Saha, S., Westlund, B., Perier, C., Burnam, L., Sluder, A., … Wolozin, B. (2005). Similar patterns of mitochondrial vulnerability and rescue induced by genetic modification of ??-synuclein, parkin, and DJ-1 in Caenorhabditis elegans. Journal of Biological Chemistry 280(52): 42655–42668.

Wang, L., Lawrence, J. C., Sturgill, T. W., & Harris, T. E. (2009). Mammalian target of rapamycin complex 1 (mTORC1) activity is associated with phosphorylation of raptor by mTOR. The Journal of Biological Chemistry 284(22): 14693–14697.

Watanabe, Y., Himeda, T., & Araki, T. (2005). Mechanisms of MPTP toxicity and their implications for therapy of Parkinson's disease. Medical Science Monitor : International Medical Journal of Experimental and Clinical Research 11(1): RA17–A23.

West, A. B., & Maidment, N. T. (2004). Genetics of parkin-linked disease. Human Genetics 114(4): 327–336.

Wu, Y., Li, X., Zhu, J. X., Xie, W., Le, W., Fan, Z., … Pan, T. (2011). Resveratrol-activated AMPK/SIRT1/autophagy in cellular models of Parkinson's disease. NeuroSignals, 19(3), 163–174. http://doi.org/10.1159/000328516

Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., … Spiegelman, B. M. (1999). Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell 98(1): 115–124.

Yoshino, H., Nakagawa-Hattori, Y., Kondo, T., & Mizuno, Y. (1992). Mitochondrial complex I and II activities of lymphocytes and platelets in Parkinson's disease. Journal of Neural Transmission. Parkinson's Disease and Dementia Section 4(1): 27–34.

Zhou, C., Huang, Y., Shao, Y., May, J., Prou, D., Perier, C., … Przedborski, S. (2008). The kinase domain of mitochondrial PINK1 faces the cytoplasm. Proceedings of the National Academy of Sciences of the United States of America 105(33): 12022–12027.

Zhu, J., & Chu, C. T. (2010). Mitochondrial dysfunction in Parkinson's disease. Journal of Alzheimer's Disease 20(SUPPL.2): S325-S334.

Zhu, J.-H., Horbinski, C., Guo, F., Watkins, S., Uchiyama, Y., & Chu, C. T. (2007). Regulation of autophagy by extracellular signal-regulated protein kinases during 1-methyl-4-phenylpyridinium-induced cell death. The American Journal of Pathology 170(1): 75–86.