**Inês de Sousa Caria**

Licenciada em Biologia Celular e Molecular

# Unanswered Role of Cholesterol Homeostasis in Parkinson's Disease

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

**Orientador:** Elsa Margarida Teixeira Rodrigues, PhD
iMed.ULisboa, Faculdade de Farmácia, Universidade de Lisboa

**Co-orientador:** Maria João de Jesus Nunes, PhD
iMed.ULisboa, Faculdade de Farmácia, Universidade de Lisboa

**Presidente:** Doutora Paula Maria Theriaga Mendes Bernardes Gonçalves

**Arguente:** Doutora Maria Helena Sequeira Cardoso

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

**Abril, 2020**

**Inês de Sousa Caria**

Licenciada em Biologia Celular e Molecular

# Unanswered Role of Cholesterol Homeostasis in Parkinson's Disease

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

**Orientador:** Elsa Margarida Teixeira Rodrigues, PhD
iMed.ULisboa, Faculdade de Farmácia, Universidade de Lisboa

**Co-orientador:** Maria João de Jesus Nunes, PhD
iMed.ULisboa, Faculdade de Farmácia, Universidade de Lisboa

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

**Abril, 2020**

**Understanding the Role of Cholesterol Homeostasis in Parkinson's Disease**

This work was supported by FEDER and national funds from Fundação para a Ciência e Tecnologia (FCT) (LISBOA-01-0145-FEDER-030194 and PTDC/MED-FSL/30194/2017).

*Para os meus pais*

# Acknowledgements

Em primeiro lugar, gostaria de dar um enorme agradecimento à Professora Doutora Elsa Rodrigues por me ter oferecido a oportunidade de realizar a minha tese sobre o seu cuidado, por me ter guiado e ter oferecido conselhos que irei seguir em todo o meu percurso científico. Na minha mente (e caixa de correio eletrónica) ainda tenho guardado um dos primeiros emails que me enviou, e que me fez sorrir depois de uma longa semana: "[…] Não desesperes! A investigação é mm assim. 80% das vezes não funciona! […]". Um conselho que me ajudou muito também nos restantes meses à medida que ia descobrindo a veracidade desta afirmação. Obrigada por toda a confiança, apoio, inspiração, carinho e por me ter ajudado a crescer como investigadora e pessoa.

Imediatamente a seguir, gostaria de dirigir os meus agradecimentos à Doutora Maria João Nunes, que foi também uma figura essencial durante este último ano. Foi uma grande orientadora e influência, sempre disponível para me ajudar e esclarecer as inúmeras dúvidas que tinha. Ensinou-me a ter mais rigor nos meus trabalhos e apelou ao meu espírito critico, mas também foi alguém com quem eu podia contar quando me sentia mais em baixo nos meus múltiplos "roadblocks".

À Professora Doutora Margarida Castro-Caldas, queria agradecer a confiança que depositou em mim, sendo uma das razões principais pela qual conheci o laboratório e a oportunidade de realizar a tese neste sítio. Nas pequenas conversas que tive no laboratório, mostrava sempre a sua preocupação comigo e disponibilidade ao dar sugestões para o meu trabalho.

À Doutora Andreia Nunes Carvalho e à Professora Doutora Maria João Gama, queria também agradecer terem-me aceitado de braços abertos no laboratório. Também se disponibilizaram sempre prontas a ajudar, quer fosse para tirar alguma dúvida nalguma experiência, ou para me fazerem relaxar e rir, nem que fosse por um bocadinho.

À minha parceira de bancada e de sala de estudo, Catarina, queria agradecer por me teres aturado em todos os meus momentos, quer fosse para desabafar durante todas as frustrações que tinha, ou partilhar uma piada ou uma conversa. O apoio que deste foi essencial para que sobrevivesse no laboratório. Queria também agradecer à Doutora Rita e Doutora Viviana, que também foram importantes no decorrer da tese e que me tentaram ajudar a sair mais do meu "casulo". Sei que não será o mesmo, mas queria dar por este meio o meu abraço (ainda que virtual).

Gostaria também de dar um enorme agradecimento aos restantes membros do grupo CellFun, que me deram as boas vindas, facilitaram a minha integração no laboratório, e pelos momentos de almoço em que me ajudavam a descomprimir, renovando as energias para enfrentar o resto do dia.

Por último e não menos importante, gostaria também de agradecer à Professora Doutora Cecília Rodrigues, por me ter recebido no seu grupo "Cellular Function and Therapeutic Targeting", permitindo-me dar então os primeiros passos na carreira de investigação.

Aos meus amigos do Secundário, Inês Pereira, Maria Freire, Ana Rita Diniz, João Faria, Diogo Ferreira e João Miguel, ainda que os nossos momentos de café (com todos!) fossem escassos ao longo do ano, não foram menos importantes em ajudar-me a espairecer e desabafar. E sabendo

eles, a minha eterna dificuldade em exprimir o que sinto, espero que por estas poucas palavras percebam a importância que tiveram neste último ano.

Aos meus amigos da Faculdade, Susana Martins, Catarina Severo, Francisca Martins, Beatriz Raposo, Beatriz Fernandes, António Queiroz e Renato Domingues, obrigada também por tudo. Por me aturarem em todos os momentos nas nossas conversas de “chat” (especialmente a minha constante partilha de imagens e noticias), pelas noites de jogos e saídas à noite. Apesar de (mais uma vez) não o conseguir exprimir bem por palavras, espero que percebam que o vosso apoio nestes últimos cinco anos foi essencial para me tornar quem sou hoje.

Passo agora aos agradecimentos à minha família, que também muito me apoiou desde pequena. Em primeiro, à minha avó Aurora, por todo o carinho e incentivo que sempre me deste. Considero-te uma pessoa muito importante na minha vida, e estou grata por poder contar contigo sempre que posso.

À minha irmã Sara, por seres quem és e por estares presente em todas as etapas da minha vida. Por seres quem mais me aturas e com quem eu posso falar, literalmente sobre tudo! Espero que saibas o quanto significas para mim, ainda que hesite em dizê-lo muitas das vezes. Sem ti, certamente não teria sobrevivido nestes últimos anos.

E por último, não poderia deixar de agradecer aos meus pais que são, sem sombra de dúvida, as pessoas mais importantes da minha vida e aos quais dedico esta tese. Desde pequena me disseram para eu seguir os meus sonhos e dar tudo o que tinha, sejam eles quais fossem, que estariam sempre do meu lado e ajudariam no que pudessem. E assim o foi! Quando acabei por decidir seguir investigação, vocês nunca saíram do meu lado. Obrigada por ouvirem todos os meus desabafos e frustrações, e de tentaram acompanhar as minhas teorias, mesmo sem perceberem o que eu dizia. Eu sei que por vezes esgoto a vossa paciência, sou teimosa e nunca me calo, mas isso é porque vocês me ensinaram a não desistir das minhas convicções e de dar sempre o meu melhor. Sem vocês eu definitivamente não estaria aqui, e por isso, vou estar eternamente grata. Espero fazer-vos orgulhosos neste dia e em todos os outros que vierem.

# Abstract

Cholesterol has a key role in neuronal function and alterations in brain cholesterol homeostasis correlate with neurodegeneration. While disruptions in cholesterol homeostasis have been clearly associated with neurodegenerative disorders such as Alzheimer's and Huntington's disease, the role of cholesterol in Parkinson's disease (PD) remains controversial. To address this question, we characterized changes in cholesterol intracellular localization and levels using N2a mouse neuroblastoma cells treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxic metabolite, 1-methyl-4-phenylpyridinium ($MPP^+$). Filipin III staining showed an increase in lysosomal accumulation of free cholesterol 16 hours after treatment with 1mM $MPP^+$, and quantification of cholesterol levels also showed a significant increase in total and free intracellular cholesterol. In agreement, we observed a significant decrease in the mRNA levels and transcriptional activity of sterol regulatory element-binding protein (SREBP) 1 and 2 proteins, which are the main regulators of fatty acids and cholesterol synthesis. Concomitantly, there is a down-regulation in the mRNA levels of SREBP-target genes, such as fatty acid synthase, hydroxymethylglutaryl-CoA reductase and a significant decreased in the mRNA levels of NPC1 and lipase A. In order to uncover signaling pathways activated upon neuronal mitochondria dysfunction, involved in mitochondria-lysosome crosstalk, we identified an early activation of the AMPK/ mTOR signaling pathway, after 6 hours of $MPP^+$ treatment. Nevertheless, in contrary to what was expected, we could not detect any alterations in the mRNA levels of TFEB and TFEB-target genes for the same time-point, that could be responsible to the previously detected increase in LAMP2 levels. To corroborate our results, we proceeded to characterized changes in cholesterol homeostasis in the brain of MPTP-treated mice (40mg/mL, i.p). Interestingly, we saw a significant decrease in NPC1 and LIPA mRNA levels 6 hours after MPTP administration, in both the midbrain and striatum. Thus, our results show that the inhibition of mitochondrial complex I leads to lysosomal accumulation, reduced SREBPs transcriptional activity and reduction of NPC1 expression, further corroborating previous reports that suggest PD as lysosomal storage disorder. Moreover, the changes in cholesterol homeostasis observed, may further contribute to the propagation of toxicity following mitochondrial dysfunction.



**Keywords:** 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), brain cholesterol, lysosomes, neurodegeneration, Parkinson's Disease, sterol regulatory element-binding protein (SREBP)

# Resumo

O colesterol tem um papel fundamental na função neuronal, e alterações na homeostase do colesterol no encéfalo têm sido relacionadas com a neurodegeneração. Enquanto que alterações no metabolismo do colesterol já foram claramente associadas a doenças neurodegenerativas como a Doença de Alzheimer e de Doença de Huntington, o papel da homeostase do colesterol na Doença de Parkinson (DP) permanece controverso. Como tal, o nosso trabalho teve como objetivo caracterizar alterações na localização intracelular e nos níveis de colesterol em células N2a de neuroblastoma de ratinho tratadas com o metabolito tóxico de 1-metil-4-fenil-1,2,3,6-tetra-hidropiridina (MPTP), 1-metil- 4-fenilpiridinio ($MPP^+$). A marcação com Filipina III permitiu observar um aumento na acumulação de colesterol livre dentro dos lisossomas 16 horas após o tratamento com 1mM $MPP^+$. Através da quantificação dos níveis de colesterol, confirmámos um aumento significativo nos níveis intracelulares de colesterol total e livre. Este aumento leva a uma redução significativa nos níveis de mRNA e na atividade transcricional das proteínas de ligação ao elemento regulador de esteróis (*sterol regulatory element-binding protein* - SREBP) 1 e 2, que são os principais reguladores da biossíntese de ácidos gordos e colesterol. Concomitantemente, há uma regulação negativa nos níveis de mRNA dos genes alvo dos SREBPs, como a sintase de ácidos gordos e a hidroximetilglutaril-CoA redutase, e ainda uma diminuição significativa dos níveis de mRNA de NPC1 e lípase A. Ao investigar as vias de sinalização ativadas após disfunção mitocondrial, e envolvidas no *crosstalk* entre mitocôndrias e lisossomas, detetámos uma ativação precoce da via de sinalização da cinase de proteínas ativada por AMP (*AMP-activated protein kinase* - AMPK) / proteína alvo da rapamicina (*mammalian target of rapamycin* - mTOR), após 6 horas de tratamento com $MPP^+$. No entanto, ao contrário do esperado, não foi possível observar nenhuma alteração nos níveis de mRNA de TFEB, e dos seus genes alvo para o mesmo tempo de tratamento, que eventualmente pudesse ser responsável pelo aumento dos níveis de LAMP2 detetados anteriormente. Para corroborar os nossos resultados, procedemos à caracterização de alterações na homeostase do colesterol no cérebro de ratinhos tratados com MPTP (40mg/mL i.p), e detetámos uma diminuição significativa nos níveis de mRNA de NPC1 e LIPA, 6 horas após a administração de MPTP, tanto no mesencéfalo quanto no estriado. Assim, os nossos resultados mostram que a inibição do complexo I mitocondrial leva à acumulação de colesterol nos lisossomas, reduzindo a atividade transcricional de SREBPs e os níveis de expressão de NPC1, corroborando estudos anteriores que sugerem que a DP poderá ser um distúrbio de armazenamento lisossomal. Além disso, as alterações observadas na homeostase do colesterol poderão contribuir ainda mais para a propagação da toxicidade após a disfunção mitocondrial.



**Palavras-chave:** 1-metil-4-fenil-1,2,3,6-tetra-hidropiridina (MPTP), colesterol no cérebro, Doença de Parkinson, lisossomas, neurodegeneração, proteínas de ligação ao elemento regulador de esteróis (SREBP)

# Table of Contents

# Index of Figures

# Index of Tables

# Abbreviations

| | |
|---|---|
| **24-OHC** | 24(S)-hydroxycholesterol |
| **25-OHC** | 25-hydroxycholesterol |
| **4E-BP1/2** | Eukaryotic initiation factor 4E-binding proteins 1/2 |
| **ABCA1** | ATP-binding cassette transporter A1 |
| **ABCG1** | ATP-binding cassette transporter G1 |
| **ACAT1/SOAT1** | Acyl-coenzyme A: cholesterol acyltransferase 1/ sterol O-acyltransferase 1 |
| **ACC** | Acetyl-CoA carboxylase |
| **AD** | Alzheimer's Disease |
| **AMPK** | AMP-activated protein kinase |
| **ApoE** | Apolipoprotein E |
| **BBB** | Brain-blood barrier |
| **bHLH** | Basic Helix-Loop-Helix |
| **BSA** | Bovine serum albumin |
| **CC** | Compound C |
| **CCCP** | Carbonyl cyanide 3-chlorophenylhydrazone |
| **CNS** | Central nervous system |
| **COPII** | Coat protein complex II |
| **CTSD** | Lysosomal protease cathepsin D |
| **CTSS** | Lysosomal proteases cathepsin S |
| **CYP46A1** | Cytochrome P450 cholesterol 24-hydroxylase |
| **eIF4E** | Eukaryotic initiation factor 4E |
| **ER** | Endoplasmic reticulum |
| **Fas** | Fatty acid synthase |
| **FPP** | Farnesyl pyrophosphate |
| **GPP** | Geranyl pyrophosphate |
| **HDL** | High-density lipoproteins |
| **HMG-CoA** | 3-hydroxy-3-methylglutaryl-coa |
| **HMGR** | 3-hydroxy-3-methylglutaryl-coa reductase |
| **HMGS** | 3-hydroxy-3-methylglutaryl-coa synthase |
| **i.p.** | Intraperitoneal |
| **Insig** | Insulin-induced gene |
| **LAMP1** | Lysosomal-associated membrane protein 1 |
| **LanS** | Lanosterol synthase |
| **LAPTM5** | Lysosomal-associated protein transmembrane 5 |
| **LBs** | Lewy bodies |
| **LDLR** | Low density lipoprotein receptor |
| **LE** | Late endosomes |
| **LY** | Lysosomes |

| | |
|---|---|
| **LIPA** | Lipase A |
| **LKB1** | Liver kinase B1 |
| **LXR** | Liver X receptor |
| **$MPP^+$** | 1-methyl-4-phenylpyridinium |
| **MPTP** | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine |
| **mTOR** | Mammalian target of rapamycin |
| **NPC1** | Niemann-Pick type C1 |
| **NPC2** | Niemann-Pick type C2 |
| **PBS** | Phosphate buffered saline |
| **PD** | Parkinson's Disease |
| **PKB or Akt** | Protein kinase B |
| **PM** | Plasma membrane |
| **PP2A** | Protein phosphatase 2A |
| **PP2C** | Protein phosphatase 2C |
| **PVDF** | Polyvinyl difluoride |
| **qPCR** | Quantitative polymerase chain reaction |
| **Raptor** | Regulator-associated protein of the mammalian target of rapamycin |
| **ROS** | Reactive oxygen species |
| **S1P** | Site-1 protease |
| **S2P** | Site-2 protease |
| **S6K1/2** | S6 kinases 1/2 |
| **SCAP** | Sterol responsive element-binding protein -cleavage activating protein |
| **Scd1** | Stearoyl coa desaturase 1 |
| **SDS** | Sodium dodecyl sulfate |
| **sGTPases** | Small GTP-binding proteins |
| **SN*pc*** | Substantia Nigra *pars compacta* |
| **sPD** | Sporadic Parkinson's Disease |
| **SRE** | Sterol responsive element |
| **SREBP** | Sterol responsive element-binding protein |
| **SSD** | Sterol-sensing domain |
| **SYT11** | Synaptotagmin-11 |
| **TFEB** | Transcription factor EB |
| **TSC2/TSC1** | Tuberous sclerosis complex 2/1 |
| **ULK1** | UNC-51 like autophagy activating kinase |

# Chapter I. Introduction and Objectives

## 1. Importance of Cholesterol in the Brain

Cholesterol is a multifaceted molecule exerting important structural and physiological roles in all cells: (i) it serves as an essential membrane component regulating the fluidity and permeability of the phospholipid bilayer; (ii) participates in signaling and membrane trafficking processes; and (iii) acts as a precursor for steroid hormones, bile acids and vitamin D, which in turn could function as lipid solubilizers or signal transducers (reviewed in: Anchisi et al., 2013; Arenas et al., 2017).

Hence, there is no doubt of the importance of this molecule, especially in the central nervous system (CNS), which comprises about 25% of the total cholesterol in the human body (reviewed in: Dietschy and Turley, 2001). Because in the adult brain the cholesterol pool is maintained in constant levels, the bulk of cholesterol accumulates during brain growth and maturation, mainly derived from myelinization (Jurevics and Morell, 1995; Quan et al., 2003; reviewed in: Dietschy and Turley, 2004). Apart from being an important constituent of myelin (Saher et al., 2005), cholesterol is crucial for dendrite outgrowth (Fan et al., 2002; Goritz et al., 2005), synaptogenesis (Mauch et al., 2001; Goritz et al., 2005), microtubule stability (Fan et al., 2002) and axonal guidance (de Chaves et al., 1997).

It is postulated that only 1% percent of total brain cholesterol is esterified and stored in lipid droplets (Bryleva et al., 2010; reviewed in: Zhang et Liu, 2015). The rest is present in an unesterified form, distributed between the sheets of myelin of oligodendrocytes and the membranes of astrocytes and neurons (reviewed in: Dietschy and Turley, 2004; Arenas et al., 2017), where is responsible for maintaining neuronal plasticity and conductivity (Saher et al., 2005). Lastly, cholesterol in membranes can be found rearranged in microdomains (or lipid rafts) that participate in signaling transduction, determining the subcellular location and function of various signaling components (reviewed in: Pfrieger, 2003).

### 1.1. Synthesis of Cholesterol in the Brain

Since the brain-blood barrier (BBB) is impermeable to the cholesterol-rich lipoproteins present in the peripheral circulation, the CNS relies mostly on *de novo* local synthesis with turnover rates different for every cell type (reviewed in: Björkhem and Meaney, 2004; Dietschy and Turley, 2004).

The complex enzymatic process, by which cells synthetize cholesterol from acetyl-CoA, is called the mevalonate pathway. It is a very resource-intense and energy expensive process, composed of more than thirty steps that occur between the endoplasmic reticulum (ER) and cytosol (reviewed in: Goldstein and Brown, 1990; Zhang and Liu, 2015; Arenas et al., 2017). In fact, it was estimated that the synthesis of only one molecule of cholesterol requires more than 100 ATP molecules (Shobab et al., 2005). The first reaction involves the condensation of acetyl-CoA to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), catalyzed by 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS). This is followed by the rate-limiting step of the mevalonate pathway in the ER, where HMG-CoA is irreversibly converted to mevalonate (or mevalonic acid) by 3-hydroxy-3-methylglutaryl-CoA

reductase (HMGR) (reviewed in: Zhang and Liu, 2015; Arenas et al., 2017). Given that HMGR plays a central role in cholesterol biosynthesis, it is subjected to a strict transcriptional and post-transcriptional regulation, being the most important drug target for cholesterol-lowering drugs designated as statins (Nakanishi et al., 1988; reviewed in: Goldstein and Brown, 1990; Waterham, 2006).

Mevalonate is then used to synthetize isopentenyl pyrophosphate (IPP), which is a basic C5 isoprene unit used to sequentially generate other isoprenoids, namely geranyl pyrophosphate (GPP) and farnesyl pyrophosphate (FPP) (reviewed in: Goldstein and Brown, 1990; Waterham, 2006; Moutinho et al., 2017). After the synthesis of FPP there are two alternative routes: (i) the non-sterol pathway where FPP starts a sequence of condensations with IPP originating a multitude of new isoprenoids with different functions, one of which is geranylgeranyl pyrophosphate (GGPP) (reviewed in: Holstein and Hohl, 2004; Moutinho et al., 2017); or (ii) the post-squalene pathway where FPP condenses with another molecule of FPP originating the first committed sterol precursor, the squalene (reviewed in: Goldstein and Brown, 1990; Holstein and Hohl, 2004; Moutinho et al., 2017).

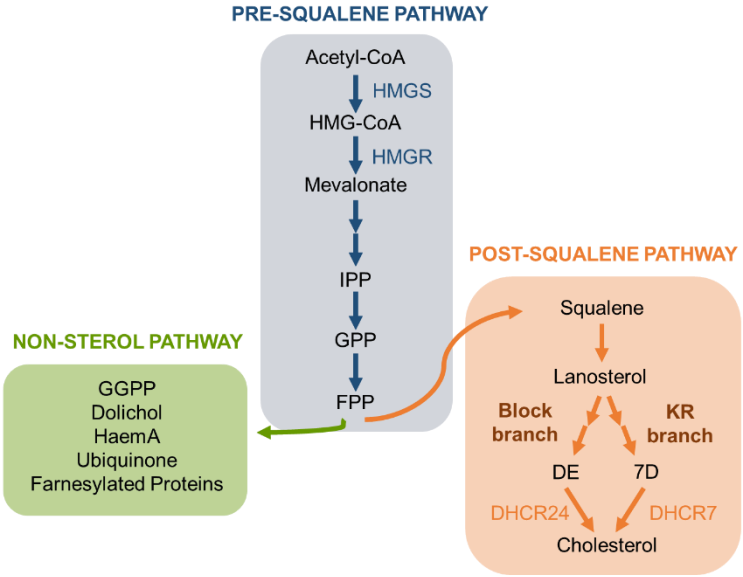


**Figure I.1. Schematic representation of the mevalonate pathway for the synthesis of cholesterol.** The mevalonate pathway is a very energy dependent process with more than 30 enzymatic steps, resulting in the synthesis of cholesterol and various isoprenoids intermediaries. Initially, a molecule of acetyl-CoA is condensed into 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by the action of 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS). After, the rate-limiting enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) catalyzes the irreversible conversion of HMG-CoA into mevalonate, followed by a sequence of enzymatic reactions leading up to the formation of farnesyl pyrophosphate (FPP). Then, there are two alternative routes: (I) the non-sterol pathway, producing various molecules involved in neuronal basic functions; or (II) the post-squalene pathway, committing to the novo synthesis of cholesterol either by the Block or the Kandutsch-Russel (KB) branch. (isopentenyl pyrophosphate (IPP), geranyl pyrophosphate (GPP); desmosterol (DE); 7-dehydrocholesterol (7D), 24-dehydrocholesterol reductase (DHCR24); 7-dehydrocholesterol reductase (DHCR7) (Adapted from: Goldstein and Brown, 1990; Moutinho et al., 2017).

The non-sterol pathway allows also the production of various isoprenoid intermediaries (reviewed in: Goldstein and Brown, 1990; Holstein and Hohl, 2004; Moutinho et al., 2017). These include haem A and ubiquinone, involved in electron transport; dolichol, necessary in glycoprotein synthesis; and isopentyladenine, present in a few transfer RNAs (reviewed in: Goldstein and Brown, 1990). Most importantly, both FPP and GGPP are used as moieties in protein prenylation of small GTP-binding proteins (sGTPases), such as Ras (from Rat sarcoma), Rho (Ras homolog) and Rab

(Ras-associated binding protein) families (reviewed in: Zhang and Casey, 1996; Hooff et al., 2010; Moutinho et al., 2017). Emphasizing the importance of isoprenoids, after being covalent attached to a lipid, Rho sGTPases participate in various basic neuronal functions, which include neuronal cell migration and dendritic arborization (Threadgill et al., 1997; reviewed in: Govek et al., 2005). Ras, on the other hand, are involved in axon growth (reviewed in: Hall and Lalli, 2010), while Rab act on vesicle transport mechanisms such as endocytosis or axonal retrograde transport (reviewed in: Ng and Tang, 2008).

As for the post-squalene pathway, after the production of lanosterol it can be further divided into two different branches of reactions. It was found that in neurons the Kandutsch-Russel branch is privileged, producing 7-dehydrocholesterol, which is finally converted into cholesterol by 7-dehydrocholesterol reductase (DHCR7). On the other hand, astrocytes contain mostly intermediaries of the Block branch, where desmosterol (DE) is metabolized by 24-dehydrocholesterol reductase (DHCR24), culminating also into the synthesis of cholesterol (Tabernero et al., 1993; Nieweg, 2009).

## 1.2. Network of Cholesterol Transport

When the brain reaches its final stages of maturation and there is a decrease in the demand of cholesterol for growth and synaptogenesis, neurons start to decline its synthesis rate and become more dependent on external cholesterol supply. This role is fulfilled predominantly by astrocytes, responsible for the secretion of high-density lipoproteins (HDL)-like, containing apolipoprotein E (ApoE) (Boyles et al., 1985; Pitas et al., 1987; Mauch et al., 2001; Gong et al., 2002; Hirsch-Reinshagen et al., 2004). As mentioned before, the uptake of cholesterol from astrocytes offers multiple advantages to neurons, since the biosynthesis of cholesterol is a lengthy process and costly in terms of energy and substrates (reviewed in: Pfrieger, 2003). Thus, after synthesis, astrocytes export cholesterol in an ApoE-dependent manner by mechanisms involving members of the ATP-binding cassette (ABC) superfamily of transporters, specifically ABCA1 and ABCG1 transporters (Hirsch-Reinshagen et al., 2004; Wahrle et al., 2004).

ABCA1 controls the loading of cholesterol and phospholipids from plasma membranes to lipid-free or lipid-poor apolipoproteins A-I (Lawn et al., 1999; Hirsch-Reinshagen et al., 2004; Wahrle et al., 2004; Vaughan and Oram, 2006). In turn, ABCG1 is thought to cooperate in the nascent particle maturation by further adding more lipids to lipidated lipoproteins (Kennedy et al., 2005; Vaughan and Oram, 2006). ApoE is considered the major apolipoprotein in the CNS (Elshourbagy et al., 1985; reviewed in: Mahley, 1988), and most of this apolipoprotein within the brain is secreted locally and independent of plasmatic ApoE levels (Linton et al., 1991). Together ApoE and ABCA1 are involved in the transport of cholesterol and other lipids to be then incorporated in neuronal membranes and myelin, helping in processes such as nerve growth and even repairing injury (Ignatius et al., 1987; Fukumoto et al., 2002; Hirsch-Reinshagen et al., 2004; reviewed in: Poirier, 1994). Both genes were also showed to be under the control of liver X receptor (LXR) (Laffitte et al., 2001; Fukumoto et al., 2002; Koldamova et al., 2003; Liang et al., 2004), which is one of the most important nuclear receptors regulating cholesterol homeostasis (reviewed in: Courtney and Landreth, 2016), facilitating the reverse cholesterol transport.

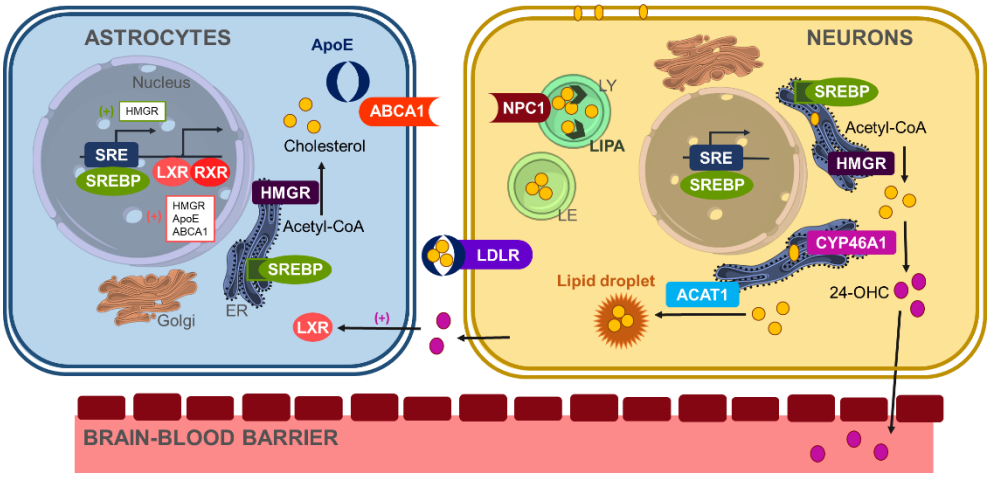


**Figure I.2. Schematic representation of the network of cholesterol transfer between astrocytes and neurons.** In the adult brain, although they still retain the ability to synthesize cholesterol, neurons mostly rely on the uptake of cholesterol from astrocyte-derived apolipoprotein E (ApoE) through interaction with low density lipoprotein receptor (LDLR) scattered in their surface. Cholesterol-loaded lipoproteins are internalized, following the endolysosomal (LE/LY) pathway, and later distributed to plasmatic membrane, and other targets. The excess cholesterol is sensed in the endoplasmic reticulum (ER) and can be converted to 24(S)-hydroxycholesterol (24-OHC) by cytochrome P450 cholesterol 24-hydroxylase (CYP46A1) to be excreted, or to cholesterol esters by acyl-coenzyme A: cholesterol acyltransferase 1 (ACAT1) and stored in the form of lipid droplets. Homeostasis is also achieved through transcriptional regulation of genes coding for proteins involved in cholesterol synthesis, like 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), by the liver X receptor (LXR) and sterol responsive element-binding proteins (SREBPs) (Adapted from: Karasinska and Hayden, 2011; Arenas et al., 2017).

In fact, after its excretion by astrocytes to the interstitial fluid, the cholesterol-enriched ApoE soon become in contact with low-density lipoprotein receptor (LDLR) and low-density lipoprotein receptor-related protein 1 (LRP1) scattered on the surface of neurons (Beisiegel et al., 1989; Wolf et al., 1992; Fan et al., 2001; reviewed in: Brown and Goldstein, 1986; Mahley, 1988). After this interaction, the ApoE-rich lipoprotein particles are then endocytosed followed by transport to late endosomes (LE) and lysosomes (LY). In the lysosomes, the cholesterol esters are hydrolyzed by lipases generating a new supply of unesterified cholesterol (Brown et al., 1975; Kowal et al., 1989; Sugii et al., 2003; reviewed in: Brown and Goldstein, 1986). This stock will presumably be transported in vesicles first to the plasma membrane (PM), followed by transport to endoplasmic reticulum (ER) and Golgi apparatus (Lange et al., 1997; Sugii et al., 2003; Wojtanik and Liscum, 2003; Infante and Radhakrishnan, 2017).

Cholesterol egress from lysosomes is mediated by Niemann-Pick type C1 (NPC1) and C2 (NPC2) proteins, proteins that are localized in LE/ LY vesicles (Higgins et al., 1999; Sugii et al., 2003; Wojtanik and Liscum, 2003). Normally, the inner membranes of endosomes are rich in cholesterol, contrasting with lysosomes that became sterol depleted. However, a deficiency of either one of these proteins will trigger cholesterol lysosomal accumulation (Pentchev et al., 1987; Carstea et al., 1997; Liscum et al., 1989). Although both proteins act in the same pathway, they have a different structure and mode of action. While NPC2 is a small soluble protein, NCP1 is a large glycoprotein consisting of 13 membrane-spanning domains, five of which represent a sterol-sensing domain (SDD) (Carstea et al., 1997; Davies and Ioannou, 2000). Initially, NPC2 binds to the hydrophobic side chain of free cholesterol directing it to NPC1, which will then form a channel, transporting the sterol across the lysosome (Sleat et al., 2004; Cheruku et al., 2006; Xu et al., 2007; Infante et al., 2008; Kwon et al., 2009).

## 1.3. Storage and Excretion of Cholesterol from the Brain

Although cholesterol is vital for numerous neuronal functions, an excess of unesterified cholesterol is toxic. Thus, neurons developed mechanisms to prevent sterol accumulation at the ER and other membranes (reviewed in: Tabas, 2002; Pfrieger and Ungerer, 2011).

One of these mechanisms is, as mentioned previously, the formation of lipid droplets (Goldstein et al., 1974; Wüstner et al., 2005), which are dynamic vesicles that protrude from the ER membrane and function as a storage site for esterified cholesterol and triglycerides (Tauchi-Sato et al., 2002; Robenek et al., 2006).

The enzyme responsible for detoxifying cholesterol by sterol esterification, meaning the addiction of a fatty acid to carbon 3 of cholesterol (reviewed in: Ikonen, 2008), is named acyl-coenzyme A: cholesterol acyltransferase 1 or sterol O-acyltransferase 1 (ACAT1/SOAT1). ACAT1 resides in the ER and is allosterically activated by the presence of high levels of free cholesterol, which helps to counteract the exceedingly low capacity of the ER to carry unesterified cholesterol (Chang et al., 1995; Chang et al., 1998), up to 1% of total cell levels (Lange et al., 1999). ACAT1 is mainly expressed in neurons (Sakashita et al., 2000), although it can be upregulated in oligodendrocytes under certain conditions, such as increased oxidative stress (Kim et al., 2011).

Besides storage, neurons also avoid accumulation of unesterified cholesterol in the ER through hydroxylation of cholesterol into 24(S)-hydroxycholesterol (24-OHC) catalyzed by the cytochrome P450 cholesterol 24-hydroxylase (CYP46A1). This is considered the major pathway for the metabolization and excretion of cholesterol from the CNS (Björkhem et al., 1997; Lund et al., 1999; Lund et al., 2003). Indeed, it was observed that animals lacking CYP46A1 had a decreased rate of cholesterol biosynthesis and increased esterification activity, probably as a compensatory mechanism for the reduced elimination (Lund et al., 2003; Mast et al., 2017). These knockout animals also have difficulty in forming spatial and motor memories, which was attributed to the lack of non-sterol intermediaries of the mevalonate pathway required for protein prenylation of sGTPases (Kotti et al., 2006). In opposition, transgenic animals overexpressing CYP46A1 had enhanced cognitive functions (Maioli et al., 2013). Indeed, our group has previously shown that the differences in cognitive function due to the modulation of CYP46A1 activity *in vivo* are likely due to changes in protein prenylation. Increased CYP46A1 expression induces prenylation and activity of sGTPases of the Rho and Rab families, in neuronal cells both *in vitro* and *in vivo* (Moutinho et al., 2015). Moreover, we have already demonstrated that CYP46A1 triggers an increase in neuronal dendritic outgrowth and dendritic protrusion density and elicits *in vitro* and *in vivo* increases of synaptic proteins in crude synaptosomal fractions. Furthermore, CYP46A1 increased tropomyosin-related kinase (Trk) receptors phosphorylation, its interaction with geranyl transferase I, and the activity of this enzyme, in a cholesterol dependent manner (Moutinho et al., 2016).

CYP46A1 location is mainly restricted to the somata and dendrites of neurons (Ramirez et al., 2008), and excitation of neurons with external stimuli caused the mobilization of the enzyme from the ER to the plasma membrane, increasing the export of 24-OHC (Sodero et al., 2012) in an ABCA1-dependent manner (Matsuda et al., 2013). So far, the promoter of CYP46A1 was found to respond to

oxidative stress (Ohyama et al., 2006) and to epigenetic modulators in a Sp transcription factors dependent manner (Milagre et al., 2008; Nunes et al., 2010; Nunes et al., 2013).

The addition of a hydroxyl group into side-chain carbons in sterols modify their solubility turning them more hydrophilic than cholesterol (Meaney et al., 2002). Nevertheless, they can freely diffuse from the CSF through the BBB, entering the circulation to be excreted through the bile in the liver (Björkhem et al., 1997; Björkhem et al., 1998). In an individual with a healthy liver, since 24-OHC is mostly derived from the brain, it is likely that its plasma levels represent the balance between synthesis and excretion. This makes 24-OHC a biomarker for metabolically active neurons (Lütjohann et al., 1996; Björkhem et al., 1997; Björkhem et al., 1998; Bretillon et al., 2000). In fact, it was observed in multiple studies that patients suffering from dementia and neurodegenerative diseases, like late onset Alzheimer's Disease (AD) and even Parkinson's Disease (PD) had relative low circulating levels of 24-OHC, marking the spread loss of neurons. Due to this, 24-OHC was also considered a potential biomarker for neurodegeneration (Bretillon et al., 2000; Kölsch et al., 2004; Huang et al., 2019).

### 1.4. Maintaining Cholesterol and Lipid Homeostasis

In order to maintain cholesterol levels in steady-state levels, a tight regulation of all proteins involved in the biosynthesis, uptake and degradation of cholesterol to avoid toxicity and cell death are maintained in the CNS (reviewed in: Zhang et Liu, 2015). One mechanism already mentioned is the regulation of cholesterol uptake in neurons via LXR activation.

So far, two isoforms of LXR were identified, LXR$\alpha$ e LXR$\beta$, and both are expressed in the CNS. They are involved in the regulation of several genes from the cholesterol and fatty acid metabolism including HMGR and squalene synthase. Moreover, as previously mentioned LXR controls the expression of ABC transporters and apolipoproteins, as well as the uptake of cholesterol by members of LDLR family (Wang et al., 2002; Whitney et al., 2002; Liang et al., 2004; Abildayeva et al., 2006). In fact, activation of LXR promotes LDLR ubiquitination by the inducible degrader of LDLR (IDOL), therefore decreasing cellular cholesterol influx (Zelcer et al., 2009).

LXR are activated by oxysterols, and 24-OHC is one of the best-studied *in vitro* LXR agonists (Lehmann et al., 1997; Shafaati et al., 2011). Corroborating this finding, it was observed in neuronal cells, that low concentrations of 24-OHC activated LXR transcriptional activity triggering a neuroprotective response (Okabe et al., 2013; reviewed in: Noguchi et al., 2014), while high doses of the same oxysterol favored a "necroptosis-like" response (reviewed in: Noguchi et al., 2014).

Another mechanism that cells have in order to maintain cholesterol homeostasis is the activation of membrane-bound transcription factors, the sterol responsive element (SRE)-binding proteins (SREBPs), responsible for the expression of lipogenic and cholesterologenic enzymes (reviewed in: Brown and Goldstein, 1999; Weber et al., 2004). In fact, it was reported that trapping as little as 1% of cholesterol at the PM rapidly triggers a switch-like response of the SREBP pathway, resulting in an increase of cholesterol synthesis and uptake and the return of PM cholesterol to optimal levels (Infante and Radhakrishnan, 2017).

SREBPs are part of a large class of transcription factors that contain basic helix-loop-helix (bHLH)-Zip domains, with the distinction of being synthesized as ER membrane-bound precursors (reviewed in: Horton et al., 2002) The prevalent isoforms are: SREBP-1a and SREBP-1c which are transcribed from a single gene by alternative promoters, and SREBP-2 expressed from an entire separate gene (Hua et al., 1995).

The latter, SREBP2, is mainly involved in the transcription of genes from the biosynthesis and uptake of cholesterol (Horton et al., 1998; Pai et al., 1998), containing SRE sequences in their promotor (Amemiva-Kudo et al., 2002; reviewed in: Shimano, 2001). Some examples are HMGR, HMGS, lanosterol synthase (LanS) and LDLR (Horton et al., 1998; Pai et al., 1998; reviewed in: DeBose-Boyd and Ye, 2018).

On the other hand, SREBP1 isoforms, specially SREBP-1c, favor the transcription of enzymes involved in the synthesis of fatty acids, including fatty acid synthase (Fas), acetyl-CoA carboxylase (ACC) and stearoyl CoA desaturase 1 (Scd1) (Amemiva-Kudo et al., 2002; reviewed in: Shimano, 2000). The SREBP1-binding site in these genes is designated as SRE-like sequences since they usually have a more diverse consensus sequence than the traditional SRE (reviewed in: Shimano, 2000). Besides, it was reported that some lipogenic enzymes like Fas have incorporated E-boxes sequences in their promoters, which could give some sensitivity towards the level of carbohydrate, glucose or insulin available within the cell (Bennett et al., 1995; Kim et al., 1995; Amemiva-Kudo et al., 2002; reviewed in: Shimano, 2001).

Together, SREBP1 and SREBP2 are transcriptionally and post-transcriptionally regulated (reviewed in: DeBose-Boyd and Ye, 2018). Both are capable of self-regulating their own expression levels in a feed-forward type mechanism, due to the presence of SRE sequences in the promoters of these genes (Sato et al., 1996; Amemiya-Kudo et al., 2000). Plus, SREBPs require the proteolytic cleavage of the membrane-bound precursor, to generate a smaller active nuclear form, known as nuclear SREBP (nSREBP) (reviewed in: Brown and Goldstein, 1999; Horton et al., 2002). Particularly, SREBP1-c is also the subject of another type of transcriptional regulation mediated by LXR, implying a correlation between the synthesis of fatty acids and oxysterol metabolism. This suggests that, if there is an over-accumulation of free cholesterol in the cell, the expression of SREBP1 by LXR will stimulate the production of fatty acids to be used in esterification processes, as means to avoid toxicity (Schultz et al., 2000; Repa et al., 2000; reviewed in: Horton et al., 2002).

SREBPs can be found in the ER membrane, oriented in “hairpin fashion” (Hua et al., 1995). Moreover, each protein can be divided into 3 functional domains: (i) a C-terminal sequence, that participates as a regulatory domain for the maturation of SREBPs and is positioned into the cytosol; (ii) a central section, that functions as anchoring region and is composed by two membrane-spanning hydrophobic segments, split apart by a loop that projects into the lumen of the ER; and (iii) a cytosol-facing N-terminal that comprises the bHLH-Zip portion responsible for dimerization, nuclear entry and transactivation, and also an acidic motif that binds coactivators (reviewed in: Brown and Goldstein, 1999; Horton et al., 2002). Some examples of known transcriptional partners are Sp1 (Sanchez et al., 1995), nuclear factor Y (NF-Y) (Dooley et al., 1998) and cAMP response element binding protein (CREB) (Dooley et al., 1999).

Under physiologic conditions, newly synthetized SREBP forms a tight complex with SREBP-cleavage activating protein (SCAP) (reviewed in: Brown and Goldstein, 1999), entrapped in the membranes of ER because of SCAP binding to resident Insulin-induced gene (Insig) anchor proteins (Yang et al., 2002). As the name depicts, SCAP is capable of sensing cholesterol and fatty acid-depletion (Hua et al., 1996; Sakai et al., 1997) and, in that case, induces SREBP maturation by interacting with its regulatory C-terminal domain (Sakai et al., 1997).

Therefore, when the cells are depleted from cholesterol and there is a fall in the levels of ER sterols (Radhakrishnan et al., 2008), SCAP interacts with coat protein complex II (COPII) machinery. This leads to the incorporation of SCAP-SREBP complex into special COPII vesicles that protrude from the ER, and migrate towards the Golgi, where the two-step cleavage processing occurs (Espenshade et al., 2002; Yang et al., 2002). Initially, a first protease that requires SCAP transport to act, and is known as Site-1 protease (S1P), cleaves SREBPs in their luminal loop. This leads to the separation of SREBP into two segments that remain bound to the membrane. Then, a second protease known as Site-2 protease (S2P) cleaves the N-terminal-containing segment within its membrane-spanning domain, originating the smaller and soluble nSREBP that is released to the cytosol. Finally, nSREBP migrates to the nucleus, where it will bind to SRE sequences in the promoters of the target genes and promote transcription (Sakai et al., 1996; reviewed in: Brown and Goldstein, 1999; Horton et al., 2002). Meanwhile, SCAPs are recycled back to the ER, where they will mediate another SREBP maturation (Nohturfft et al., 1999).

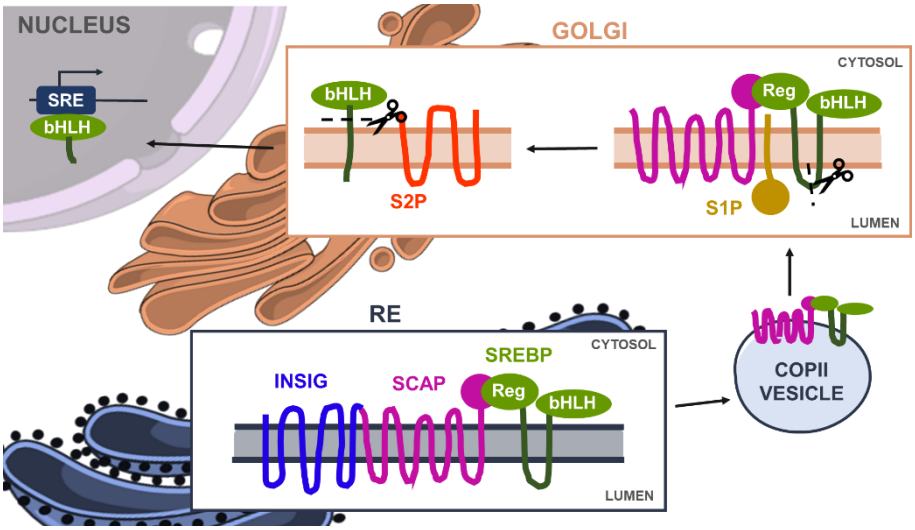


**Figure I.3. Schematic representation of sterol responsive element-binding proteins (SREPBs) two-step proteolytic activation.** Under physiological conditions, SREBPs are retained in the endoplasmic reticulum (ER) membranes thought the association of SREBP-cleavage activating protein (SCAP) with Insig. However, when the ER sterol content falls beyond a threshold point, SCAP-SREBP complex is incorporated into specific coat protein complex II (COPII) vesicles that migrate towards the Golgi apparatus. In this, SREBP is cleaved sequentially by Site-1 and Site-2 proteases (S1P and S2P), releasing its N-terminal segment containing the active basic helix-loop-helix (bHLH)-Zip motif, also known as nuclear SREBP (nSREBP). In the nucleus, nSREBP binds to sterol responsive element (SRE) sequences and promotes the expression of several enzymes involved in fatty acids and cholesterol synthesis. (Adapted from: Horton et al., 2002; Ikonen, 2008; DeBose-Boyd and Ye, 2018).

As seen above, sterols are responsible for finely adjusting all this process, by exerting a negative feedback over SCAP through its sterol-sensing domain (SSD) (Hua et al., 1996; Nohturfft et al., 1998). Firstly, excess cholesterol in the ER can bind directly to SCAP, promoting the

conformational change to bind Insig (Adams et al., 2004; Radhakrishnan et al., 2007). This action restraints the binding site of SCAP, that is recognized by COPII proteins, thus leading to dissociation of SREBP-SCAP from COPII machinery (Sun et al., 2005). Secondly, because sterols reduce the activity of SCAP, they also selectively inhibit cleavage by S1P shutting off SREBP maturation (Nohturfft et al., 1999 DeBose-Boyd et al., 1999; reviewed in: Brown and Goldstein, 1999). Since S2P only acts after cleavage of SREBP by S1P, it is also indirectly regulated (reviewed in: Brown and Goldstein, 1999). Plus, it was shown that oxysterols, like 25-hydroxycholesterol (25-OHC), similarly promote SCAP attachment to Insig, but do it by connecting directly to the Insig protein (Adams et al., 2004; Radhakrishnan et al., 2007).

Another important protein that contains a SSD, is HMGR, whose activity is regulated post-transcriptionally by sterols. Indeed, excess sterols in the ER prompt Insig to bind and ubiquitinate HMGR, marking it for degradation by proteasome and consequently blocking the mevalonate pathway (Sever et al., 2004; reviewed in: Goldstein et al., 2006). Lastly, it was shown that different sterols can affect the establishment of HMGR-Insig and SCAP-Insig complexes in a different manner. While lanosterol was shown to stimulate HMGR-Insig formation, it cannot promote the necessary SCAP conformational change. The opposite happens with cholesterol, which does not influence HMGR attachment to Insig. Finally, oxysterols can induce formation of both complexes (Brown et al., 2002; Song et al., 2005; reviewed in: Goldstein et al., 2006).

## 2. AMPK/mTOR Signaling Pathway in Lipid and Cholesterol Metabolism

### 2.1. AMPK

There are various molecules that could be considered regulators of cell metabolism. One of which is the evolutionary conserved serine/threonine protein kinase AMP-activated protein kinase (AMPK), capable of receiving both intra- and extracellular signals, and acting in order to restore both local and whole-body energy homeostasis (reviewed in: Garcia and Shaw, 2017; Curry et al., 2018). Because of its essential role in maintaining glucose and lipid levels, several groups have investigated the role of AMPK in the brain. Neurons, in particularly, require a fine tuning of their energy status, since they consume a huge bulk of energy for synaptic activity, and cannot synthesize and store the necessary glycogen, relying in neighboring cells to provide nutrients (reviewed in: Poels et al., 2009).

AMPK is a heterotrimeric enzymatic complex with three important subunits: (I) a catalytic α-subunit, that contains both an auto-inhibitory domain and the active site for AMPK activation, the Thr-172 residue (Hawley et al., 1996; reviewed in: Hardie et al., 2003; Curry et al., 2018); (II) a β-regulatory domain that acts as a scaffold, binding the α- and y-subunits together by its C-terminal, and helps to translocate AMPK to cellular membranes (Woods et al., 1996; reviewed in: Hardie et al., 2003; Curry et al., 2018); and (III) a regulatory γ-subunit that binds AMP, ADP or ATP, depending on the metabolic state of the cell, regulating the catalytic function of the complex (Xiao et al., 2007; reviewed in: Hardie et al., 2003; Curry et al., 2018). Moreover, each subunit has its own range of isoforms with tissue-specific roles in the activity and stability of AMPK (reviewed in: Ross et al., 2016).

There are various upstream kinases that activate AMPK under different mechanisms, and the first discovered was the liver kinase B1 (LKB1) in response to a rise in the classical AMP/ATP ratio

(Woods et al., 2003; Shaw et al., 2004). In a state of energy abundance, the γ-domain binds to the available ATP molecules and blocks the site for AMPK activation (Xiao et al., 2007; Chen et al., 2012). On the other hand, under stressful conditions, like glucose starvation, the AMP/ATP ratio quickly elevates, and the abundant AMP readily binds to γ-subunit. Consequently, the γ-subunit suffers a conformational change that exposes Thr-172 in the α-subunit, making it available to be further phosphorylated (Xiao et al., 2007; Chen et al., 2012; Landgraf et al., 2013), thus initiating various catabolic pathways to restore the levels of ATP (reviewed in: Garcia and Shaw, 2017), and promote cell survival (Culmsee et al., 2001; reviewed in: Poels et al., 2009; Curry et al., 2018).

However, despite previous studies stating that AMP binding to the γ-subunit promotes phosphorylation of Thr172 via directly stimulating the activity of the upstream kinases, AMP does not seem to have a direct influence on Thr-172 residue phosphorylation by LKB1 (Sanders et al., 2007). Instead, AMP causes significant allosteric activation of AMPK already phosphorylated on Thr172 residue, while also protecting it from dephosphorylation of Th172 by phosphatases, like protein phosphatase 2A (PP2A) and 2C (PP2C) (Xiao et al., 2007; Sanders et al., 2007). Meanwhile, LKB1 was observed to be constitutively active and indifferent to several AMPK agonists (Woods et al., 2003; Sakamoto et al., 2004). Thus, depending exclusively on the metabolic state of the cell, AMPK activation only occurs in instances of decreased dephosphorylation, meaning in states where there is plenty ADP (Sanders et al., 2007).

Besides the classical AMP/ATP ratio, AMPK can also be regulated through the cytoplasmatic $Ca^{2+}$ concentration. In fact, the calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2) is thought to be the dominant upstream kinase in neurons, regulating AMPK in reaction to $Ca^{2+}$ signaling cascade during neuronal activity (Hurley et al., 2005; Hawley et al., 2005; Woods et al., 2005). Lastly, TGF-β activated kinase 1 (TAK1) is another kinase described to phosphorylate AMPK, triggered by cytokine receptors activation by different signals involved in cell growth, inflammation and apoptosis (Momcilovic et al., 2006).

The mode of function of AMPK is relatively simple: it increases energy levels by inducing energy-producing mechanisms, like fatty acid β-oxidation and glucose uptake, while negatively regulating energy-dispendious processes, such as synthesis of proteins, fatty acids and cholesterol. Particularly, the synthesis of new proteins and cell growth are processes that require huge bulks of energy, maintained by the mammalian target of rapamycin complex 1 (mTORC1). Thus, AMPK acts on mTOR to inhibit its activity and prevent ATP depletion (reviewed in: Garcia and Shaw, 2017).

However, AMPK can have a direct role in suppressing lipid and cholesterol biosynthesis, unrelated to mTOR inhibition, through phosphorylation of SREBP-1c and SREBP2 (Zhou et al., 2001; Li et al., 2011; reviewed in: Garcia and Shaw, 2017). This way, when AMPK is active, it can bind and directly phosphorylate both SREBPs, in their precursor and nuclear form, blocking their cleavage and subsequent nuclear translocation, ultimately inhibiting the expression of their respective target genes. Because SREBPs genes regulate themselves in an SRE-dependent manner, AMPK is also preventing the feed-forward transcription of their own genes (Li et al., 2011). Additionally, AMPK was also discovered to directly phosphorylate and inactivate ACC1 and HMGR, regulating this manner the rate

limiting steps of fatty acid and cholesterol synthesis, respectively (Zhou et al., 2001; reviewed in: Hardie and Carling, 1997; Garcia and Shaw, 2017).

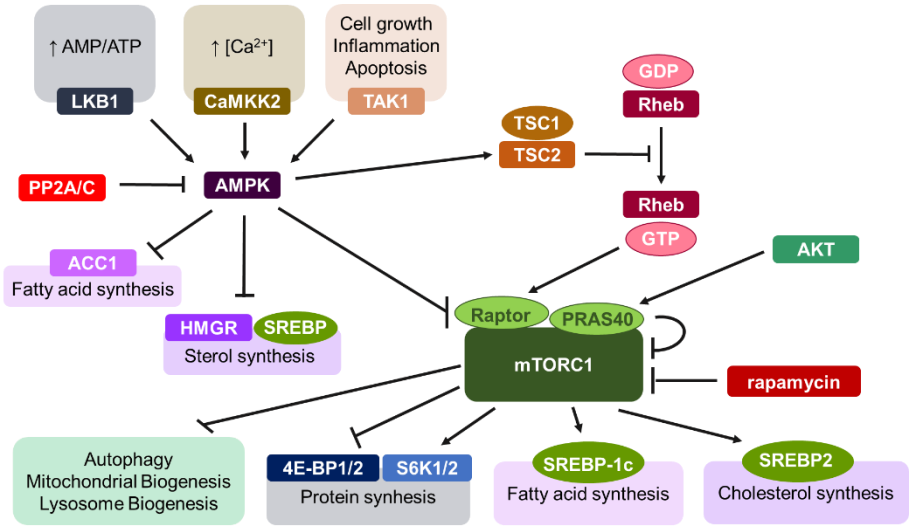


**Figure I.4. Diagram of interactions involved in AMP-activated protein kinase/ mammalian target of rapamycin complex 1 (AMPK/mTOR) signaling pathway.** There are various signals that can trigger AMPK activation by upstream kinases, like high content in AMP or intracellular Ca2+ which activate liver kinase B1 (LKB1) and calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK2), respectively. In order to restore energy levels, the activated AMPK will directly phosphorylate and inhibit sterol responsive element-binding proteins (SREBPs), acetyl-CoA carboxylase 1 (ACC1) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) activity, preventing energy-dispendious processes such as fatty acid and sterol synthesis. AMPK is also responsible for blocking mTORC1 both by phosphorylation of its Raptor unit and by phosphorylating and activating tuberous sclerosis complex 2/1 (TSC2/TSC1). When the energy status of the cell is replenished, mTORC1 can be activated either by protein kinase B (Akt) phosphorylation on proline-rich Akt substrate of 40 kDa (PRAS40) subunit or by loss of AMPK inhibition. In the latter, TSC2/TSC1 complex cannot longer suppress Rheb activity, which in turn activates mTORC1. Thus, resulting in fatty acid and sterol synthesis by stimulating SREBP-1c and SREBP2 maturation, and protein synthesis by phosphorylating and activating ribosomal protein S6 kinases 1/2 (S6K1/2) while blocking eukaryotic initiation factor 4E (eIF4E)-binding proteins 1/2 (4E-BP1/2). Conversely, the active mTORC1 inhibits autophagy, and mitochondrial and lysosomal biogenesis. Lastly, phosphatases like protein phosphatase 2A/C (PP2A/C) can inactive AMPK while rapamycin is known to suppress mTORC1. (Adapted from: Laplante and Sabatini, 2012; Lipton and Sahin, 2014; Garcia and Shaw, 2017).

## 2.2. mTOR

Like AMPK, mTOR is also a phylogenetically conserved serine/threonine kinase that participates in numerous mechanisms in response to the metabolic state of the cell (reviewed in: Laplante and Sabatini, 2012; Lipton and Sahin, 2014). However, both complexes have almost opposite effects, because mTORC1 acts to promote cell growth and proliferation, by inhibiting various catabolic mechanisms, like autophagy (Hosokawa et al., 2009), and stimulating anabolic events, including upregulation of mitochondrial biogenesis (Cunningham et al., 2007) and increase in cholesterol and fatty acid synthesis through the activation of SREBPs (Düvel et al., 2010; reviewed in: Laplante and Sabatini, 2012).

mTOR can be found as two different protein complexes, mTORC1 and mTORC2, that only share mTOR itself, the mammalian lethal with sec13 protein 8 (mLST8) unit and the inhibitory DEP domain containing mTOR-interacting protein (DEPTOR). Then, each complex has a specific structure and act on specific signalization pathway (reviewed in: Laplante and Sabatini, 2012; Lipton and Sahin, 2014).

mTORC2 is further constituted by the rapamycin-insensitive companion of mTOR (Rictor), the mammalian stress-activated MAP kinase-interacting protein 1 (mSIN1) and proteins observed with Rictor 1 (PROTOR 1) and 2 (PROTOR 2) (Sarbassov et al., 2004; reviewed in: Hoeffer and Klann, 2010; Lipton and Sahin, 2014). Some examples of downstream targets are the protein kinase B (PKB or Akt) and Cα (PKCα), and the serum- and glucocorticoid-regulated kinase 1 (SGK1), involved in cell survival and cytoskeleton organization (Sarbassov et al., 2004; reviewed in: Kurdi et al., 2018).

On the other hand, mTORC1 is composed additionally by the regulator-associated protein of the mammalian target of rapamycin (Raptor), and by the proline-rich Akt substrate of 40 kDa (PRAS40) (reviewed in: Hoeffer and Klann, 2010; Lipton and Sahin, 2014). Because of the Raptor unit, mTORC1 is the only complex described to have a high sensitivity to the allosteric inhibitor rapamycin, which acts by interrupting the formation of this complex (Kim et al., 2002; Jacinto et al., 2004; reviewed in: Hoeffer and Klann, 2010).

The mode by which mTORC1 controls protein synthesis is by direct phosphorylation of the ribosomal protein S6 kinases 1 (S6K1) and 2 (S6K2), and the eukaryotic initiation factor 4E (eIF4E)-binding proteins 1 (4E-BP1) and 2 (4E-BP2), each regulating a different step during initiation of cap-dependent translation. While mTORC1 phosphorylation of S6K1/2 leads to their activation, 4E-BP1/2 phosphorylation results in their blockage and release from eIF4E present at the 5' cap of mRNAs, thus promoting gene transcription (reviewed in: Hoeffer and Klann, 2010; Laplante and Sabatini, 2012; Lipton and Sahin, 2014). mTORC1 can also control phosphatases activity, like PP2A, which in turn regulate mTORC1 substrates, creating a feedback loop that regulates initiation rates (reviewed in: Hoeffer and Klann, 2010).

As mentioned previously, mTOR, specifically mTORC1, is a downstream target of AMPK, subject to negative regulation. Therefore, when there is a low energy level, AMPK is activated, and phosphorylates Raptor unit, inhibiting mTORC1 function (Gwinn et al., 2008). Another mechanism by which AMPK suppresses mTORC1 activity is by phosphorylating and activating the tuberous sclerosis complex 2 (TSC2), which is upstream of the mTORC1 signaling cascade. Both TSC2 and associated TSC1 are tumor suppressors that act jointly as GTPase-activating protein (GAP) on Rheb (Ras homolog enriched in brain). Thus, activated TSC2/TSC1 inhibit the activity of Rheb, consequently blocking mTORC1. Conversely, when there is abundance of energy in the cell, mTORC1 is activated, stimulating various anabolic events (Inoki et al., 2003; Inoki et al., 2003; Li et al., 2004; reviewed in: Laplante and Sabatini, 2012; Lipton and Sahin, 2014). Lastly, one mechanism described to regulate mTORC1 activity independent of AMPK and TSC2, is the binding of AKT, a target of mTORC2, to its substrate PRAS40, releasing from its inhibitory pressure (Sancak et al., 2007; Haar et al., 2007).

One of these anabolic events is the activation of lipid synthesis by SREBP-1c, which is done in an insulin-dependent manner (reviewed in: DeBose-Boyd and Ye, 2018). Initially, insulin activates mTORC1, which then up-regulates SREBP-1c activity in three different ways: (I) by stimulating SREBP-1c expression through a complex containing LXR (Li et al., 2010; Tian et al., 2016); (II) by enhancing SREBP1-c proteolytic cleavage by an unknown target of phosphorylation of S6K1 (Düvel et al., 2010; Owen et al., 2012); and (III) though phosphorylation of the phosphatase lipin-1, preventing

its migration to the nucleus and alleviating its inhibitory effect over SREBP-1c expression of lipogenic enzymes (Peterson et al., 2011).

Moreover, recently it was observed that mTORC1 also influences cholesterol synthesis by SREBP2, in a process depending on membrane trafficking. When mTORC1 is highly active, it blocks autophagic processes and restricts endosomal recycling in the plasma membrane. This means that there are low levels of cholesterol in lysosomes and they are not enough to suppress SREBP2 maturation in the ER. However, when mTORC1 activity is severely reduced, there is a stimulation of autophagy and uptake of cholesterol that enters the LE/LY pathway. Thus, leading to a rise in ER cholesterol levels and inhibition of SREBP2 proteolytic cleavage (Eid et al., 2017).

## 3. Parkinson's Disease (PD)

PD is considered the second most common neurodegenerative disorder after AD, affecting more than six million people worldwide (reviewed in: Doria et al., 2016), with a rising prevalence given the aging of the world population (De Rijk et al., 1997; reviewed in: Doria et al., 2016).

### 3.1. Hallmarks of PD

The pathological hallmarks of PD are the selective death of midbrain dopaminergic neurons in the Substantia Nigra *pars compacta* (SNpc) and the accumulation of abnormal proteinaceous inclusions in the cytoplasm of the surviving neurons, designated as Lewy bodies (LBs) (reviewed in: Dauer and Przedborski, 2003; Poewe et al., 2017). Since there is a marked loss of the nigrostriatal neurons that are rich in neuromelanin, other feature found to be typical of PD brains is depigmentation of the SNpc. Due to the same reason there is also a characteristic loss of dopamine in the putamen, where dopaminergic neurons project (reviewed in: Dauer and Przedborski, 2003).

Because the nigrostriatal pathway is severely damaged and is dopamine-poor, PD mainly manifests itself as a progressive motor disorder with symptoms including resting tremors or involuntary movements (also called dyskinesias), muscle rigidity (stiffness), slow movements and changes in posture, also collectively known as Parkinsonism (reviewed in: Dauer and Przedborski, 2003; Doria et al., 2016). Nevertheless, it can be accompanied by non-motor symptoms like sleep disturbances, dementia, alterations in behavior and depression, that may precede the diagnosis, which is based mainly based in motor symptoms, and worsens the quality of life of PD patients (O'Sullivan et al., 2008; reviewed in: Chaudhuri and Schapira, 2009).

Usually, the diagnosis employs a combination of magnetic resonance imaging (MRI) and positron emission tomography (PET), to help establish the degree of the dopaminergic lesion, as well as rule out other syndromes with overlapping symptoms to PD (reviewed in: Stoessl et al., 2014). Still, the difficulty in diagnosing PD in time, can be attributed to the fact that when the physical symptoms start to appear, about 60% of the dopaminergic neurons in SNpc are already severely damaged, and approximately 80% of the dopamine in the putamen is missing. Moreover, the definitive diagnosis requires the identification of the pathological marks mentioned, namely LBs and SNpc neuronal loss, which are done in post-mortem brains (reviewed in: Dauer and Przedborski, 2003).

To this day, PD is still not curable, and treatments are mostly focused on the relief of the motor symptoms with the administration of L-dopa as the most popular therapy, for replenishing the lack of dopamine in the striatum (reviewed in: Dauer and Przedborski, 2003; Poewe et al., 2017).

Most of the diagnosed cases of PD are sporadic PD (sPD) and are considered idiopathic since there is doubt about the influence of environmental agents (like pesticides), metabolism alterations and genetic factors. Still, one of the major risks for developing sPD is aging, with the onset typically at 60 years old, and with the incidence multiplying with each further decade (reviewed in: Dauer and Przedborski, 2003). This is attributed in part to the fact that, as people get older, it increases the possibility for accumulation of mutations in mitochondrial DNA (mtDNA) and oxidative stress (Bender et al., 2006; Kraytsberg et al., 2006), while there is also impairment of quality control processes, namely the ubiquitin-proteasome and autophagy machinery (reviewed in: Kaushik and Cuervo, 2015). Plus, the link between mitochondria dysfunction and the development of sPD is well documented, since it was found that PD patients had a severe deficit in the activity of NADH dehydrogenase, present in the complex I of the electron transport chain, as seen in samples of substantia nigra from post-mortem brains (Schapira et al., 1990), as well as fibroblasts (Mytilineou et al., 1994) and blood platelets samples (Haas et al., 1995).

Only 5% to 10% of the cases are attributed to have a familial origin (reviewed in: Poewe et al., 2017). The first case of inherited PD described a mutation in gene coding for α-synuclein, the major component of LBs (Polymeropoulos et al., 1997; reviewed in: Dauer and Przedborski, 2003). Since then, several genetic studies allowed the identification of other PD-causing mutations, in genes involved in mitochondrial quality control, such as PTEN-induced kinase (PINK) (Valente et al., 2004), Parkinson disease protein 2 (Parkin) (Kitada et al., 1998) and Parkinson disease protein 7 (DJ-1) (Bonifati et al., 2003); and autophagy, such as leucine-rich repeat kinase 2 (LRRK2) (Paisán-Ruíz et al., 2004), providing clues about the underlying mechanisms of the disease.

### 3.2. From Mitochondria Injury to Lysosomal Dysfunction

As mentioned before, one of pathological hallmarks of PD is the accumulation of LBs, which are spherical cytoplasmatic aggregates scattered through the brain and composed of numerous proteins like parkin, ubiquitin and neurofilaments (reviewed in: Dauer and Przedborski, 2003). Still, its main component is α-synuclein (Spillantini et al., 1998), a small cytosolic protein that is highly expressed in the CNS. Under physiological conditions, α-synuclein exists as small molecular weight oligomers, like dimers and trimers, near the presynaptic terminal of neurons and in astrocytes (Maroteaux et al., 1988; Jakes et al., 1994; Mori et al., 2002; reviewed in: Dauer and Przedborski, 2003). It is thought to have a role in maintaining synaptic function, through interaction with dopamine transporter, modulation of the size of the presynaptic vesicles and regulation of the rate of cycling the readily released pool (Abeliovich et al., 2000; Lee et al., 2001; Murphy et al., 2000). Plus, it appears to have an influence in cellular membrane dynamics and trafficking machinery since it reversibly attaches to lipid membranes and transport vesicles (Jensen et al., 1998; Jensen et al., 1999; Eliezer et al., 2001). Nevertheless, the conformation of physiological and pathological α-synuclein remains a controversial issue.

However, in the case of PD, there are known pathogenic mutations in the α-synuclein gene that promote the formation of non-fibrillar oligomers, or protofibrils, which in its turn are considered the most toxic species of α-synuclein (Conway et al., 1998; Conway et al., 2000; Rochet et al., 2000). Given the propensity of α-synuclein to interact with synaptic vesicles, it is believed that in its profibril form, it acts in a way to permeabilize these vesicles, thus provoking the release of dopamine into the cytoplasm (Lashuel et al., 2002). Afterwards, dopamine in the cytoplasm can suffer auto-oxidation, generating the highly reactive superoxide and other reactive oxygen species (ROS). ROS can further promote cellular damage by reacting with nucleic acids, proteins and membrane lipids (reviewed in: Dauer and Przedborski, 2003). The free oxygen radicals can also provoke the inactivation of tyrosine hydroxylase, which is an enzyme involved in dopamine synthesis, further increasing the deficit of this neurotransmitter (Ara et al., 1998).

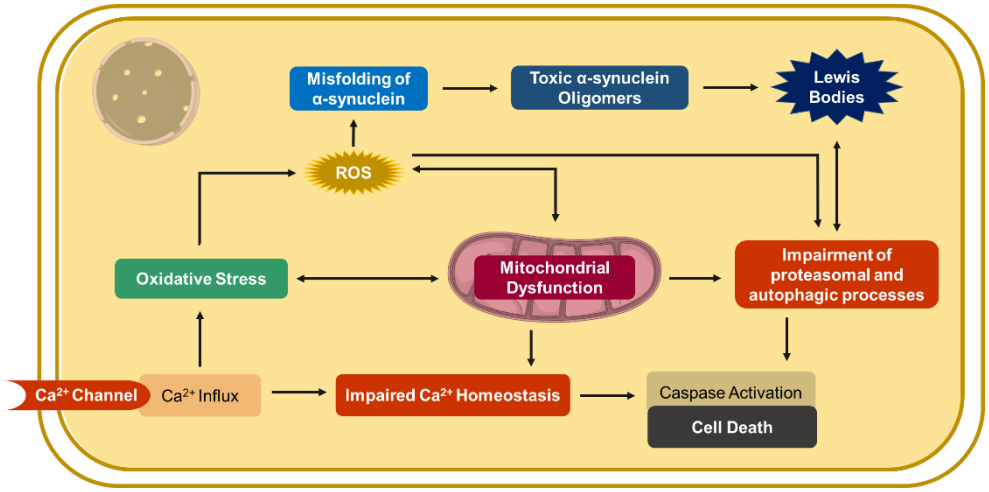


**Figure I.5. Diagram of interactions from pathways involved in Parkinson's Disease (PD).** In PD, there are various mechanisms that can be altered and contribute to the development of the disease. One of this is loss of mitochondrial function which leads to oxidative stress and influx of Ca2+ into the cell. The production of reactive oxygen species (ROS) can result in the misfold of α-synuclein and formation of toxic oligomers, which can further lead to formation of Lewis Bodies. The accumulation of protein aggregates is implicated in the impairment of both proteasomal and autophagic processes, also contributed by the mitochondrial dysfunction and the toxic oxygen species. Particularly, ROS can induce permeabilization of lysosomes releasing caspases into the cytoplasm and triggering cell death. (Adapted from: Poewe et al., 2017).

Other characteristic of PD, already referred above, is the severe mitochondrial dysfunction observed in neurons, glia and even platelets from diagnosed patients. Analysis of these types of samples demonstrated a block in the complex I of the electron transport chain, resulting in decrease of ATP levels and in a rise in oxidative stress (reviewed in Doria et al., 2016). Then, just like in a vicious cycle, ROS can also target the electron transport chain itself, leading to even more oxidative damage and further severing mitochondrial function. In addition, the depletion of energy from mitochondria loss of function, causes an increase in the levels of $Ca^{2+}$ in the cytoplasm, thus causing the leakage of dopamine from the synaptic vesicles and activating caspase cascade, ultimately leading to apoptosis (reviewed in: Dauer and Przedborski, 2003).

Given the number of degenerated mitochondria accumulated and the presence of ROS and Lewis bodies, it has also been shown that there is an impairment of both proteasomal and autophagic proteolysis in PD, since these are the main mechanisms for the degradation of cellular components

(reviewed in: Wong and Cuervo, 2010; Arduíno et al., 2011). For instance, the mitochondrial-derived oxygen species can induce lysosomal membrane permeabilization (LMP), leaking various enzymes to the cytoplasm, which results in lysosomal depletion, defective clearance and accumulation of undegraded autophagosomes (Dehay et al., 2010). Plus, if there is a considerable amount of both proteins and organelles needed to be removed, vesicular trafficking can be also impaired, further leading to a decrease in the clearance of autophagosomes (Arduíno et al., 2012). LMP can also be directly responsible for the triggering of cell death, since one of the lysosomal enzymes released to the cytoplasm is cathepsin D, a known pro-apoptotic enzyme (Dehay et al., 2010). Thus, autophagic process is also very relevant to the pathology of PD, since it functions as a protective mechanism against toxicity and allows protein/ organelle quality control by selectively removing atypical protein aggregates, like the LB, and even entire cytoplasmatic organelles, such as compromised mitochondria (reviewed in: Wong and Cuervo, 2010; Arduíno et al., 2011).

Nevertheless, the initial insult that triggers the neurodegeneration process involved in PD is still unknown, being hypothesized to be the accumulation of protein aggregates, the mitochondrial dysfunction or even a combination of both. Regardless, it will result in the loss of cell functions, including energy production, increase in oxidative stress, block in vesicle trafficking, loss of quality control mechanisms and impairment of autophagic processes, ultimately leading to neuroinflammation and cell death (reviewed in: Dauer and Przedborski, 2003).

### 3.3. Study of Parkinson's Disease

As mentioned before, PD is a complex and multifactorial disease. Despite the recent efforts, the mechanisms that underlie the development of this disease remain to be understood, thus, highlighting the need to study and understand the mechanisms behind the development and progression of the disease.

#### 3.3.1. MPTP-treated Mice as an Animal Model of PD

The use of animal models in the study of PD has been essential to discover the pathophysiologic mechanisms involved in the development of the disease and to unveil new therapeutic approaches to minimize its symptoms. Currently, in the *in vivo* research of the disease, there are available both drug-induced and genetic models, each with its own advantages depending on the objective of the study (reviewed in: Bové and Perier, 2012; Blandini and Armentero, 2012).

In order to learn specific key molecular pathways involved in the development of PD, especially in familial PD, the use of transgenic models is recommended. These can replicate the formation of fibrils resembling those seen in LBs, like in the case of the common α-synuclein overexpression model, and even some neuropathological features such as the motor impairment (Lee et al., 2002; Giasson et al., 2002). On the downside, little is recorded about the loss of dopaminergic neurons (reviewed in: Dauer and Przedborski, 2003; Blandini and Armentero, 2012). On the other hand, although the toxin models do not recapitulate all the neurological symptoms like the formation of the pathogenic inclusions, they demonstrate a more consistent impairment of the nigrostriatal

pathway, even if by different mechanisms of action and behavioral changes (reviewed in: Meredith and Rademacher, 2011; Blandini and Armentero, 2012).

Regarding toxin animal models, there are several toxins used to mimic the features of PD in laboratory studies, ranging from known herbicides and insecticides, namely paraquat and rotenone, and pharmacological agents such as 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (reviewed in: Dauer and Przedborski, 2003; Bové and Perier, 2012). There are also different animal models that can be used, comprising primates, rats, mice and even worms (reviewed in: Przedborski et al., 2001; Bové and Perier, 2012). Thus, the importance of the selection of the animal model and the neurotoxin used to approach the study of the disease.

In order to study cell death mechanisms in PD, the most common model used is the administration of MPTP to mice (reviewed in: Bové and Perier, 2012), although there are several parameters that can alter the sensitivity to the neurotoxin. Different strains of mice demonstrate different magnitudes of dopaminergic lesion, and within the same strain, the gender, age and body weight may have an influence in the results (reviewed in: Jackson-Lewis and Przedborski, 2007). Also, the type of administration of MPTP (route and duration) and the dose used are factors that must be accounted for (Sonsalla and Heikkila, 1986; reviewed in: Jackson-Lewis and Przedborski, 2007).

After administration, MPTP reaches the brain through the systemic circulation. Because it is a highly lipophilic toxin, it can easily diffuse across the BBB. In the brain, the astrocytes and serotonergic cells uptake MPTP, where the enzyme monoamine oxidase B (MAO-B) catalyzes the production of 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$). $MPDP^+$ is an unstable molecule, so it is quickly converted into the active metabolite 1-methyl-4-phenylpyridinium ($MPP^+$) that is further released to the extracellular space (reviewed in: Dauer and Przedborski, 2003; Bové and Perier, 2012). Since $MPP^+$ has high affinity to the dopamine transporter, it is selectively uptake by surrounding dopaminergic neurons (Bezard et al., 1999). Inside, $MPP^+$ can: (I) accumulate in mitochondria through a mechanism dependent on the mitochondrial transmembrane potential (Hoppel et al., 1987); (II) cooperate with the vesicular monoamine transporter-2 (VMAT2), leading to its build up in synaptic vesicles (Liu et al., 1992); or (III) interact with negative charged enzymes in the cytoplasm of neurons (Klaidman et al., 1993; reviewed in: Dauer and Przedborski, 2003).

The accumulation of the toxin in synaptic vesicles seems to be neuroprotective, preventing its access to mitochondria (Liu et al., 1992). Indeed, following the entering to the mitochondria matrix, $MPP^+$ propagates toxicity by blocking the oxidation of glutamate or pyruvate by the complex I multi-subunit enzyme NADH-ubiquinone oxireductase of the electron transport chain (Nicklas et al., 1985; reviewed in: Bové and Perier, 2012). Consequently, this causes the loss of mitochondrial membrane potential, accumulation of ROS, depletion of ATP and finally triggers mitochondrial apoptotic cascade (reviewed in: Dauer and Przedborski, 2003; Bové and Perier, 2012). This mode of action is parallel to what has been seen in post-mortem brains of PD patients, where it was observed a substantial impairment of mitochondrial function from the inhibition of complexes I-IV of the electron transport machinery (reviewed in Bové and Perier, 2012; Doria et al., 2016). Thus, highlighting the usefulness of MPTP in studies with mouse models of PD.

There are three types of regimens used when administering MPTP in mice: (I) acute, that triggers non-apoptotic neurodegeneration and used to study inflammation, in which the animal is injected intraperitoneal (i.p.) four times in the same day, each with 20mg/kg MPTP (Jackson-Lewis et al., 1995; reviewed in: Bové and Perier, 2012); (II) sub-acute, leading to a more gradual loss of dopaminergic neurons in an apoptotic manner and used to study molecular pathways of cell death, that involves the injection of 30mg/kg i.p. during 5 succeeding days (Tatton and Kish, 1997; reviewed in: Bové and Perier, 2012); and (III) chronic, that replicates the slow impairment of the nigrostriatal pathway and the only regimen that induces the formation of protein aggregates, where, as one example, MPTP can be delivered along 21 days with the help of a minipump (Bezard et al., 1997; reviewed in: Meredith and Rademacher, 2011). However, the parameters of these regimens are not completely strict, as each laboratory group designs the best protocol for its envisioned studies. Indeed, in our lab, to observe the respond in the early stages of MPTP treatment, we adopted a modified acute regimen where MPTP is administered in a single dose of 40mg/kg i.p. and sacrificed the animals 3h or 6h later (Castro-Caldas et al., 2009; Castro-Caldas et al., 2012; Rosa et al., 2018).

### 3.3.2. $MPP^+$-treated Cell lines for the Study of PD

The use of cell culture models in studying neurodegenerative diseases offers many advantages in comparison to animal models. Besides allowing the investigation of the role of an isolated cell type in a condition that will mimic the disease environment, cell-based studies enable the study of a specific molecule or pathway within the cell, and whether or not its effect is protective or toxic in the context of the disease. Plus, they facilitate the screening of potential drugs and pharmacological targets, while also being relatively easy to manipulate without the high-costs and ethical problems associated with animal models (reviewed in: Falkenburger and Schulz, 2006; Schlachetzki et al., 2013; Falkenburger et al., 2016).

For the study of PD, cells can also be genetically and pharmacologically manipulated. Between the variety of neurotoxins that can be employed, $MPP^+$ is once again the most common, since it mimics the effects seen in post-mortem brains of PD patients (reviewed in: Bové and Perier, 2012). Thus, the use of cell lines treated with $MPP^+$, like the human-derived HEK293 and SH-SY5Y cell lines, and mouse-derived N2a cell line, are also very useful in in studies of PD that research pathways that are possibly altered in the disease, including the depletion of energy, dysregulation of $Ca^{2+}$ homeostasis, oxidative stress and apoptotic cell death mechanisms (reviewed in: Schmidt and Ferger, 2001).

## 4. New Clues about the Role of Cholesterol in PD

As seen in the Chapter 1.1., cholesterol is a very abundant molecule in the human brain, performing roles in synaptogenesis, axonal guidance, modulation of dendrite outgrowth, and even synthesis of myelin sheets during brain development. So, given its importance, any perturbations in cholesterol homeostasis could lead to disruption of neurotransmission, loss of synaptic plasticity, neurodegeneration and even the onset of neurodegenerative diseases. Nevertheless, the role of cholesterol in PD remains controversial and vastly unexplored.

There are already various reports that could potentially show a link between PD and disturbances in cholesterol homeostasis. For example, a genome-wide association study of sporadic PD (Do et al. 2011), identified SREBP1-coding gene (*SREBF1*) as a genetic risk factor for this disease. Interestingly, Ivatt and coworkers (2014), identified in a genome-wide RNAi screen, this risk *locus* as a regulator of mitophagy, proposing a role for lipids in the regulation of mitochondrial homeostasis. Moreover, impairment of cholesterol synthesis is suggested to be involved in PD (Musanti et al., 1993), while parkin, an E3 ubiquitin ligase encoded by PARK2, which is mutated in some patients with early onset PD, regulates systemic lipid metabolism (Kim et al., 2011).

Another important finding is that α-synuclein, the major constituent of the LB, has two cholesterol-binding domains, one of which binds to cholesterol with high affinity (Fantini et al., 2011). Plus, Bar-On and colleagues (2006), demonstrated that cholesterol-extracting agents, namely methyl-β-cyclodextrin, reduced the accumulation of α-synuclein in neuronal cell bodies and synapses in B103 neuroblastoma cells, previously transfected with human α-synuclein. Using the same experimental conditions, these authors observed that statins, which are cholesterol synthesis inhibitors, also decreased the aggregation of α-synuclein in detergent insoluble fractions, while the supplementation of the media with cholesterol had the opposite effect (Bar-On et al., 2008). A similar effect was observed *in vivo* by Koob et al. (2010), since transgenic mice overexpressing α-synuclein in neurons and treated with statins had fewer inclusions of this protein in comparison with controls.

Paul et al. (2017), observed that hypercholesterolemia in mice could cause oxidative stress and lead to impairment of the nigrostriatal pathway. In the same study, they observed that MPTP-treated mice fed with a high cholesterol diet show an increased vulnerability to the loss of dopaminergic neurons, further aggravating the motor impairment given by the neurotoxin alone. Similarly, in an *in vitro* study, Raju and team (2017), saw that the addition of cholesterol to differentiated SH-SY5Y cells treated with $MPP^+$, potentiated the neurotoxicity and reduced viability, probably by decreasing mitochondrial membrane potential. Likewise, Eriksson et al. (2017), also observed accumulation of cholesterol in a lysosomal-like pattern in pre-apoptotic BE(2)-M17 cells treated with $MPP^+$, comparing it to a similar profile after treatment with U18666A, a known intracellular cholesterol transport inhibitor.

## 5. Aims of the Study

Neurodegenerative diseases such as PD are a truly global challenge and one of the leading medical and societal challenges faced by European Union society. To date, there are no effective treatments that can slow or halt the disease, and currently approved drugs only temporary ameliorate motor impairment. Therefore, it is imperative that drug discovery attempts to identify new potential therapeutic targets to open new therapeutic avenues for this disease. Therefore, the main goal of this thesis was to identify novel disease-modifying targets in PD, focusing on neuronal cholesterol homeostasis. Specifically, we wanted to explore *in vitro* and *in vivo* how mitochondrial dysfunction could affect cholesterol intracellular levels and localization, and whether such changes could have a neuroprotective or deleterious role in the development of the disease.

In order to accomplish these goals, our work was divided into three main tasks:

I. Characterization of the effect of $MPP^+$ treatment in N2a neuroblastoma cells by evaluating changes in total, free and esterified cholesterol levels, cholesterol intracellular distribution, SREBPs transcriptional activity, and the expression levels of various key genes and proteins involved in cholesterol synthesis, catabolism, uptake and transport.

II. Characterization of the effect of MPTP administration on the nigro-striatal region of mice brain, by evaluating changes in total, free and esterified cholesterol levels and the expression levels of various key genes and proteins involved in cholesterol synthesis, catabolism, uptake and transport.

III. Identification of signaling pathways activated upon mitochondria dysfunction that could be responsible for alterations in cholesterol homeostasis, focusing on the activation of AMPK/mTOR signaling pathway.

# Chapter II. Materials and Methods

## 1. Materials

### 1.1. Reagents and Supplements

Dulbecco's Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from Corning (Life Sciences, MA, USA). Opti-Minimum Essential Medium (Opti-MEM), penicillin-streptomycin mix, L-glutamine and TripLE Express were acquired from GIBCO (Thermo Fisher Scientific Inc, MA, USA). Amido Black Staining Solution 2X, Filipin III from *Streptomyces filipinensis*, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 1-methyl-4-phenylpyridinium ($MPP^+$), were bought from Sigma Aldrich Inc (MO, USA). Compound C (CC) was obtained from CalBiochem (CA, USA). Proteinase inhibitors, DNAse I Recombinant Kit and β-galactosidase Reporter Gene Assay (chemiluminescent) was purchased from Roche Applied Science (Penzberg, Germany). Albumin Fraction IV used for diluting antibodies used in Western Blot was acquired from PanReac AppliChem (ITW Reagents Division, Darmstadt, Germany). Bovine serum albumin (BSA) used in protein quantification, polyvinyl difluoride (PVDF) membranes and SuperSignal™ West Femto were obtained from Thermo Fisher Scientific Inc. 30% Acrylamide/Bis-acrylamide and Bio-Rad Protein Assay Reagent was obtained from BioRad Laboratories Inc. (CA, USA). Sodium dodecyl sulfate (SDS) 20% and NZYTech Reverse Transcriptase Kit was acquired from NZYTech Lda. (Lisbon, Portugal). Lipofectamine 3000 and Amplex Red Cholesterol Assay Kit were purchased from Invitrogen™ (Thermo Fisher Scientific Inc). NucSpot 470 Nuclear Stain was obtained from Biotium (CA, USA). WesternBright™ ECL was purchased from Advansta (CA, USA). TRIzol Reagent was bought from Ambion (Thermo Fisher Scientific Inc.).

### 1.2. Antibodies

The primary antibodies used in Western Blot analysis were purchased as follow: mouse anti-SREBP1 and rabbit anti-SREBP2 from Abcam (Cambridge, UK); rabbit anti-mTOR, rabbit anti-*p*mTOR, rabbit anti-AMPKα and rabbit anti-*p*AMKα from Cell Signaling Technology Inc. (MA, USA); mouse anti-β-actin from Sigma-Aldrich Inc. Both secondary antibodies, goat anti-rabbit horseradish peroxidase and goat anti-mouse horseradish peroxidase, were acquired from BioRad Laboratories Inc. For the immunocytochemistry assays, the primary antibody rat anti-LAMP2 was acquired from Santa Cruz Biotechnology Inc., whereas the secondary goat anti-rat Alexa Fluor 594 was obtained from Abcam (Cambridge, UK).

## 2. Methods

### 2.1. Animal Treatments and Dissection

The animal experiments described below were conducted under the Institutional, Portuguese and European guidelines (Diário da República, n.º 151/2013, Série I de 2013-08-07; and 2010/63/EU

European Council Directive), and the methods approved by the Direção Geral de Alimentação e Veterinária and Órgão Responsável pelo Bem-Estar Animal (ORBEA) of Faculty of Pharmacy, Universidade de Lisboa. The twelve-week-old male C57BL/6 wild-type mice used in the trials were purchased form Harlan, and kept under regimented conditions on a 12h light-dark cycle, at constant temperature (22-24$^0$C) and humidity (50-60%), with free access to a standard diet and water *ad libitum*, in the FFULisboa Animal House – Campus Lumiar. Then, the animals were divided into groups depending on the duration of MPTP treatment (3h and 6h). The animals treated with the neurotoxin received a single acute i.p. injection of 40mg/kg body weight and were later sacrificed according to each group mentioned, whereas control mice were injected i.p. with a saline solution and sacrificed 6h after i.p. injection. All mice were anesthetized with isoflurane, decapitated, the midbrain and striatum brain regions were dissected, frozen in liquid nitrogen, and stored at -80ºC until needed.

### 2.2. Cell Culture

The N2a mouse neuroblastoma cell line were grown in T75 flasks (Falcon®, Corning, NY, USA), at 37ºC, 5% $CO_2$ in humidified atmosphere, in 1:1 (v/v) Opti-MEM/ high glucose DMEM media, supplemented with 10% heat inactivated FBS, 2mM L-glutamine, penicillin (100U/mL) and streptomycin (100ug/mL). Cell sub-culturing and harvesting were achieved by enzymatic dissociation when cells reached a confluency of approximately 80%, with 2mL of TrypLE Express. Cells were counted with the help of a hemocytometer, diluted in fresh medium and reseeded at the desired concentration. The treatments were only done in the following day, after changing to fresh medium.

### 2.3. Preparation of Total Tissue and Cell Extracts

In order to obtain total cell extracts, cells were washed twice with PBS, and lysed in lysis buffer (50mM Tris-HCl pH7.4, 180mM NaCl, 1Mm EDTA, 1% Triton-X 100, 1mM DTT, 10mM NaF, 1.25mM $Na_3VO_4$ and 1X protease inhibitors). Total tissue extracts from mouse midbrain and striatum were prepared from a pool of two different MPTP-treated mice brains, which were mixed, homogeneized and resuspended in Cell lysis buffer 1X (Cell Signaling Technology) supplemented with 1mM DTT, 1.25mM $Na_3VO_4$ and 1X protease inhibitors). Afterwards, the samples were sonicated four times for 4 seconds each, followed by a centrifugation at 13 000 rpm for 15 minutes at 4ºC. The supernatants were recovered and kept at -80ºC until further use. Quantification of both total protein levels was done by Bradford Quantification using Bio-Rad Protein Assay Reagent.

### 2.4. Western Blot

Before immunoblotting, the samples were denatured for 5 minutes at 95ºC using a mixture of 1:1 (v/v) sample/ SDS denaturing buffer (0.25M Tris-HCl pH6.8, 4% SDS, 4% glycerol, 1% β-mercaptoethanol and bromophenol blue). Afterward, proteins were resolved on a 7.5% or 10% SDS-polyacrylamide (37.5:1 crosslinker ratio) gel electrophoresis (SDS-PAGE) with a fixed current of 35mA. The gel was then blotted to an activated PVDF membrane with a constant current of 500mA for 2 hours. For activation the PVDF membrane was wetted in ethanol for 1 minute, transferred to water for 2 minutes, and equilibrated for 5 minutes in transfer buffer. For immunodetection, the membranes

were blocked in 5% dry non-fat milk in Tris-buffered saline-tween (TBS-T, 20mM Tris-HCl pH 7.6, 150mM NaCl, 0.1% Tween-20) for 1 hour and incubated overnight at 4ºC (with agitation in a cold chamber) with the specific antibodies: mouse anti-SREBP1 (1:400 3% BSA), rabbit anti-SREBP2 (1:400 3% BSA), rabbit anti-*p*mTOR (1:500 5% BSA), rabbit anti-mTOR (1:1000 5% BSA), rabbit anti-*p*AMPKα (1:1000 3% BSA), rabbit anti-AMPKα (1:1000 5% BSA) and mouse anti-β-actin (1:40000 3% BSA). The incubation with the secondary antibody, goat anti-rabbit horseradish peroxidase (1:5000 5% non-fat dry milk) or goat anti-rabbit horseradish peroxidase (1:5000 5% non-fat dry milk), occurred in the following day for at least 1 hour at room temperature with agitation. Between each of these steps, the membranes were washed three times with TBS-T for 15 minutes each. The membranes were revealed using WesternBright™ ECL (Advansta, USA) and SuperSigmal™ West Femto (Thermo Fisher Scientific, USA) reagents in ChemiDocTM MP Imaging System (Bio-Rad, USA). Between incubations with different antibodies, the membranes were stripped with an acidic stripping solution (0.4M glycine, 0.2% SDS, 17mM Tween-20, 2.9M acetic acid, pH2.2). Both the evaluation of β-actin expression and Amido Black staining were used as a loading control. Results were analyzed using Image Lab Software Version 5.0 build 18 (Bio-Rad Laboratories, USA) and are displayed as fold induction from control samples.

### 2.5. RNA Extraction

After harvesting, the cells were washed two times with PBS 1X, resuspended in TRIzol Reagent (Ambion, Thermo Fisher, USA) and stored at -80ºC. Later, the lysate was collected and incubated at room temperature for 5 minutes. For each mL of TRIzol used, 0.2mL of chloroform was added. The samples were then agitated vigorously for 15 seconds, incubated for 2 to 3 minutes at room temperature and centrifuged at 12000 x *g* for 15 minutes at 4ºC. The aqueous phase was recovered and added to 0.5mL of isopropanol for each mL of TRIzol used, followed by an incubation for 10 minutes at room temperature. Samples were centrifuged at 12000 x *g* for 10 minutes at 4ºC and the resulting pellet was washed with 70% ethanol and airdried. Finally, the RNA extracts were resuspended in DEPC-treated water and incubated for 10 minutes in a warm bath at 55ºC. The RNAs were quantified using Nanodrop spectrophotometer (Thermo Scientific, USA). Once again, RNA samples were kept at -80ºC. Brain tissue from MPTP-treated mice was resuspended and dissociated in TRIzol and RNA isolated as previously mentioned. Each samples of RNA from midbrain or striatum corresponds to a single MPTP-treated mouse.

### 2.6. Quantitative Polymerase Chain Reaction (qPCR)

Before proceeding to cDNA synthesis, RNA samples were digested with DNAse I Recombinant enzyme (Roche, Germany). For this, 1µg of total RNA was diluted to a final volume of 8µL, and 1µL Incubation Buffer 10X (Roche, Germany), 0.1U/µL DNAse I and DEPC-treated water was added to this solution to reach a final volume of 10µL. The samples were then heated at 37ºC for 20 minutes, followed by 10 minutes at 75ºC, in a VWR Thermal Cycler (VWR International LLC, USA). For cDNA synthesis, 5ng/µL random hexamers and 0.5mM dNTP mix was added to the DNAse-treated samples. After 5 minutes incubation at 65ºC, 100U of NZY Tech Reverse Transcriptase

enzyme (NZYTech) diluted in Reaction Buffer 1X (NZYTech) and 2µL of Reaction Buffer 10X were added to the mix to reach a final volume of 20µL. The samples were then exposed to 25ºC for 10 minutes, followed by 50 minutes at 50ºC. In the last step, the samples were subjected to 85ºC for 5 minutes. When cooled down, the samples were stored at -20ºC. Next, a master mix of SensiFAST™ SYBR® Hi-ROX Kit (Bioline, UK) and primers was prepared for each target gene according to Table 2.1.

**Table II.1.** Sequences of primers used in RT-PCR analysis.

| Gene | Species | Sequence (5'-3') |
|---|---|---|
| ACAT1 | Mouse | GAA GGC TCA CTC ATT TGT CAGA (fwd)<br>GTC TCG GTA AAT AAG TGT AGG CG (rev) |
| ACC1 | Mouse | GAT GAA CCA TCT CCG TTG GC (fwd)<br>GAC CCA ATT ATG AAT CGG GAG TG (rev) |
| ApoE | Rat | CTT CTG GGA TTA CCT CGC CT (fwd)<br>GTC CTC CAT CAG TAC CGT CAG (rev) |
| CTSD | Mouse | GCT TCC GGT CTT TGA CAA CCT (fwd)<br>CAC CAA GCA TTA GTT CTC CTCC (rev) |
| CTSS | Mouse | CGA CGC CAG CCA TTC CTC CTT (fwd)<br>AGA GTC CCA TAG CCA ACC ACA AGA (rev) |
| CYP46A1 | Mouse | CGT GTG CTC CAA GAT GTG TT (fwd)<br>GGA CTC GTG ACA ATG ACT GAG (rev) |
| Eef | Mouse | ACA CGT AGA TTC CGG CAA GT (fwd)<br>AGG AGC CTT TTC CCA TCTC (rev) |
| Fas | Mouse | GGC ATC ATT GGG CAC TCC TT (fwd)<br>GCT GCA AGC ACA GCC TCT CT (rev) |
| HMGR | Mouse | CCG GCA ACA AA AGA TCT GTG (fwd)<br>ATG TAC AGG ATG GCG ATG CA (rev) |
| HMGS | Mouse | GTC TCC TTG CTT TGC TTC (fwd)<br>GGA CAG AGA ACT GTG GTC TCC (rev) |
| LAMP1 | Mouse | GAC GGT GAC CAG AGC GTT C (fwd)<br>GTG GGC ACT AGG GCA TCAG (rev) |
| LanS | Mouse | CCC TGA ACT ATG TGG CTCT (fwd)<br>ATA GGG TGT TGA GTC CTT CC (rev) |
| LAPTM5 | Mouse | AGC ACC TGG AGG CTG GGA AGTC (fwd)<br>TTG CCA TCA GAG CAG TGG CTC AT (rev) |
| LDLR | Mouse | GGA CTC AGG GCC TCT GTC TG (fwd)<br>AGC AGG CTG GAT GTC TCT GT (rev) |
| LIPA | Mouse | TTT AGT CTT GGC TCC CGTG (fwd)<br>CCA AAC ATG TCC TTG AGA AGAG (rev) |
| MVK | Mouse | ATC GGT ATT AAG CAG GTG TG (fwd)<br>GAT TGC CAG GTA CAG GTA GA (rev) |
| NPC1 | Mouse | TGG AGA GTG TGG AAT TGC GAC (fwd)<br>GGA CAC CTG GAC AGG AAC TG (rev) |
| Scd1 | Mouse | TTC TTG CGA TAC ACT CTG GTG C (fwd)<br>CGG GAT TGA ATG TTC TTG TCGT (rev) |
| SREBP1 | Rat | GCA GGA AAC TGA GAG ACC CC (fwd)<br>GTA CCC ACT GGC CTT CTC AC (rev) |
| SREBP2 | Mouse | GCG TTC TGG AGA CCA TGGA (fwd)<br>ACA AAG TTG CTC TGA AAA CAA ATCA (rev) |
| SYT11 | Mouse | ATA CGC CCC AGC TTT GAT GT (fwd)<br>CTT GTA TGG CGG GGT CTT GT (rev) |
| TFEB | Mouse | CCA CCC CAG CCA TCA ACAC (fwd)<br>CAG ACA GAT ACT CCC GAA CCTT (rev) |

In a 384 well PCR plaque, 2µL of sample cDNA diluted 1:18 in Milli-Q water and 3µL of real-time target gene mix, containing SensiFAST™ SYBR® Hi-ROX 1x and the specific primers, was added in each well. qPCR was done in a QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific, USA) with the cycling program of three stages determined as follow: (I) initial denaturation

at 95ºC for 2 minutes; (II) amplification stage of 40 cycles of 5 seconds at 95ºC (denaturation) and 30 seconds at 60ºC (anneling and extension); and (III) melting curve analysis. The mRNA levels were normalized to the level of Eef, and results were presented as fold induction over controls, through the ΔΔCt method.

### 2.7. Luciferase Gene Reporter Assay

Cells were transfected using Lipofectamine 3000 (Invitrogen™, USA) as a lipid-based transfection reagent, designed to improve the efficiency of transfection in hard to transfect cells and increase its reproducibility. Thus, the protocol used was the one given by the manufacturer with a few minor changes. For each microgram of DNA, 2μL of Lipofectamine 3000 was used. As such, initially two solutions using Opti-Mem medium were prepared. One containing 2μL Lipofectamine 3000, and the other containing a mix of 2μL P3000 reagent, 1μg pLDLR-luciferase reporter plasmid (Castoreno et al., 2005) and 0.1μg CMV-β-galactosidase plasmid. The pLDLR-luciferase reporter plasmid contains the SRE of the LDLR promotor upstream of the luciferase gene (Castoreno et al., 2005). Both solutions were mixed in 1:1 (v/v) ratio and left for incubation at room temperature for 15 minutes. The harvested cells were added carefully to the transfection solution, and cells were plated at a density of 20 000 cells transfected with 50 nanograms of DNA in each well of a 96-well plaque. In the following day, the growth medium was replaced, and cells were treated. At the end of the treatment cells were washed one time with PBS 1X, resuspended in β-galactosidase Reporter Gene Assay Lysis Buffer 1X (Roche Applied Science, Germany) and frozen at -80ºC. Luciferase activity was measured by adding 50μL Luciferase Buffer (20mM Tris-acetate pH7.8, 33mM DTT, 0.1mM EDTA, 3.74mM $MgSO_4$, 0.27mM Coenzyme A, 0.47mM luciferin and 0.53mM ATP) to 10μL of lysate. β-galactosidase activity was read using β-galactosidase Reporter Gene Assay Chemiluminescent kit (Roche) as instructed by the manufacturer. Briefly, 25μL β-gal Substrate 1X was mixed with 10μL of cell extracts and 2.5μL β-galactosidase Reporter Gene Assay Lysis Buffer 1X. After incubation at room temperature for 1 hour with agitation, the enzyme activity was read by adding 12.5μL of Initiation Reagent. All readings were performed in FB 12 luminometer (Berthold Detection Systems, Germany). Normalization of the luciferase activity was based on β-galactosidase activity and are presented as fold induction over control samples.

### 2.8. Amplex Red Cholesterol Assay

The Amplex Red Cholesterol Assay Kit (Invitrogen™, USA) was used to evaluate possible alterations to the levels of free, esterified and total cholesterol within control and treated cells. Thus, the protocol followed was performed according to the manufacturer instructions. After harvesting, the cells were washed, resuspended in Reaction Buffer 1X and stored at -80ºC. To prepare the total cell extracts, samples were sonicated four times for 4 seconds each and centrifuged at 13 000rpm for 10 minutes at 4ºC. In a 96-well plaque, for each sample, the recovered supernatant was diluted 10x in Reaction Buffer 1X, to a final volume of 50μL. In parallel, a cholesterol calibration curve and a negative control without cholesterol were prepared. Reaction was initiated with the addition of 50μL of a solution containing 300μM Amplex Red reagent, 2U/mL horseradish peroxidase, 2U/mL cholesterol

oxidase and 0.2U/mL cholesterol esterase in Reaction Buffer 1X. In order to measure the free cholesterol levels, 50µL of the same solution but without cholesterol esterase was added to the samples. The levels of esterified cholesterol were obtained by subtracting the values of free cholesterol from total cholesterol levels. After incubating for 30 minutes at 37ºC, the microplate was read in Glowmax Multi+ Detection System (Promega Corporation, USA) at 590nm. The background fluorescence was corrected by subtracting the negative control and normalization of the results was obtained through protein quantification using Bio-Rad Protein Assay Reagent. The Amplex Red Cholesterol Assay kit was also used to quantify the cholesterols levels in midbrain and striatum of MPTP-treated mice. These samples were the same total extracts used before in Western Blot, and that were prepared using a lysis buffer containing DTT. Since Amplex Red is unstable in the presence of thiols like DTT, a dilution of 1:50 in Reaction Buffer 1x was used for the quantifications. The remaining protocol was as above described.

### 2.9. Immunocytochemistry

Firstly, the cells were washed three times with PBS 1X and fixed for 10 minutes at room temperature with 4% paraformaldehyde in PBS. Cells were then washed with PBS and incubated with blocking solution (10% fetal bovine serum, 0,05% Tween-20 in PBS) for 45 minutes at room temperature. After blocking, cells were incubated with the primary antibody anti-LAMP2 (1:200 in blocking solution) overnight at 4ºC. In the following day, the cells washed with PBS and incubated for 2 hours at room temperature with a donkey anti-rat Alexa Fluor 594 (1:200), and 50ng/µL Filipin III from *Streptomyces filipinensis* (Sigma Aldrich) in blocking solution. Afterwards, the nuclei were stained with NucSpot 470 Nuclear Stain 1X (Biotium, USA) in PBS for 30 minutes at room temperature, and mounted. All images were acquired in an AxioScope.A1 microscope with an AxioCam HR camera (Zeiss, Germany), using 400x magnification in at least ten representative random fields. The time of exposure was set for treated samples. Lastly, quantification of total fluorescence and colocalization was performed with ImageJ 1.52i software (National Institutes of Health, USA) and results are shown as fold induction over control samples.

### 2.10. Statistical Analysis

All data obtained was analyzed using Prism 5.0 software (GraphPad Software Inc., USA). Results are expressed as mean ± SEM values. Statistical analysis was done using Student's unpaired t-test or one-way ANOVA test with the Tukey or Dunnet post-hoc test. Means with a *p* value under 0.05 were considered statistically significant.

## 1. Characterization of the effect of MPP+ treatment in cholesterol homeostasis in neuronal cells

### 1.1. MPP+ treatment increases cholesterol levels in N2a neuroblastoma cells

In order to determine if a PD-like mitochondrial dysfunction affects cellular cholesterol homeostasis, we used a model system of N2a neuroblastoma cells treated with the neurotoxin MPP+. As mentioned previously, MPP+ mode of action parallels mitochondria dysfunction observed in post-mortem brains of PD patients, by blocking the multi-subunit enzyme NADH-ubiquinone oxireductase in the complex I of the electron transport chain and causing a decrease in mitochondrial oxidative phosphorylation and increase in oxidative stress (Nicklas et al., 1985; reviewed in: Bové and Perier, 2012). To do so, we treated undifferentiated N2a neuroblastoma cells with vehicle or 100µM or 250µM of MPP+ for 6h and 24h, and quantified levels of total, free and esterified cholesterol using the Amplex Red Cholesterol Assay kit (Figure III.1.).

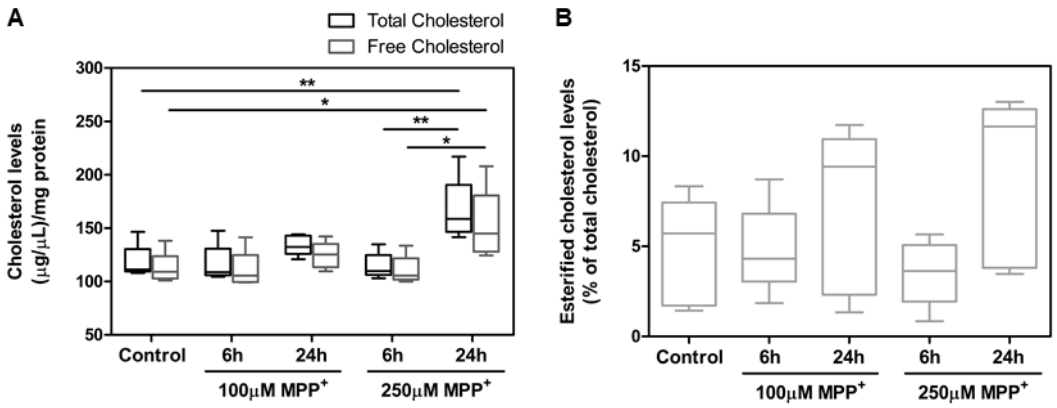


**Figure III.1. MPP+ treatment increases cholesterol levels in N2a neuroblastoma cells.** Quantification of total, free **(A)** and esterified **(B)** cholesterol in N2a neuroblastoma cells treated with vehicle or 100µM or 250µM of MPP+ for 6h and 24h. Cholesterol levels were determined using the Amplex Red Cholesterol Assay. The results are expressed as µg/µL of cholesterol per mg of total protein **(A)** and % of total cholesterol **(B)** and represent mean values ± SEM obtained from five independent experiences. (*p<0.05; **p<0.01).

We can observe that treatment with 250µM MPP+ for 24h, led to a rise in the levels of total and free cholesterol ($p<0.01$, $p<0.05$, respectively). We also determined if there were alterations in the amount of esterified cholesterol in cells, which is the biologically inert form of cholesterol that is stored in lipid droplets in order to avoid toxicity (Bryleva et al., 2010; reviewed in: Zhang et Liu, 2015). Similarly, our results show a tendency to an increase in the levels of unesterified cholesterol at the same experimental conditions (from approximately 6% in vehicle-treated cells, to 12% of esterified cholesterol in the cells treated with 250µM MPP+). Therefore, MPP+ treatment in N2a neuroblastoma cells induces the accumulation of cholesterol in dose and time-dependent manner.

## 1.2. The transcriptional activity of SREBPs is reduced in MPP$^+$-treated N2a neuroblastoma cells

Since we found that MPP$^+$ treatment caused an overall increase in cholesterol levels in N2a neuroblastoma cells, we hypothesize that this could be due to an increase in cholesterol synthesis. As mentioned before, the expression of lipogenic and cholesterogenic enzymes are under the strict control of the ER membrane-bound transcription factors SREBP1 and SREBP2, respectively (reviewed in: Brown and Goldstein, 1999; Weber et al., 2004). SREBPs require a two-step proteolytic cleavage to generate a smaller active form, capable of entering the nucleus and bind with SRE sequences in the promoters of the target genes (reviewed in: Brown and Goldstein, 1999; Horton et al., 2002).

To investigate if MPP$^+$ treatment leads to changes in SREBPs transcriptional activity, we performed a reporter assay with the pLDLR-luc plasmid, containing the SRE-sequence of the LDLR promoter upstream of the luciferase coding-gene (Castoreno et al., 2005, Figure III.2.A.). N2a cells were transfected with the pLDLR-luc reporter plasmid and 24h afterwards, cells were treated with vehicle or with 100µM or 250µM MPP$^+$ for 6h and 24h (Figure III.2.B.).

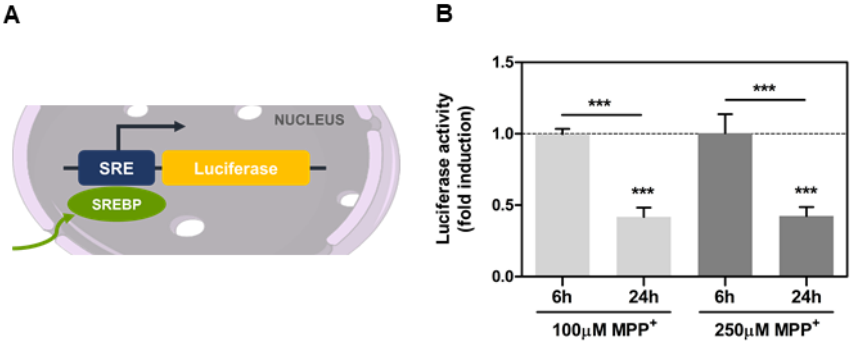


**Figure III.2. SREBP transcriptional activity is reduced after MPP$^+$ treatment. (A)** N2a neuroblastoma cells were transfected with pLDLR-luciferase plasmid, containing the luciferase coding-gene downstream to the SRE-sequence of the LDLR promotor. **(B)** Relative promoter activity in cells treated with 100µM or 250µM MPP$^+$ for 6h or 24h is expressed as fold induction over the promoter activity in vehicle-treated cells. Normalized luciferase activities were expressed as mean values ± SEM of duplicates from six independent experiences (***p<0.001).

Interestingly, 24h after MPP$^+$ treatment, we could observe a significant decrease in luciferase activity ($p<0.001$), of approximately 55%, independently of the concentration of MPP$^+$ used. Since we observe a significant increase in free cholesterol at this precise time point, the decrease in SREBPs transcriptional activity appears to be a feedback response to the rise in intracellular cholesterol levels.

To further confirm our results, we determined by Western Blot analysis the protein levels of the truncated forms of SREBP1 and SREBP2 in N2a neuroblastoma cells treated for 6h and 16h with vehicle or 250µM and 1mM MPP$^+$ (Figure III.3.). Interestingly, our results show a significant decrease in nSREBP1 levels in a time-dependent manner. Indeed, there is a rapid decrease of roughly 40% in nuclear levels of SREBP1 6h after MPP$^+$ treatment (250µM MPP$^+$ $p<0.001$; 1mM MPP$^+$ $p<0.01$), while treatment for 16h leads to a decrease to 20% of control values (for both concentrations $p<0.001$) (Figure III.3.A.). On the other hand, our analysis of nSREBP2 protein levels only demonstrate a tendency to be decreased after 16h of MPP$^+$ treatment. Overall, these results suggest that MPP$^+$

treatment in neuronal cells increases cholesterol levels, which leads to the retention of SREBPs in the ER, therefore decreasing their transcriptional activity.

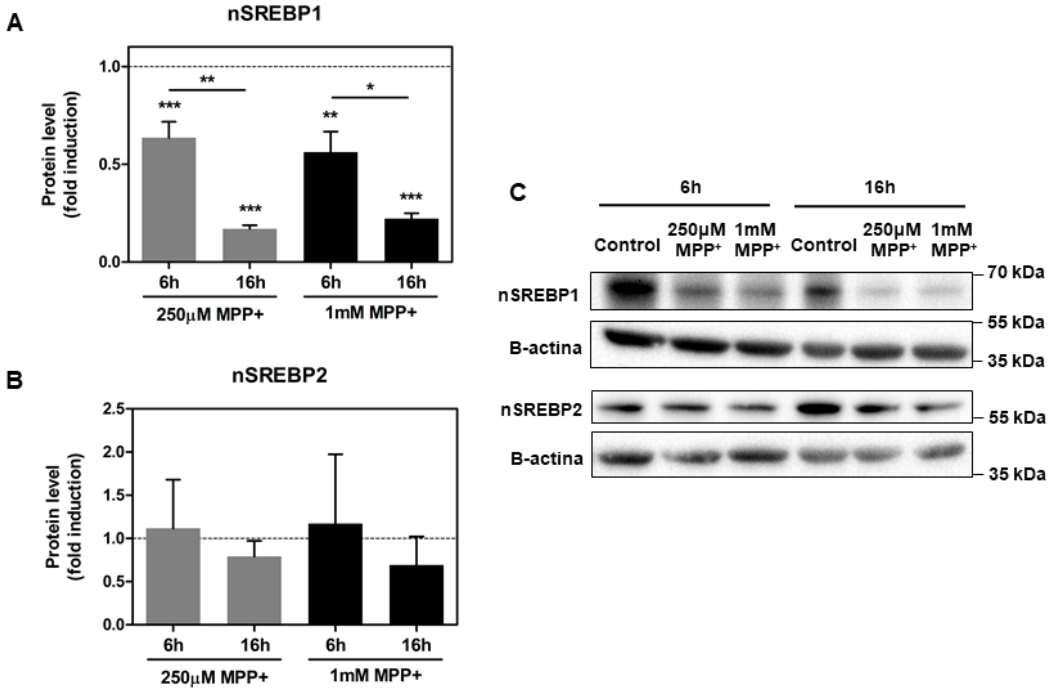


**Figure III.3. MPP⁺ treatment reduces the levels of SREBP1 and SREBP2 truncated forms in N2a neuroblastoma cells.** Western Blot analysis of the SREBP1 **(A)** and SREBP2 **(B)** truncated forms after treatment for 6h or 16h with 250μM or 1mM of MPP⁺. The representative immunoblots for nSREBP1, nSREBP2 and β-actin are shown in **(C)**. β-actin was used as loading control and the results are expressed as mean values ± SEM of five different experiments. (**p<0.01; ***p<0.001).

## 1.3. N2a neuroblastoma cells treated with MPP+ have altered SREBP1 and SREBP2 mRNA levels as well as their respective targets

Given our former results, we decided to verify if the MPP⁺ treatment could also be directly affecting SREBP mRNA expression levels, besides its maturation process, and if the decrease in nSREBPs could be affecting mRNA levels of lipogenic and cholesterogenic genes. To this effect, we decided to quantify mRNA levels of both SREBP1 and SREBP2 and its canonical target genes, by qPCR analysis, after treating N2a cells with 250μM and 1mM MPP⁺ for 16h, which was the time point where we observed the most striking changes in the levels of nSREBP (Figure III.4.A and B.). Our results show that the mRNA levels of SREBP1 are significantly reduced, to about 50% of control levels, in the cells treated with 250μM MPP⁺ ($p<0.01$). Likewise, our analysis also demonstrates that there is a reduction of approximately 25% in the expression of SREBP2 in cells treated with the same concentration of MPP⁺ ($p<0.05$).

Next, we decided to evaluate the mRNA levels of some downstream targets of both SREBP1 and SREBP2, to observe if the overall synthesis of lipids (Figure III.4.A.) and cholesterol (Figure III.4.B.) was affected in MPP⁺-treated cells. Firstly, we could observe that there is an accentuated downregulation of fatty acid biosynthesis-related genes, as demonstrated by the decrease to approximately 45% and 35% of control levels, in the mRNA levels of Fas (250μM MPP⁺ $p<0.001$; 1mM

MPP+ p<0.01), and Scd1 (250µM and 1mM of MPP+ p<0.001), respectively. Both genes are well known to be under the regulation of SREBP1 transcription factor (Amemiva-Kudo et al., 2002; reviewed in: Shimano, 2000). On the other hand, our qPCR analysis also revealed a decrease in several known SREBP2 downstream targets. While HMGR expression was clearly declined by 50% at both MPP+ concentrations tested (p<0.001), HMGS and LDLR mRNA levels were also significantly reduced after 250µM MPP+ treatment.

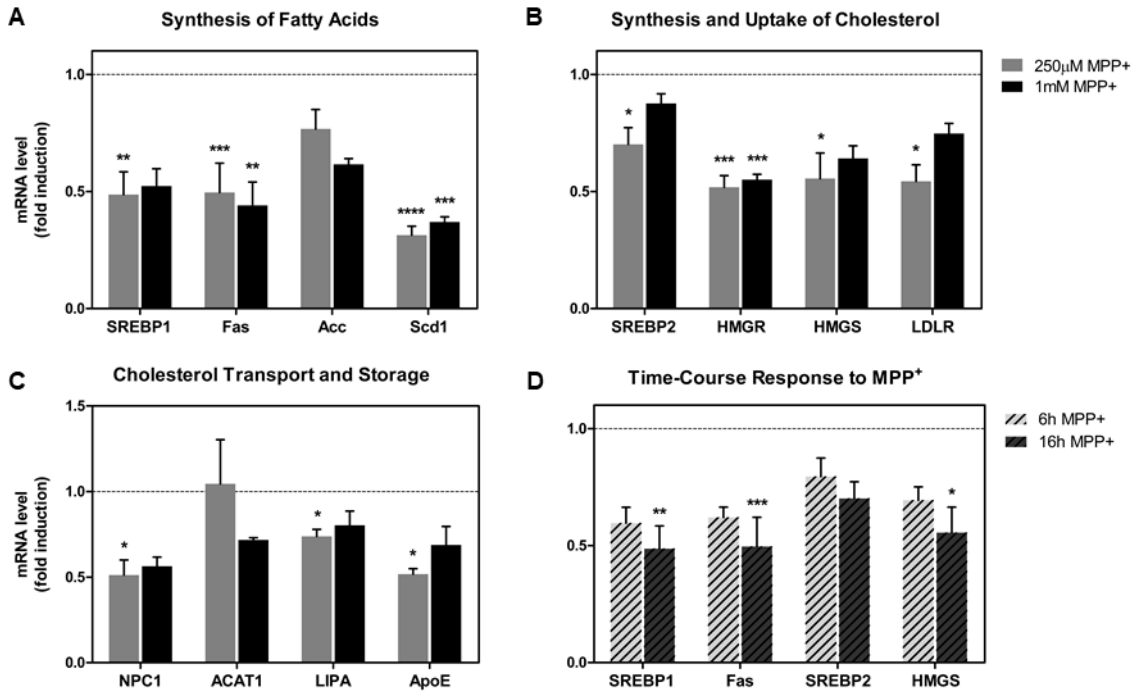


**Figure III.4. MPP+ treatment in N2a neuroblastoma cells downregulates SREBP1 and SREBP2 expression, and their respective targets, in a time-dependent manner.** qPCR analysis of the mRNA levels of **(A)** SREBP1 involved in fatty acids synthesis, and **(B)** SREBP2 in the biosynthesis and uptake of cholesterol, as well as their downstream targets, in neuronal cells treated with 250µM or 1mM of MPP+ for 16h. mRNA levels of genes of interest involved in cholesterol transport and storage were also evaluated **(C)**. **(D)** Time-course analysis in N2a cells that were treated with 250µM MPP+ for 6h or 16h. Normalization was achieved using the internal standard Eef (eukaryotic translation elongation factor). Data is represented as mean values ± SEM from at least five individual experiments and is expressed as fold induction over control (*p<0.05; **p<0.01; ***p<0.001, ****p<0.0001).

We also decided to investigate if the genes involved in transport and storage of cholesterol were also affected by MPP+ treatment, which could also explain why there is an accumulation of cholesterol within the cells after MPP+ treatment (Figure III.4.C.). Therefore, we determined the mRNA levels of NPC1 that mediates cholesterol export from lysosomes and is implicated in the lysosomal storage disease Niemman-Pick type C disease (reviewed in: Wheeler and Sillence, 2019); ACAT1 and lipase A (LIPA), involved in cholesterol esterification (Chang et al., 1995; Chang et al., 1998) and hydrolization of cholesteryl esters (reviewed in: Li and Zhang, 2019), respectively; and ApoE, a apolipoprotein involved in cholesterol export from cells (reviewed in: Poirier, 1994). Our qPCR analysis revealed that the mRNA levels of genes involved in the transport of cholesterol, namely NPC1 and ApoE mRNA levels were almost reduced in half after 250µM MPP+ (p<0.05), while LIPA expression was decrease to about 75% of control levels (p<0.05).

Lastly, we also performed a time-course analysis in N2a neuroblastoma cells treated with vehicle or 250μM $MPP^+$ for 6h, to detect if the changes in the expression of lipid-related proteins, could be detected earlier during $MPP^+$ treatment (Figure III.4.D.). We observed that the levels of transcripts for all genes tested (SREBP1, Fas, SREBP2 and HMGS) tended to be already reduced at 6h of treatment but were only significantly decreased at the longer time-point, as previously described. Thus, our results so far demonstrate that probably due to the decrease in nSREBPs levels after $MPP^+$ treatment, the expression of both SREBP and its downstream targets is significantly downregulated.

### 1.4. $MPP^+$ stimulates lysosomal cholesterol accumulation in neuronal cells

Recent studies have suggested that PD shares a number of characteristics with lysosomal storage disorders such as Gaucher's disease (reviewed in: Klein and Mazzulli, 2018). Moreover, a cohort study discovered that 56% of PD patients have at least one putative damaging variant in a lysosomal storage disorders gene, and 21% are carrying multiple alleles (Robak et al., 2017). Among these, NPC1 variants have been identified (Josephs et al., 2004; Kluenemann et al., 2013). Plus, as previously mentioned, loss of function mutations in either the NPC1 or NPC2 genes gives rise to the accumulation of unesterified cholesterol in late endosomes/lysosomes due to impaired egress, and consequently to lysosomal dysfunction and neuronal cell death. Since our results have shown that $MPP^+$ treatment leads to a decrease in NPC1 transcripts, we decided to assess if there were any changes in intracellular cholesterol distribution. So, once again we treated the N2a cells for 16h with 250μM and 1mM of $MPP^+$ and labeled cells with filipin III and immunostained for LAMP2 (Figure III.5.).

Filipin III from *Streptomyces filipinensis* is a natural fluorescent antibiotic that only binds to free cholesterol and allows detecting patterns of cholesterol accumulation (reviewed in: Maxfield and Wüstner, 2012). Meanwhile, labeling lysosomes with LAMP2 allows to determine if cholesterol is sequestered in the lysosomes.

The microscopy images of filipin III staining shows that treatment with 1mM $MPP^+$ increases, in a statistically significant manner, the relative fluorescent per cell in approximately 170% ($p<0.05$), which reflects an increase in free cholesterol levels (examples marked by white arrows). Furthermore, treatment with the neurotoxin increases LAMP2 staining by 55% ($p<0.01$), which suggested an increase in the number lysosomes or enlargement of lysosomal (LAMP2-positive) vesicles. Interestingly, our results also shown there is a tendency for an increase in the co-localization between filipin III and LAMP2 staining, which may reflect an accumulation of free cholesterol in lysosomes (Figure III.5.D.). These values are interesting not only because they corroborate the increased in free cholesterol levels determined before using the Amplex Red Cholesterol assay, but also indicate that the accumulated unesterified cholesterol is in lysosomes.

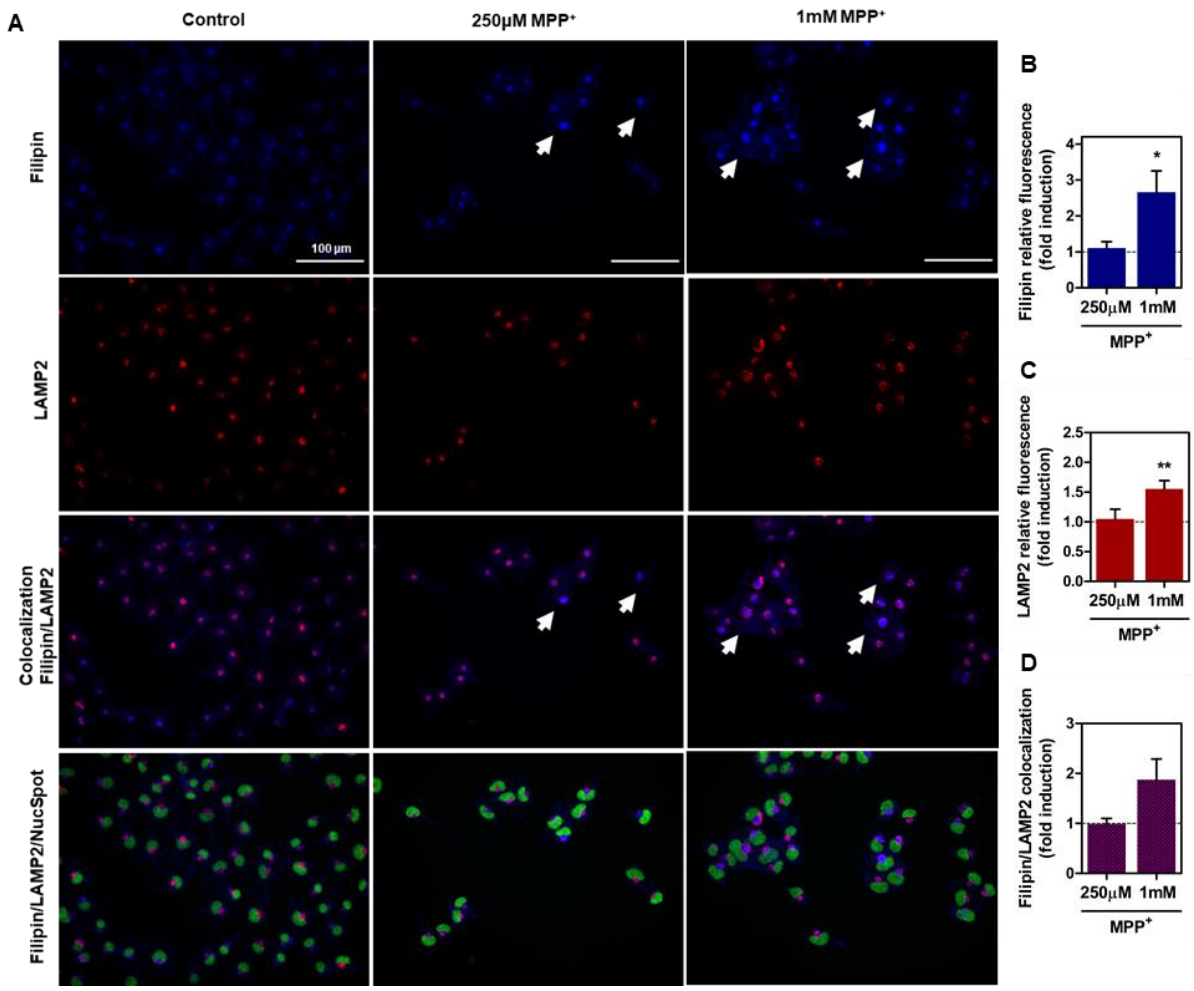


**Figure III.5. MPP⁺ treatment induces unesterified cholesterol accumulation in the LE/LY pathway. (A)** N2a neuroblastoma cells treated with vehicle or 250µM and 1mM of MPP⁺ for 16h. After fixation, cells were labeled with filipin III and NucSpot, and immunostained with LAMP2. Images are representative of filipin III (blue), LAMP2 (red) and nuclei (green), and co-localized pixels of LAMP2 with filipin III (purple). White arrows mark examples of enlarged free cholesterol aggregates and increased colocalization of free cholesterol in lysosomes. Scale bar: 100 µm. The quantification of relative fluorescence for filipin III **(B)**, LAMP2 **(C)** and colocalization images **(D)**, was done using the values of average fluorescence normalized to total cell number. Data is represented as fold induction from control samples in mean values ± SEM, from fourteen independent experiences. (*p<0.05; **p<0.01).

## 1.5. CCCP treatment does not replicate lysosomal cholesterol accumulation as seen with MPP⁺

Drug-induced impairment of mitochondrial function has been shown to elicit stress signaling pathways that regulate lysosomal biogenesis, and that could eventually be involved on the increase in cholesterol levels in lysosomes. Therefore, we decided to investigate if our results were due to the specific use of MPP⁺, or if other compounds that also caused impairment of mitochondria function had the same effect in cholesterol accumulation. In order to investigate this premise, we treated N2a neuroblastoma cells for 16h with vehicle or with 10µM and 20µM of CCCP, which is a mitochondrial uncoupler vastly used to induce mitophagy (Fernández-Mosquera et al., 2017), and labeled cells with filipin III and immunostained for LAMP2

Interestingly, the relative fluorescence calculated for LAMP2 did not present any statistical relevant differences between controls and CCCP-treated cells (Figure III.6.A. and C.). Similarly, when

observing filipin staining for all samples, we could not observe the distinct phenotype of cholesterol accumulation (Figure III.6.A), corroborated by the lack of any significant differences in the average fluorescent intensity per cell (Figure III.6.B.). Furthermore, there were also no statistically significant differences in filipin/LAMP2 colocalization values (Figure III.6.D).

Altogether, our results show that different mitochondrial drugs used to mimic mitochondrial oxidative defects, namely $MPP^+$ inhibiting complex I and the mitochondrial uncoupler CCCP, led to different cellular outcomes in terms of neuronal cholesterol accumulation, particularly in the lysosomal pathway.

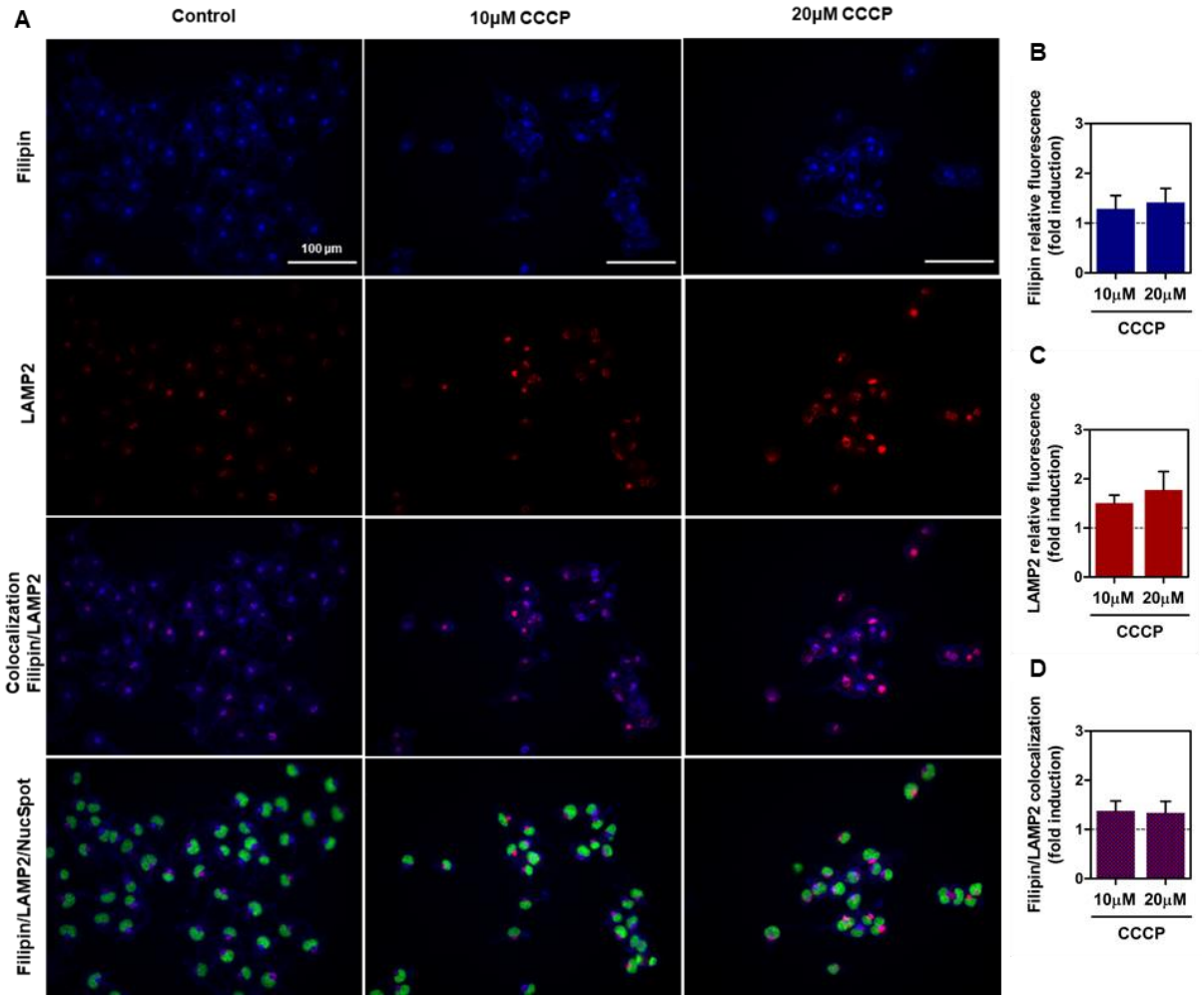


**Figure III.6. CCCP treatment does not induce the accumulation of free cholesterol. (A)** The N2a neuroblastoma cells were treated with DMSO and 10μM or 20μM of CCCP for 16h, fixed and stained with filipin III, NucSpot and immunostained with LAMP2. Images are representative of filipin III (blue), LAMP2 (red), nuclei (green) and co-localized pixels of filipin III and LAMP2 (purple). Scale bar: 100 μm. The quantification of relative fluorescence for filipin III **(B)**, LAMP2 **(C)** and colocalization images **(D)**, was done using the values of average fluorescence normalized to total cell number. Data is represented as fold induction from control samples in mean values ± SEM, from three different experiences.

## 2. Characterization of changes in cholesterol homeostasis in MPTP-treated mice

### 2.1. Cholesterol levels are not altered in the nigrostriatal region of MPTP-treated mice

After observing changes in cholesterol homeostasis after $MPP^+$ treatment in the N2a neuroblastoma cells, we decided to investigate if similar alterations could be observed in an *in vivo* model of PD, in this case mice subjected to MPTP administration. Accordingly, we firstly analyzed whether MPTP was able to also trigger cholesterol accumulation, using samples of striatum and midbrain of mice treated with a single i.p. dose of 40mg/kg MPTP or saline, and sacrificed 3h or 6h after its administration (Figure III.7.). The selected time-point is due to the close temporal correlation between formation of $MPP^+$, which peaks at 3h after MPTP treatment, and $MPP^+$ inhibition of mitochondrial respiration in the CNS (Saporito et al., 2000). As before, the quantification of total, free and esterified cholesterol was done via Amplex Red Cholesterol assay.

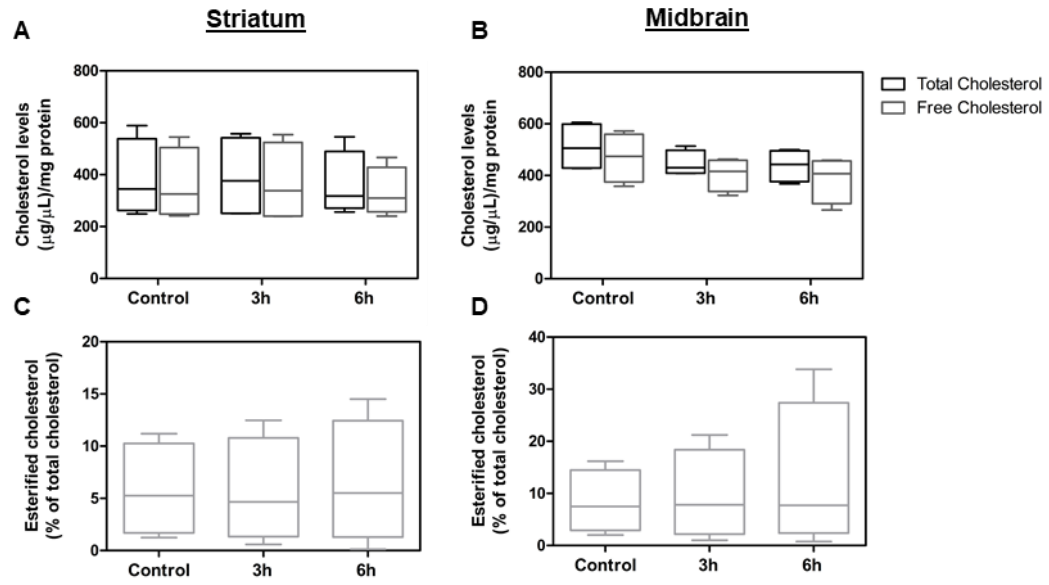


**Figure III.7. MPTP does not alter cholesterol content in mouse midbrain.** Quantification of total, free and esterified cholesterol levels was done using Amplex Red Cholesterol Assay, in samples of striatum **(A, C)** and midbrain **(B, D)** of mice treated with an acute single dose of 40 mg/kg, i.p. of MPTP or saline and sacrificed 3h or 6h after MPTP administration. Results are represented as mean values ± SEM in µg/µL of cholesterol per mg of total protein **(A, B)** and % of total cholesterol **(C, D)**, and were obtained from four individual experiments.

Our results show that MPTP does not induce any significant changes in the levels of total, free and esterified cholesterol at the time points analyzed. Since the cholesterol accumulation induced by $MPP^+$ treatment in N2a cells was only observed with longer treatments, it will be interesting to analyze putative changes in cholesterol levels in mice treated with MPTP for 24h or longer.

### 2.2. MPTP treatments alters the profile of cholesterol-related genes and proteins in striatum and midbrain in mice

Since the ER membrane is one of the most cholesterol-poor membranes in cells, small changes in the ER membrane cholesterol levels, that cannot be measured with Amplex Red Cholesterol Assay, can rapidly be sensed by the cell, and translated in the activation/ deactivation of SREBPs. Plus, although $MPP^+$-depend cholesterol accumulation in N2a cells was only observed 16h

after treatment, at an earlier time point, we could already observe the trend to the decrease in the mRNA levels of SREBP1, Fas, SREBP2 and HMGS genes (Figure III.4.D.). Due of this fact, we decided to use once more the samples of midbrain and striatum from mice treated with an i.p. injection of 40mg/kg MPTP or saline, and sacrificed 3h or 6h later, and quantified the mRNA levels of various genes involved in cholesterol biosynthesis, transport and storage, and lipid synthesis by qPCR analysis (Figure III.8.).

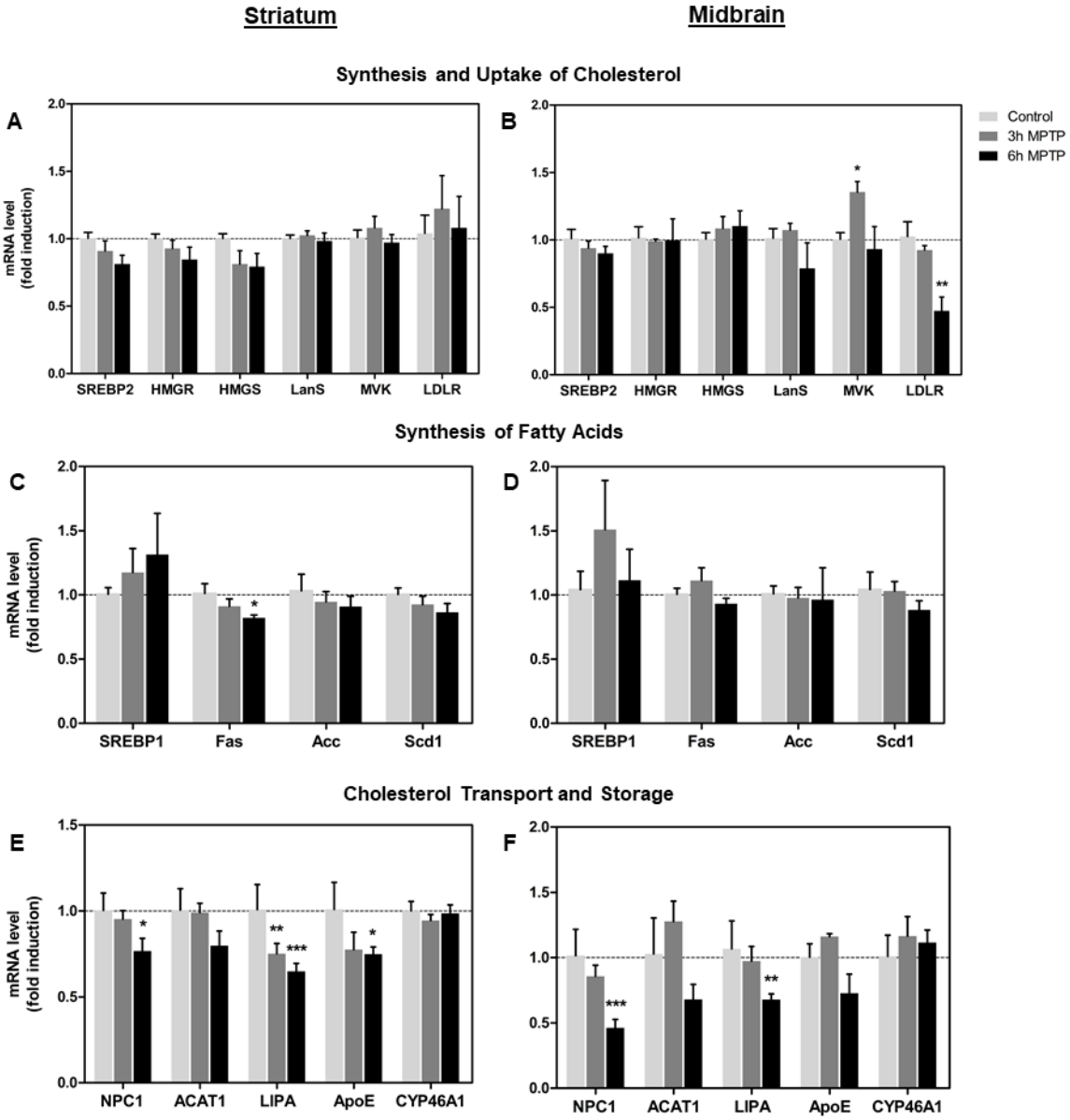


**Figure III.8. MPTP treatment affects the mRNA levels of genes involved in synthesis and transport of lipids in the midbrain and striatum.** The mice were treated with a single i.p. injection of 40mg/kg of MPTP or saline, and sacrificed after 3h or 6h. qPCR analysis of striatum and midbrain show expression levels of proteins involved in synthesis and uptake of cholesterol **(A, B)**, biosynthesis of fatty acids **(C, D)**, as well as transport and storage of cholesterol **(E, F)**. Normalization was achieved using the internal standard Eef. Data is represented as mean values ± SEM from at least five independent experiments. (*p<0.05, **p<0.01, ***p<0.001).

In the striatum samples, we can observe that there is a time-dependent decrease in the mRNA levels of Fas, a SREBP1-target gene, being significantly reduced by 20% (p<0.05), 6h after MPTP treatment, when compared with controls. Also, NPC1 and ApoE have a significant decrease of 25% in

their mRNA levels at 6h of MPTP treatment ($p<0.05$), while LIPA mRNA levels where already significantly decreased to 75% at 3h ($p<0.01$), and further reduced to 65% of control values 6h after MPTP administration ($p<0.001$).

In the midbrain samples we could observe a similar profile, with a time-dependent significant decrease in the levels of NPC1 and LIPA transcripts when compared with controls. NPC1 mRNA expression decreased 55% after 6h of MPTP treatment ($p<0.001$), while there is a decrease of about 30% in LIPA mRNA levels, in the same samples ($p<0.01$). Moreover, we could detect a transient but significant increase in MVK mRNA levels that are increased 1.4 fold over control, 3h after the treatment ($p<0.05$), returning to control levels 3h later. On the other hand, LDLR mRNA levels show a tendency to be already decreased at the shorter time point, reaching a significant decrease of approximately 50% of control 6h after MPTP treatment ($p<0.01$).

Interestingly, MPTP administration *in vivo*, recapitulates in the nigro-striatal pathway, some of the mRNA changes observed in N2a cells after $MPP^+$ treatment, namely a decrease in the expression of genes involved in fatty acid and cholesterol-related processes, such as Fas, LDLR, NPC1 and LIPA.

## 3. Assessment of altered AMPK/mTOR signaling pathway by $MPP^+$ treatment in N2a neuroblastoma cells

### 3.1. $MPP^+$ treatment leads to the activation of the AMPK/ mTOR signaling pathway

Our results so far suggest that mitochondrial dysfunction induced by $MPP^+$ leads do an increase in cellular cholesterol levels, probably due to its accumulation in the endo-lysosomal pathway. Independently, this interdependence of mitochondria and lysosomes has already been underscored by other lines of evidence. For instance, it has been shown that under acute mitochondrial stress, AMPK is activated, promoting autophagosome formation and transcription factor EB (TFEB)/MITF-dependent lysosomal biogenesis, thus programming the cell to clean-up the dysfunctional mitochondria (Fernández-Mosquera et al., 2017). On the other hand, as mentioned before, AMPK activation results in mTORC1 inhibition (reviewed in: Garcia and Shaw, 2017). mTORC1 is a major coordinator of anabolism, promoting the synthesis of proteins, cholesterol, and nucleotides, among other essential building blocks for cell growth and proliferation (reviewed in: Laplante and Sabatini, 2012). However, the reach of AMPK goes beyond mTORC1 because it often also regulates key enzymes of anabolic pathways. For example, it directly phosphorylates and negatively regulates HMGCR, the limiting step of the cholesterol synthesis pathway, and represses the synthesis of fatty acids by phosphorylating ACC (Zhou et al., 2001; reviewed in: Hardie and Carling, 1997; Garcia and Shaw, 2017).

Thus, we decided to focus in investigating the activation of the AMPK/mTOR signaling pathway, not only given its importance in regulating lipid metabolism, but also because there were already various articles describing changes in the activation of this pathway within the context of PD, namely using the $MPP^+$/MPTP neurotoxin (Choi et al., 2010; Dehay et al., 2010; Arduíno et al., 2012; Sakamoto et al., 2017; Jovanovic-Tucovic et al., 2019). For that reason, N2a cells were treated with

vehicle or 250μM and 1mM of $MPP^+$ for 16h and Western Blot analysis was used to determine the levels of AMPK, mTOR and also their respective phosphorylated forms as a mean to analyze their activation (immunoblots represented in Figure III.9.C.).

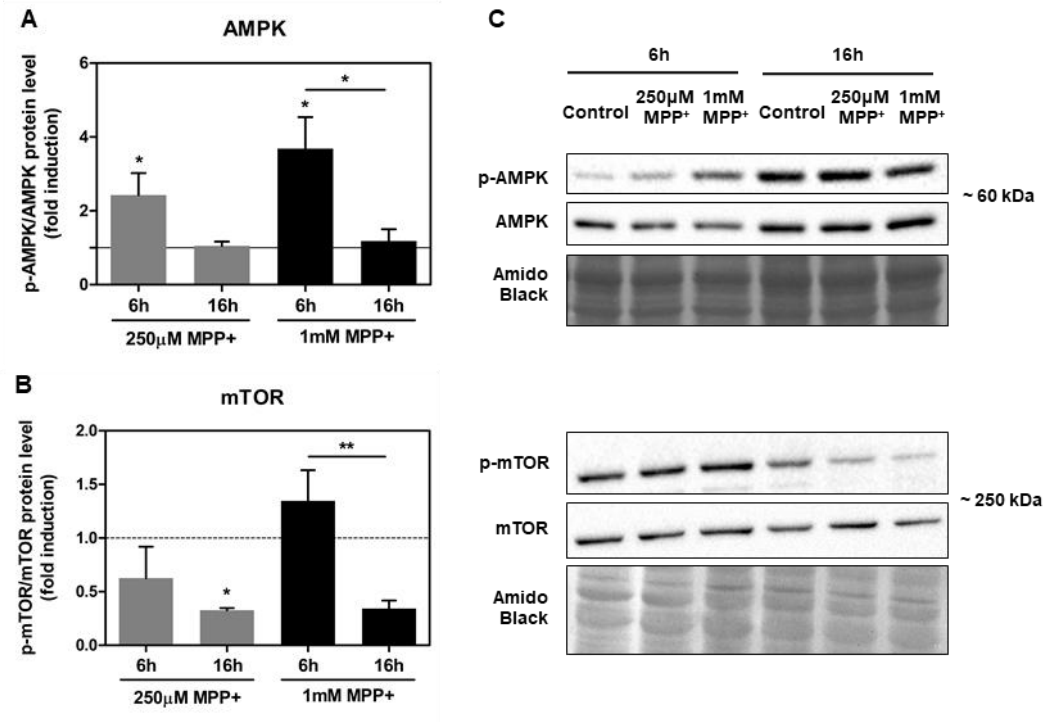


**Figure III.9. $MPP^+$ treatment initially stimulates AMPK activation, consequently leading to long-term inhibition of mTOR in N2a neuroblastoma cells.** Western Blot analysis of p-AMPK/AMPK **(A)** and p-mTOR/mTOR **(B)** protein levels ratio after treatment with vehicle or 250μM or 1mM of $MPP^+$ for 6h or 16h. **(C)** Representative immunoblots for p-AMPK, AMPK, p-mTOR and mTOR. The Amido Black staining was used as loading control. Data is expressed as mean values ± SEM of four different experiments. (*p<0.05; **p<0.01).

As expected, we observed an early and transient phosphorylation of AMPK after $MPP^+$ treatment. Indeed, when neuronal cells were treated with 250μM and 1mM $MPP^+$ for 6h, the p-AMPK/AMPK protein ratio was significantly increased by approximately 2.4 and 3.5 fold, respectively, when compared with untreated samples ($p<0.05$). However, AMPK phosphorylation levels return to basal levels 16h after treatment (1mM $MPP^+$ $p<0.05$).

In contrast, analysis of the phosphorylation levels of mTOR in the same samples, show a significant decrease p-mTOR/mTOR ratio 16h after treatment with 250μM $MPP^+$. A similar tendency was observed when cells were treated with 1mM $MPP^+$. Our results may imply that an early activation of AMPK could be responsible for mTOR inhibition at a later time-point.

### 3.2. $MPP^+$ treatment modifies the expression of several lysosomal proteins

In the previous results, we observed that the $MPP^+$ treatment in neuronal cells caused an increase in cholesterol and LAMP2 levels, and most likely the accumulation of cholesterol within lysosomes, concomitant with a decrease in NPC1 mRNA levels. As mentioned before, the increase in LAMP2 could be a consequence of an increase in lysosomal biogenesis, or due to enlargement of LAMP2-positive vesicles. Activation of lysosomal biogenesis after acute mitochondrial stress, has been previously shown (Fernández-Mosquera et al., 2017). This was also observed in similar

pharmacologic models of mitochondrial respiratory chain inhibition in cultured cells, in which AMPK signaling is necessary for lysosomal biogenesis, by inhibiting mTORC1, which in turn activates TFEB (Roczniak-Ferguson et al., 2012; Settembre et al., 2012). Plus, our last results, show an activation of the AMPK/mTOR signaling pathway, consistent with the putative activation of TEFB. Therefore, we evaluated by qPCR analysis the mRNA levels of TEFB, and TEFB-lysosomal target genes in N2a neuroblastoma cells treated with vehicle or with 250µM or 1mM $MPP^+$ for 6 or 16h (Figure III.10.). As TEFB targets, we selected lysosomal-associated membrane protein 1 (LAMP1), lysosomal-associated protein transmembrane 5 (LAPTM5); the lysosomal proteases cathepsins S (CTSS) and D (CTSD); and synaptotagmin-11 (SYT11) which is regulator of endocytosis and vesicle recycling in neurons, and was identified as a PD-associated gene and a parkin substrate (Wang et al., 2018).

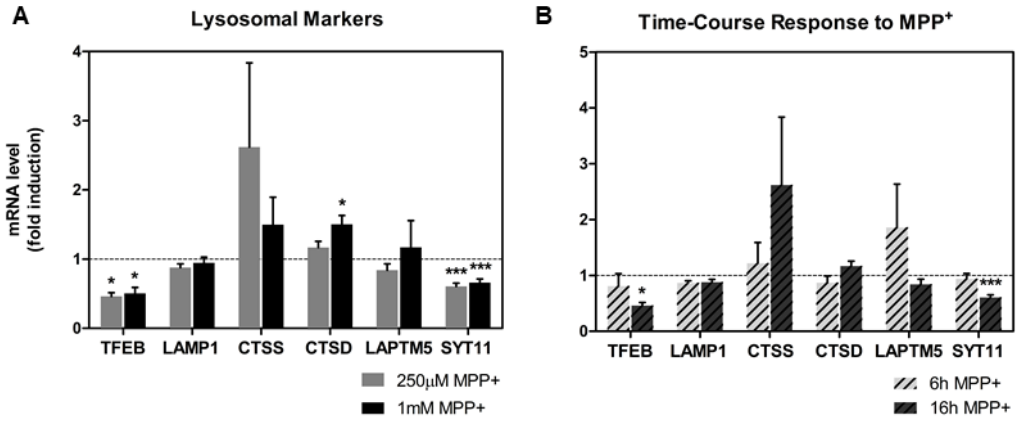


**Figure III.10. N2a neuroblastoma cells treated with $MPP^+$ present altered mRNA levels of multiple lysosomal proteins. (A)** qPCR analysis shows the gene expression of transcription factor EB (TFEB), lysosomal-associated membrane protein 1 (LAMP1), cathepsin S (CTSS) and D (CTSD), lysosomal-associated protein transmembrane 5 (LAPTM5), and synaptotagmin-11 (SYT11), in N2a neuroblastoma cells treated with 250µM or 1mM of $MPP^+$ for 6h or 16h. **(B)** qPCR analysis of a time-course response to $MPP^+$ in N2a cells treated with 250µM of $MPP^+$ during 6h or 16h. Normalization was achieved using the internal standard Eef. Results are represented as mean values ± SEM from at least five individual experiments. (*p<0.05; ***p<0.001).

Surprisingly, our results show a marked reduction in TFEB and SYT11 mRNA levels in comparison with control samples, independent of the dose of $MPP^+$ used. TFEB mRNA levels were significantly reduced by about 50% ($p<0.05$), whereas SYT11 decreased approximately 35% ($p<0.001$). On the other hand, CTSD mRNA levels in samples treated with 1mM $MPP^+$ had an increase of 2.6 fold, in comparison with control ($p<0.05$).

Since TFEB-target genes transcriptional activation have been described to be rapidly increased upon respiratory chain complex I inhibition, eventually returning to baseline levels after 12 h treatment (Fernández-Mosquera et al., 2017), we treated N2a cells with vehicle or 250µM $MPP^+$ during 6h (Figure III.10.B.). Interestingly, after 6h of treatment, TFEB and TFEB-target genes mRNA levels did not present any statistically significant differences between controls and $MPP^+$-treated cells.

We also decided to evaluate the expression levels of the same lysosomal markers in samples of midbrain and striatum of mice treated with a single acute injection of 40mg/kg of MPTP or saline, and sacrificed after 3h and 6h (Figure III.11.).

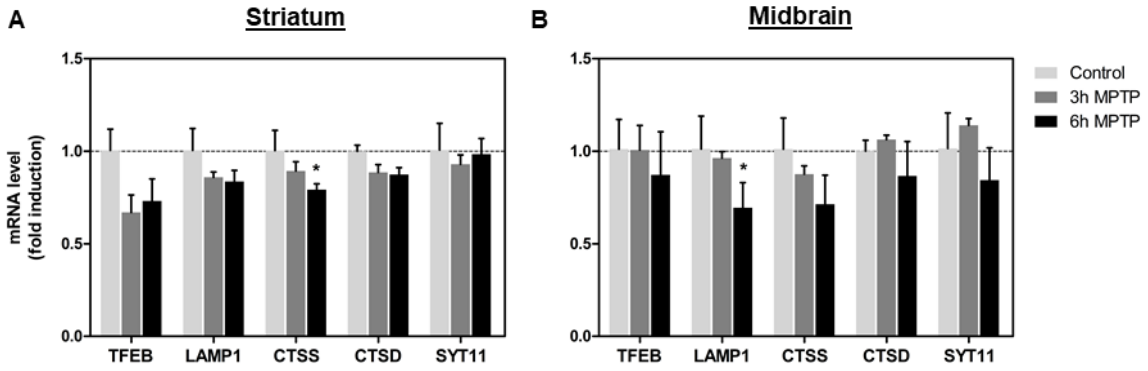


**Figure III.11. MPTP downregulates the expression of various lysosomal markers in the striatum (A) and midbrain (B) of MPTP-treated mice.** The mice were treated with a single acute dose of 40mg/kg MPTP or saline during 3h or 6h. qPCR analysis shows the gene expression of transcription factor EB (TFEB), lysosomal-associated membrane protein 1 (LAMP1), cathepsin S (CTSS) and D (CTSD), lysosomal-associated protein transmembrane 5 (LAPTM5), and synaptotagmin-11 (SYT11). Normalization was achieved using the internal standard Eef. Results are represented as mean values ± SEM from two independent treatments (*p<0.05).

In the striatum, CTSS gene showed a significant decrease in its mRNA levels after 6h of MPTP treatment, decreasing to about 80% of vehicle-treated mice ($p<0.05$). On the other hand, LAMP1 gene was the only gene to have a statistically significant change in its mRNA levels in the midbrain MPTP-treated mice, retaining only about 70% of control levels after 6h of treatment ($p<0.05$). Our results show that, in contrast to what was initially hypothesized, the activation of AMPK induced by $MPP^+$ does not lead to an increase in lysosomal biogenesis.

### 3.3. Inhibition of AMPK signaling pathway by compound C did not prevent lysosomal cholesterol accumulation in $MPP^+$-treated cells

Lastly, to understand if the increase in cholesterol levels induced by $MPP^+$ treatment was related to the previously observed activation of the AMPK/ mTOR pathway, we decided to verify if blocking the initial activation of AMPK, we could prevent the cholesterol accumulation. The AMPK inhibitor selected was dorsomorphin or compound C (CC), despite its specificity not being restricted to this target (reviewed in: Dasgupta and Seibel, 2018), it is still widely used to study the inhibition of AMPK signaling pathway. To investigate this, we pre-treated N2a cells with or without 20μM of CC for 1 h, following a treatment with 1mM of $MPP^+$ for 16h. After, cells were fixed, labelled with filipin and immunostained for LAMP2 (Figure III.12.A.).

Surprisingly, CC treatment led to a tendency in increasing lysosomal cholesterol accumulation in N2a cells, inducing a cellular phenotype of cholesterol storage like $MPP^+$ (Figure III.12.B.). Moreover, inhibiting AMPK also led to a statistically significant increase LAMP2 average fluorescence (Figure III.12.C.). Thus, pre-treating cells with CC in order to block AMPK activation before the $MPP^+$ treatment, does not counteract the $MPP^+$-induced lysosomal cholesterol accumulation.

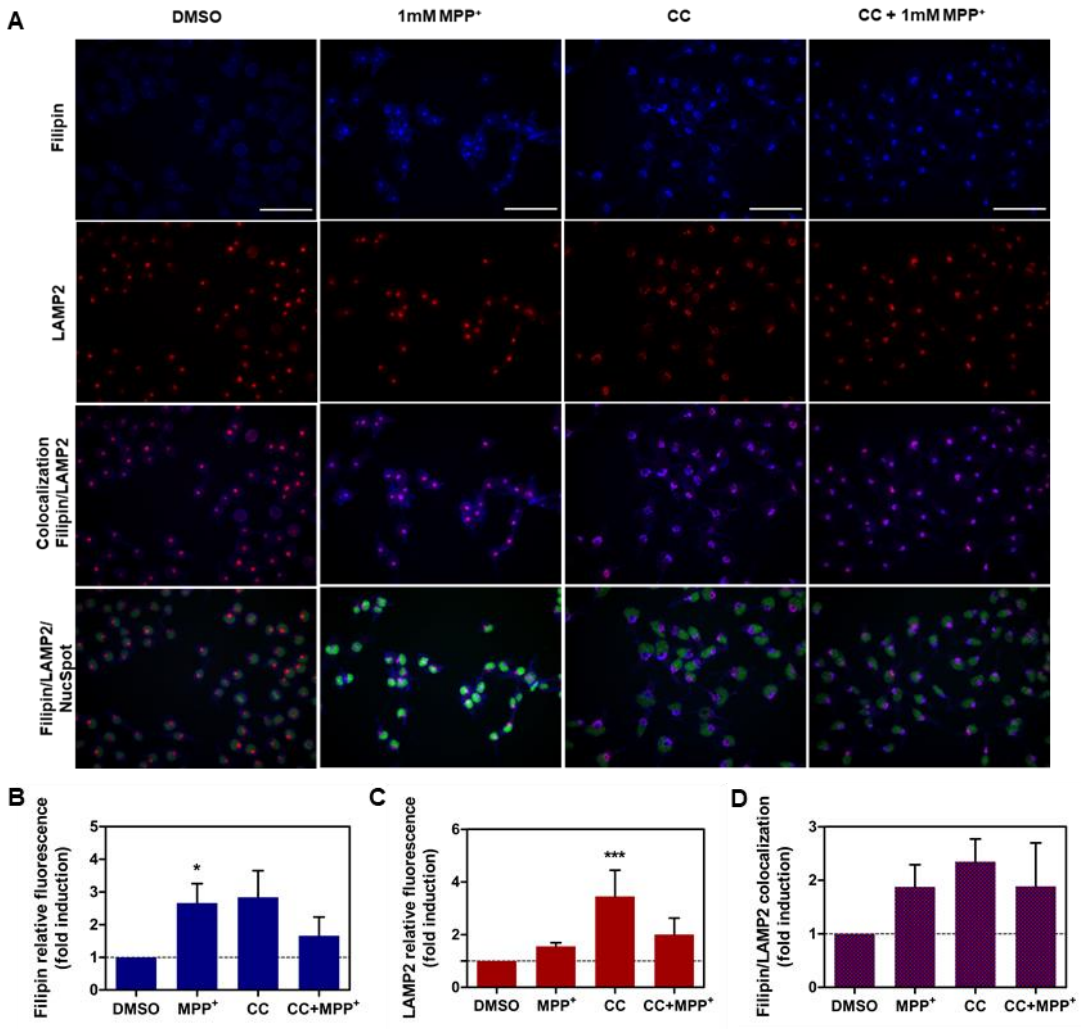


**Figure III.12. The AMPK inhibitor Compound C decreases the buildup of free cholesterol in lysosomes caused by MPP⁺ treatment. (A)** N2a neuroblastoma cells were first treated with 20μM of CC or DMSO for 1 h, followed by 1mM of MPP⁺ for 16h. Then, cells were fixed, labeled with filipin III and NucSpot, while immunostained with LAMP2. Images are representative of filipin III (blue), LAMP2 (red), nuclei (green) and co-localized pixels of filipin III and LAMP2 (purple). Scale bar: 100μm. The values of average fluorescence normalized to total cell number were used for quantification of relative fluorescence for filipin III **(B)**, LAMP2 **(C)** and the colocalization images **(D)**. Data is represented as mean values ± SEM from at least three independent experiences (*p<0.05; ***p<0.001).

# Chapter IV. Discussion

Almost one quarter of all cholesterol in the human body is contained in the CNS, where is important for the formation of synapses, axonal guidance, dendrite outgrowth and synthesis of myelin. Given its role in maintaining normal brain function, it is known that alterations in cholesterol homeostasis can lead to neurodegeneration or even the onset of neurodegenerative diseases. The use of neurotoxins that cause PD-like mitochondrial dysfunction has proven successful in finding potential disease-modifying targets. Thus, during this thesis, we decided to explore how mitochondrial dysfunction caused by MPTP/$MPP^+$, a widely used neurotoxin for research of PD, could affect cholesterol homeostasis and if such changes would be beneficial or detrimental for the development of the disease.

Our results showcase a correlation between the three pathologic mechanisms in the context of PD, namely mitochondrial dysfunction, intracellular cholesterol accumulation, and eventually impairment in clearance mechanisms. By quantifying the levels of total, free and esterified cholesterol, we show that $MPP^+$ treatment in neuroblastoma cells leads to cholesterol accumulation in a dose and time-dependent manner, and that cholesterol accumulated mainly in late endosomes/ lysosomes. Previous reports have already explored the interdependence of cholesterol accumulation and altered lysosomal function in other pathologic contexts. For instance, fibroblasts with a mutation in the NPC1 gene, or treated with U18666A, both causing overload of cholesterol in late endosomes/ lysosomes characteristic of Niemman's Pick Type C disease, have overall increased cholesterol levels, and lysosomal dysfunction (Sobo et al., 2007). Plus, several *in vitro* models of heterozygous and homozygous GBA1 mutations, which increase the risk for the development of PD, present lysosomal dysfunction accompanied by increased total cholesterol levels when compared to wild type GBA1 cells, as well as increased levels of cholesterol esters (Magalhaes et al., 2016). Interestingly, a previous study had already reported lysosomal cholesterol accumulation in $MPP^+$-treated BE(2)-M17 neuroblastoma cells (Eriksson et al., 2017).

In parallel to an increase in cholesterol levels, we observed, for the first time, that $MPP^+$ treatment leads to a decrease in SREBPs expression and transcriptional activity that appear to be a feedback response to the rise in intracellular cholesterol levels, as well as the downregulation of some of their downstream targets. Amongst these genes, we observed a significant decrease in the mRNA levels of HMGR, which codes for the rate-limiting enzyme of cholesterol synthesis. Besides being transcriptionally regulated by SREBP2, HMGR is also directly post-transcriptionally regulated by the intracellular sterol content, sensed by an SDD motif. Interestingly, our results match a previous study that had reported decreased cholesterol biosynthetic capability in skin fibroblasts of PD patients, accessed by measuring the activity of HMGR. In the same study, it was also reported reduced cholesterol esterifying activity (Musanti et al., 1993). However, we only observed a tendency to have decreased mRNA levels of ACAT1 when N2a cells were treated with 1mM $MPP^+$ for 16h.

In addition, gene expression analysis revealed that LDLR, NPC1 and ApoE mRNA levels in $MPP^+$-treated cells were all significantly reduced, hinting for mitochondrial dysfunction as a trigger for cholesterol trafficking impairment. Moreover, altered cholesterol trafficking could also be a mechanism involved in neurodegeneration caused by administration of MPTP in *in vivo* models, since we observed that the midbrain and striatum of MPTP-treated mice also had altered mRNA levels of NPC1 and LIPA (in striatum and midbrain), ApoE (in striatum for 6h) and LDLR (in midbrain) expression. However, it is to mention that at the early time-points analyzed, MPTP treatment did not led to any statistically significant differences in the levels of cholesterol in the midbrain and striatum of MPTP-treated mice. Conversely, we observed accumulation of cholesterol in neuronal cells with more prolonged time of treatment. Therefore, we hypothesize that the long-term inhibition of mitochondrial complex I and the extended dysfunctional cholesterol trafficking are important factors contributing to the rise in cholesterol content.

Afterwards, we also decided to evaluate if $MPP^+$ treatment in neuronal cells activated AMPK/ mTOR signaling pathway, and if its activation could be somehow responsible for the phenotypic changes observed, such as the increase in LAMP2 levels and lysosomal cholesterol accumulation. As mentioned before, AMPK has an important role in regulating the metabolic state of the cell, by favoring mechanisms that would originate energy, such as glucose uptake and fatty acid β-oxidation, suppressing various energy-dispendious processes, like synthesis of proteins (reviewed in: Garcia and Shaw, 2017). To block the latter, AMPK phosphorylates and inhibits the activity of mTORC1, slowing down the synthesis of fatty acids and cholesterol, since mTORC1 has been shown to regulate the activity of SREBP1 (Peterson et al., 2011) and SREBP2 (Eid et al., 2017). In an mTORC1-independent manner, AMPK was also described to intervene directly in the inhibition of lipid synthesis by direct phosphorylation of SREBPs, ACC1 and HMGR (Zhou et al., 2001; Li et al., 2011; reviewed in: Hardie and Carling, 1997; Garcia and Shaw, 2017). Recurring to Western Blot analysis of total and phosphorylated forms of AMPK and mTORC1 proteins, we observed that $MPP^+$ treatment in neuronal cells triggered an early activation of AMPK, 6h after $MPP^+$ treatment. These results were expected, since AMPK is known to be activated during stressful conditions like oxidative stress, which is a common outcome following $MPP^+$-derived mitochondrial dysfunction. Corroborating, SH-SY5Y cells treated with 2mM $MPP^+$ were reported to also have increased levels of p-AMPK as soon as only 2h after treatment. Conversely, AMPK activation was prevented by pre-treatment with antioxidants (N-acetylcysteine and butylated hydroxyanisole). Thus, $MPP^+$-derived production of ROS contributes directly to AMPK activation (Jovanovic-Tucovic et al., 2019). The relation of mitochondrial defects and alterations in the autophagic system, which are regulated by AMPK activation, is also well documented. For example, hybrid cells harboring sPD patient mitochondria, with decreased complex I function, presented impaired autophagic activity marked by the excessive accumulation of autophagosomes (Arduíno et al., 2012). Likewise, it was observed that $MPP^+$-treated BE-M17 cells had a dose-dependent accumulation of undegraded autophagosomes, preceding cell death. Interestingly, the reduced autophagic flux was attributed to a consequence of lysosomal membrane permeabilization, directly associated with the formation of ROS caused by the $MPP^+$-inhibition of the mitochondrial electron chain transport (Dehay et al., 2010). Independently, knockout models of various

genes coding for mitochondrial proteins also displayed increased number and larger dysfunctional lysosomes by a ROS-dependent mechanism (Demers-Lamarche et al., 2016). Therefore, it is plausible that $MPP^+$-inhibition of mitochondrial complex I leads to the generation of ROS, which will affect AMPK phosphorylation and affect permeabilization of lysosomal membranes. As a result, the number of dysfunctional lysosomes on the cell increases, which could underlie the rise in intracellular cholesterol levels.

Additionally, we observed inhibition of mTOR activity after 16h of $MPP^+$ treatment, which we proposed to be related to the initial activation of AMPK. We anticipated these results, because treatment with rotenone, another drug used in studies of PD given its effect on blocking complex I of mitochondria, lead to a time and dose-dependent inhibition of mTORC1 phosphorylation in primary cortical mice neurons (Zhou et al., 2015). Plus, in another study using SH-SY5Y cells, $MPP^+$ triggered an early AMPK activation within 2h, while mTORC1 inhibition was delayed until 16h after treatment (Jovanovic-Tucovic et al., 2019).

Interestingly, fibroblasts heterozygous and homozygous mutants of GBA1 that displayed, as mentioned previously, increased levels of cholesterol and number lysosomes, also had lower levels of p-70S6K1, a mTORC1 downstream target, than fibroblasts wild type GBA1 (Magalhaes et al., 2016). Likewise, cells knockout for p18, which belongs to a scaffold complex necessary for mTORC1 activation, or cells re-expressing p18 but treated with rapamycin, a known mTORC1 inhibitor that stimulates autophagy, also reported a rise in cholesterol content attributed to the accumulation of late endosomes that would accommodate cholesterol (Takahashi et al., 2012). More so, an active mTORC1 has been shown to stimulate the expression and maturation of SREBP-1c and SREBP2 (Peterson et al., 2011; Eid et al., 2017), and in our results we observe a decreased expression of SREBP1/2 expression, as well as their downstream targets, leading to reduced synthesis of both fatty acids and cholesterol. Thus, given these lines of evidence, we propose that mTORC1 inhibition after $MPP^+$ treatment is related to the observed increased levels of cholesterol, lysosomal cholesterol accumulation, and lastly, the reduced lipid biosynthesis.

Because we previously detected an initial phosphorylation of AMPK that could be responsible for the later mTORC1 suppression, we decided to see if by blocking AMPK activity, we could prevent the alterations in cholesterol content. Despite our hypothesis, we observed that pre-treating cells with compound C, a common AMPK inhibitor, did not prevent the $MPP^+$-induced lysosomal cholesterol accumulation. However, it is to mention that, compound C is also a potent inhibitor of other kinases, and so our results cannot be attributed only to blocking AMPK function (reviewed in: Dasgupta and Seibel, 2018).

Both lysosomes biogenesis and the autophagic processes, which are involved in $MPP^+$-dependent neurodegeneration, are regulated by the transcription factor EB (TFEB) (Sardiello et al., 2009; Settembre et al., 2011). Under physiologic conditions, TFEB is a basic helix-loop-helix leucine zipper transcription factor that remains in close association with lysosomes membranes due to phosphorylation by mTORC1. However, during stress-conditions, where AMPK suppresses mTORC1 activity, this kinase can longer confine TFEB location. This results in TFEB migration to the nucleus, where it will stimulate the expression of various proteins involved in lysosomal function (Roczniak-

Ferguson et al., 2012; Settembre et al., 2012). Overall, AMPK promotes the autophagic process by three different mechanisms. Firstly, AMPK starts the macroautophagic signaling cascade through the direct activation of UNC-51 like autophagy activating kinase (ULK1) and by blocking mTORC1, which could also function as ULK1 inhibitor (Lee et al., 2010; Kim et al., 2011). After, ULK1 phosphorylates Beclin-1, leading the formation of autophagosomes (Russell et al., 2013). Secondly, both the mitochondrial stress and AMPK are responsible for increasing the transcriptional activity of TFEB, thus inducing the formation of lysosomes that will merge with the newly made autophagosomes (Fernández-Mosquera et al., 2017; reviewed in: Curry et al., 2018). Once again, since we observed AMPK activation after 6h of treatment, we also expected that TFEB and TFEB-target expression could be increased for the same time-point. Nevertheless, we did not observe any statistically significant differences between $MPP^+$-treated cells and controls, meaning that the activation of AMPK was not leading to an increase in lysosomal biogenesis. Plus, we detected a down-regulation of TFEB and its lysosomal targets at a later time-point. This has been observed in cells treated with rotenone and the mitochondrial uncoupler CCCP, indicating that the extended inhibition of mitochondrial function suppresses lysosomal biogenesis (Fernandez-Mosquera et al., 2017). Yet, when we treated neuronal cells with CCCP to investigate if different mitochondrial drugs, that lead to oxidative stress, could mimic similar lysosomal cholesterol accumulation as $MPP^+$, we observed that this particular outcome was specific to the neurotoxin-inhibition of the mitochondrial complex I. Interestingly, AMPK was found to be a molecular target of methyl-β-cyclodextrin, which is a compound used to deplete cholesterol from cells and has an effect in ameliorating cholesterol accumulation in lysosomes in NPC1 mutants (Dai et al., 2017).

In summary, we demonstrate that mitochondria dysfunction leads to downregulation of SREBPs expression and activity in neuronal cells. Given their role in regulating fatty acids and cholesterol biosynthesis and the profound alterations in sterol homeostasis observed after $MPP^+$ treatment, we suggest that targeting SREBP could be beneficial in PD. Specially, since *SREBF1*, SREBP1-coding gene, was previously identified as a risk factor for the disease (Do et al. 2011), and even a regulator of mitophagy (Ivatt et al., 2014). Similarly, SREBP2 was also discovered to stimulate expression of some autophagic genes (Seo et al., 2011), and overexpression of SREBP2 restored some of the autophagic dysfunction in fat-overloaded hepatocytes (Cheng et al., 2018). Thus, with this line of evidence, it would be interestingly to evaluate if the overexpression of either SREBP1 or SREBP2 could ameliorate mitochondria function and cell survival, by analyzing ATP levels and cell death parameters. We are also interested in investigating the role of SREBP1 in mitophagy, which could be done by accessing the stabilization of PINK1 in cells overexpressing SREBP1 and subjected to $MPP^+$ treatment.

Furthermore, we discovered that the inhibition of mitochondrial complex I leads to lysosomal dysfunction and reduced NPC1 expression, further supporting previous reports that suggest PD as a lysosomal storage disorder, similar to Gaucher's disease (reviewed in: Klein and Mazzulli, 2018). Indeed, it was shown that 56% of PD patients presented at least one putative damaging variant in a lysosomal storage disorders gene, and 21% carried multiple alleles (Robak et al., 2017). These include GBA1 mutations, which are considered the greatest risk factor for developing PD (Sidransky et

al., 2009), and even NPC1 variants (Josephs et al., 2004; Kluenemann et al., 2013). Although the clear association between the NPC1 gene and PD remains unknow (Deng et al., 2014), based on our results, we proposed continuing investigating NPC1 as another risk factor for PD. Therefore, it would be interesting to determine if heterozygous mice for the NPC1 mutated gene, which to not present a clinical NPC phenotype, are more or less susceptible to MPTP treatment.

We also found evidence that $MPP^+$ treatment in neuronal cells signals the activation of AMPK/ mTOR signaling pathway. Thus, it would be interesting to overexpress TEFB in $MPP^+$-treated cells and observe if this could prevent the alterations in cholesterol homeostasis and modulate cholesterol levels by overexpressing SREBP1/2, and analyze also lysosomal biogenesis. Indeed, overexpression of TFEB plasmid in BE-M17 cells was capable of attenuating $MPP^+$-induced cell death by promoting the formation of lysosomes (Dehay et al., 2010).

Lastly, although we failed to observe if the role of cholesterol could be beneficial or detrimental to development of PD, based on our results and other evidence already presented, we propose that the changes in cholesterol homeostasis observed, such as the increase in cholesterol levels, are actually contributing to the propagation of toxicity after $MPP^+$-induced mitochondrial dysfunction.

# Chapter V. References


Abeliovich, A., Schmitz, Y., Fariñas, I., Choi-Lundberg, D., Ho, W.-H., Castillo, P. E., … Rosenthal, A. (2000). Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system. Neuron, 25(1), 239–252.

Abildayeva, K., Jansen, P. J., Hirsch-Reinshagen, V., Bloks, V. W., Bakker, A. H. F., Ramaekers, F. C. S., … Mulder, M. (2006). 24 (S)-Hydroxycholesterol Participates in a Liver X Receptor-controlled Pathway

Adams, C. M., Reitz, J., Brabander, J. K. De, Feramisco, J. D., Li, L., Brown, M. S., & Goldstein, J. L. (2004). Cholesterol and 25-Hydroxycholesterol Inhibit Activation of SREBPs by Different Mechanisms

Amemiya-Kudo, M., Shimano, H., Hasty, A. H., Yahagi, N., Yoshikawa, T., Matsuzaka, T., … Yamada, N. (2002). Transcriptional activities of nuclear SREBP-1a, -1c , and -2 to different target promoters of lipo

Amemiya-Kudo, M., Shimano, H., Yoshikawa, T., Yahagi, N., Hasty, A. H., Okazaki, H., … Yamada, N. (2000). Promoter analysis of the mouse sterol regulatory element-binding protein-1c gene. J. Biol. Chem.</

Anchisi, L., Dessì, S., Pani, A., & Mandas, A. (2013). Cholesterol homeostasis: a key to prevent or slow down neurodegeneration. Front. Physiol., 3, 486.

Ara, J., Przedborski, S., Nanine, A. B., Jackson-Lewis, V., Trifiletti, R. R., Horwitz, J., & Ischiropoulos, H. (1998). Inactivation of tyrosine hydroxylase by nitration following exposure to peroxynitrite and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Proc. Natl. Acad. Sci. U. S. A., 95(13), 7659–7663.

Arduíno, D. M., Esteves, A. R., Cortes, L., Silva, D. F., Patel, B., Grazina, M., … Cardoso, S. M. (2012). Mitochondrial metabolism in Parkinson's disease impairs quality control autophagy by hampering micro

Arduíno, D. M., Esteves, A. R., Silva, D. F. F., Martins-Branco, D., Santos, D., Pimentel, D. F. G., & Cardoso, S. M. (2011). Therapeutic Intervention at Cellular Quality Control Systems in Alzheimer's and Parkinson's Diseases. Curr. Pharm. Des., 17(31), 3446–3459.

Arenas, F., Garcia-Ruiz, C., & Fernandez-Checa, J. C. (2017). Intracellular Cholesterol Trafficking and Impact in Neurodegeneration. Front. Mol. Neurosci., 10, 382.

Bar-On, P., Crews, L., Koob, A. O., Mizuno, H., Adame, A., Spencer, B., & Masliah, E. (2008). Statins reduce neuronal alpha-synuclein aggregation in in vitro models of Parkinson's disease. J. Neuroche

Bar-On, P., Rockenstein, E., Adame, A., Ho, G., Hashimoto, M., & Masliah, E. (2006). Effects of the cholesterol-lowering compound methyl-beta-cyclodextrin in models of alpha-synucleinopathy. J. Neurochem., 98(4), 1032–1045.

Beisiegel, U., Weber, W., Ihrke, G., Herz, J., & Stanley, K. K. (1989). The LDL-receptor-related protein, LRP, is an apolipoprotein E-binding protein. Nature, 341(6238), 162–164.

Bender, A., Krishnan, K. J., Morris, C. M., Taylor, G. A., Reeve, A. K., Perry, R. H., … Turnbull, D. M. (2006). High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. Nat. Genet., 38(5), 515–517.

Bennett, M. K., Lopez, J. M., Sanchez, H. B., & Osborne, T. F. (1995). Sterol Regulation of Fatty Acid Synthase Promoter. J. Biol. Chem., 270(43), 25578–25583.

Bezard, E., Dovero, S., Bioulac, B., & Gross, C. E. (1997). Kinetics of nigral degeneration in a chronic model of MPTP-treated mice. Neurosci. Lett., 234(1), 47–50.

Bezard, E., Gross, C. E., Fournier, M.-C., Dovero, S., Bloch, B., & Jaber, M. (1999). Absence of MPTP-induced neuronal death in mice lacking the dopamine transporter. Exp. Neurol., 155(2), 268–273.

Björkhem, I., Lütjohann, D., Breuer, O., Sakinis, A., & Wennmalm, Å. (1997). Importance of a Novel Oxidative Mechanism for Elimination of Brain Cholesterol. Turnover of cholesterol and 24(S)-hydroxycholesterol in rat brain as measured with 18O2 techniques in vivo and in vitro. J. Biol. Chem., 272(48), 30178–30184.

Björkhem, I., Lütjohann, D., Diczfalusy, U., Ståhle, L., Ahlborg, G., & Wahren, J. (1998). Cholesterol homeostasis in human brain: turnover of 24S-hydroxycholesterol and evidence for a cerebral origin of most of this oxysterol in the circulation. J. Lipid Res., 39(8), 1594–1600.

Björkhem, I., & Meaney, S. (2004). Brain Cholesterol: long secret life behind a barrier. Arterioscler. Thromb. Vasc. Biol., 24(5), 806–815.

Blandini, F., & Armentero, M.-T. (2012). Animal models of Parkinson's disease. FEBS J., 279(7), 1156–1166.

Bonifati, V., Rizzu, P., van Baren, M. J., Schaap, O., Breedveld, G. J., Krieger, E., … Heutink, P. (2003). Mutations in the DJ-1 Gene Associated with Autosomal Recessive Early-Onset Parkinsonism. Science, 299(5604), 256–259.

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

Boyles, J. K., Pitas, R. E., Wilson, E., Mahley, R. W., & Taylor, J. M. (1985). Apolipoprotein E associated with astrocytic glia of the central nervous system and with nonmyelinating glia of the peripheral nervous system. J. Clin. Invest., 76(4), 1501–1513.

Bretillon, L., Sidén, Å., Wahlund, L.-O., Lütjohann, D., Minthon, L., Crisby, M., … Björkhem, I. (2000). Plasma levels of 24S-hydroxycholesterol in patients with neurological diseases. Neurosci. Lett., 293(2), 87–90.

Brown, A. J., Sun, L., Feramisco, J. D., Brown, M. S., & Goldstein, J. L. (2002). Cholesterol Addition to ER Membranes Alters Conformation of SCAP, the SREBP Escort Protein that Regulates Cholesterol Metabolism. Mol. Cell, 10(2), 237–245.

Brown, M. S., Dana, S. E., & Goldstein, J. L. (1975). Receptor-dependent hydrolysis of cholesteryl esters contained in plasma low density lipoprotein. Proc. Natl. Acad. Sci. U. S. A., 72(8), 2925–2929.
Brown, M. S., & Goldstein, J. L. (1999). A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. Proc. Natl. Acad. Sci. U. S. A., 96(20), 11041–11048.
Brown, M. S., & Goldstein, J. L. (1986). A Receptor-Mediated Pathway for Cholesterol Homeostasis. Science, 232(4746), 34–47.
Bryleva, E. Y., Rogers, M. A., Chang, C. C. Y., Buen, F., Harris, B. T., Rousselet, E., … Chang, T.-Y. (2010). ACAT1 gene ablation increases 24(S)-hydroxycholesterol content in the brain and ameliorates amyloid pathology in mice with AD. Proc. Natl. Acad. Sci. U. S. A., 107(7), 3081–3086.
Carstea, E. D., Morris, J. A., Coleman, K. G., Loftus, S. K., Zhang, D., Cummings, C., … Tagle, D. A. (1997). Niemann-Pick C1 Disease Gene: Homology to Mediators of Cholesterol Homeostasis. Science, 277(5323), 228–231.
Castoreno, A. B., Wang, Y., Stockinger, W., Jarzylo, L. A., Du, H., Pagnon, J. C., … Nohturfft, A. (2005). Transcriptional regulation of phagocytosis-induced membrane biogenesis by sterol regulatory element binding proteins. Proc. Natl. Acad. Sci. U. S. A., 102(37), 13129–13134.
Castro-Caldas, M., Carvalho, A., Rodrigues, E., Henderson, C., Wolf, C., Rodrigues, C., & Gama, M. (2012). Tauroursodeoxycholic acid prevents MPTP-induced dopaminergic cell death in a mouse model of Parkinson's disease. Mol. Neurobiol., 46(2), 475–486.
Castro-Caldas, M., Neves Carvalho, A., Peixeiro, I., Rodrigues, E., Lechner, M., & Gama, M. (2009). GSTpi expression in MPTP-induced dopaminergic neurodegeneration of C57BL/6 mouse midbrain and striatum. J. Mol. Neurosci., 38(2), 114–127.
Chang, C. C. Y., Chen, J., Thomas, M. A., Cheng, D., Priore, V. A. Del, Newton, R. S., … Chang, T.-Y. (1995). Regulation and Immunolocalization of Acyl-Coenzyme A:Cholesterol Acyltransferase in Mammalian Cells as Studied with Specific Antibodies*. J. Biol. Chem., 270(49), 29532–29540.
Chang, C. C. Y., Lee, C.-Y. G., Chang, E. T., Cruz, J. C., Levesque, M. C., & Chang, T.-Y. (1998). Recombinant Acyl-CoA: cholesterol Acyltransferase-1 (ACAT-1) Purified to Essential Homogeneity Utilizes Cholesterol in Mixed Micelles or in Vesicles in a Highly Cooperative Manner*. J. Biol. Chem., 273(52), 35132–35141.
Chaudhuri, K. R., & Schapira, A. H. V. (2009). Non-motor symptoms of Parkinson's disease: dopaminergic pathophysiology and treatment. Lancet Neurol., 8(5), 464–474.
Chen, L., Wang, J., Zhang, Y.-Y., Yan, S. F., Neumann, D., Schlattner, U., … Wu, J.-W. (2012). AMP-activated protein kinase undergoes nucleotide-dependent conformational changes. Nat. Struct. Mol. Biol., 19(7), 716–718.
Cheng, C., Deng, X., & Xu, K. (2018). Increased expression of sterol regulatory element binding protein-2 alleviates autophagic dysfunction in NAFLD. Int. J. Mol. Med., 41(4), 1877–1886.
Cheruku, S. R., Xu, Z., Dutia, R., Lobel, P., & Storch, J. (2006). Mechanism of Cholesterol Transfer from the Niemann-Pick Type C2 Protein to Model Membranes Supports a Role in Lysosomal Cholesterol Transport*. J. Biol. Chem., 281(42), 31594–31604.
Choi, J.-S., Park, C., & Jeong, J.-W. (2010). AMP-activated protein kinase is activated in Parkinson's disease models mediated by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Biochem. Biophys. Res. Commun., 391(1), 147–151.
Conway, K. A., Harper, J. D., & Lansbury, P. T. (1998). Accelerated in vitro fibril formation by a mutant alpha-synuclein linked to early-onset Parkinson disease. Nat. Med., 4(11), 1318–1320.
Conway, K. A., Harper, J. D., & Lansbury, P. T. (2000). Fibrils formed in vitro from alpha-synuclein and two mutant forms linked to Parkinson's disease are typical amyloid. Biochemistry, 39(10), 2552–2563.
Courtney, R., & Landreth, G. E. (2016). LXR Regulation of Brain Cholesterol : From Development to Disease. Trends Endocrinol. Metab., 27(6), 404–414.
Culmsee, C., Monnig, J., Kemp, B. E., & Mattson, M. P. (2001). AMP-Activated Protein Kinase is Highly Expressed in Neurons in the Developing Rat Brain and Promotes Neuronal Survival Following Glucose Deprivation. J. Mol. Neurosci., 17(1), 45–58.
Cunningham, J. T., Rodgers, J. T., Arlow, D. H., Vazquez, F., Mootha, V. K., & Puigserver, P. (2007). mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. Nature, 450(7170), 736–740.
Curry, D. W., Stutz, B., Andrews, Z. B., & Elsworth, J. D. (2018). Targeting AMPK Signaling as a Neuroprotective Strategy in Parkinson's Disease. J. Parkinsons. Dis., 8(2), 161–181.
Dai, S., Dulcey, A. E., Hu, X., Wassif, C. A., Porter, F. D., Austin, C. P., … Zheng, W. (2017). Methyl-β-cyclodextrin restores impaired autophagy flux in Niemann-Pick C1-deficient cells through activation of AMPK. Autophagy, 13(8), 1435–1451.
Dasgupta, B., & Seibel, W. (2018). Compound C/Dorsomorphin: Its Use and Misuse as an AMPK Inhibitor. In Methods Mol. Biol. (Vol. 1732, pp. 195–202).
Dauer, W., & Przedborski, S. (2003). Parkinson's Disease: Mechanisms and Models. Neuron, 39(6), 889–909.
Davies, J. P., & Ioannou, Y. A. (2000). Topological Analysis of Niemann-Pick C1 Protein Reveals That the Membrane Orientation of the Putative Sterol-sensing Domain Is Identical to Those of 3-Hydroxy-3-methylglutaryl-CoA Reductase and Sterol Regulatory Element Binding Protein Cleavage-activating. J. Biol. Chem., 275(32), 24367–24374.
de Chaves, E. I. P., Rusiñol, A. E., Vance, D. E., Campenot, R. B., & Vance, J. E. (1997). Role of Lipoproteins in the Delivery of Lipids to Axons during Axonal Regeneration*. J. Biol. Chem., 272(49), 30766–30773.

de Rijk, M. C., Tzourio, C., Breteler, M. M. B., Dartigues, J. F., Amaducci, L., Lopez-Pousa, S., … Roca, W. A. (1997). Prevalence of parkinsonism and Parkinson's disease in Europe: the EUROPARKINSON collaborative study. J. Neurol. Neurosurg. Psychiatry, 62(1), 10–15.
DeBose-Boyd, R. A., & Ye, J. (2018). SREBPs in Lipid Metabolism, Insulin Signaling, and Beyond. Trends Biochem. Sci., 43(5), 358–368.
Dehay, B., Bové, J., Rodríguez-Muela, N., Perier, C., Recasens, A., Boya, P., & Vila, M. (2010). Pathogenic Lysosomal Depletion in Parkinson's Disease. J. Neurosci., 30(37), 12535–12544.
Demers-Lamarche, J., Guillebaud, G., Tlili, M., Todkar, K., Bélanger, N., Grondin, M., … Germain, M. (2016). Loss of Mitochondrial Function Impairs Lysosomes. J. Biol. Chem., 291(19), 10263–10276.
Deng, H., Xiu, X., & Jankovic, J. (2014). Genetic convergence of Parkinson's disease and lysosomal storage disorders. Mol. Neurobiol., 51(3), 1554–1568.
Dietschy, J. M., & Turley, S. D. (2004). Cholesterol metabolism in the central nervous system during early development and in the mature animal. J. Lipid Res., 45(8), 1375–1397.
Dietschy, J. M., & Turley, S. D. (2001). Cholesterol metabolism in the brain. Curr. Opin. Lipidol., 12(2), 105–112.
Do, C. B., Tung, J. Y., Dorfman, E., Kiefer, A. K., Drabant, E. M., Francke, U., … Eriksson, N. (2011). Web-based genome-wide association study identifies two novel loci and a substantial genetic component for Parkinson's disease. PLoS Genet., 7(6), e1002141.
Dooley, K. A., Bennett, M. K., & Osborne, T. F. (1999). A Critical Role for cAMP Response Element-binding Protein (CREB) as a Co-activator in Sterol-regulated Transcription of 3-Hydroxy-3-methylglutaryl Coenzyme A Synthase Promoter*. J. Biol. Chem., 274(9), 5285–5291.
Dooley, K. A., Millinder, S., & Osborne, T. F. (1998). Sterol Regulation of 3-Hydroxy-3-Methylglutaryl-coenzyme A Synthase Gene through a Direct Interaction Between Sterol Regulatory Element Binding Protein and the Trimeric CCAAT-binding Factor/Nuclear Factor Y*. J. Biol. Chem., 273(3), 1349–1356.
Doria, M., Maugest, L., Moreau, T., Lizard, G., & Vejux, A. (2016). Contribution of cholesterol and oxysterols to the pathophysiology of Parkinson's disease. Free Radic. Biol. Med., 101, 393–400.
Düvel, K., Yecies, J. L., Menon, S., Raman, P., Lipovsky, A. I., Souza, A. L., … Manning, B. D. (2010). Activation of a Metabolic Gene Regulatory Network Downstream of mTOR Complex 1. Mol. Cell, 39(2), 171–183.
Eid, W., Dauner, K., Courtney, K. C., Gagnon, A., Parks, R. J., Sorisky, A., & Zha, X. (2017). mTORC1 activates SREBP-2 by suppressing cholesterol trafficking to lysosomes in mammalian cells. Proc. Natl. Acad. Sci. U. S. A., 114(30), 7999–8004.
Eliezer, D., Kutluay, E., Bussel, R. J., & Browne, G. (2001). Conformational properties of alpha-synuclein in its free and lipid-associated states. J. Mol. Biol., 307(4), 1061–1073.
Elshourbagy, N. A., Liao, W. S., Mahley, R. W., & Taylort, J. M. (1985). Apolipoprotein E mRNA is abundant in the brain and adrenals, as well as in the liver, and is present in other peripheral tissues of rats and marmosets. Proc. Natl. Acad. Sci. U. S. A., 82(1), 203–207.
Eriksson, I., Nath, S., Bornefall, P., Giralso, A. M. V., & Öllinger, K. (2017). Impact of high cholesterol in a Parkinson's disease model: Prevention of lysosomal leakage versus stimulation of α-synuclein aggregation. Eur. J. Cell Biol., 96(2), 99–109.
Espenshade, P. J., Li, W.-P., & Yabe, D. (2002). Sterols block binding of COPII proteins to SCAP, thereby controlling SCAP sorting in ER. Proc. Natl. Acad. Sci. U. S. A., 99(18), 11694–11699.
Falkenburger, B. H., & Schulz, J. B. (2006). Limitations of cellular models in Parkinson's disease research. J. Neural Transm., 70, 261–268.
Falkenburger, B. H., Saridaki, T., & Dinter, E. (2016). Cellular models for Parkinson's disease. J. Neurochem., 139, 121–130.
Fan, Q.-W., Ichiro, I., Asou, H., Katsuhiko, Y., & Michikawa, M. (2001). Expression and regulation of apolipoprotein E receptors in the cells of the central nervous system in culture: A review. J. Am. Aging Assoc., 24(1), 1–10.
Fan, Q., Yu, W., Gong, J.-S., Zou, K., Sawamura, N., Senda, T., … Michikawa, M. (2002). Cholesterol-dependent modulation of dendrite outgrowth and microtubule stability in cultured neurons. J. Neurochem., 80(1), 178–190.
Fantini, J., Carlus, D., & Yahi, N. (2011). The fusogenic tilted peptide (67-78) of α-synuclein is a cholesterol binding domain. Biochim. Biophys. Acta, 1808(10), 2343–2351.
Fernández-Mosquera, L., Diogo, C. V, Yambire, K. F., Santos, G. L., Sánchez, M. L., Bénit, P., … Raimundo, N. (2017). Acute and chronic mitochondrial respiratory chain deficiency differentially regulate lysosomal biogenesis. Sci. Rep., 7, 45076.
Fukumoto, H., Deng, A., Irizarry, M. C., Fitzgerald, M. L., & Rebeck, G. W. (2002). Induction of the Cholesterol Transporter ABCA1 in Central Nervous System Cells by Liver X Receptor Agonists Increases Secreted Aβ Levels*. J. Biol. Chem., 277(50), 48508–48513.
Garcia, D., & Shaw, R. J. (2017). AMPK: mechanisms of cellular energy sensing and restoration of metabolic balance. Mol. Cell, 66(6), 789–800.
Giasson, B. I., Duda, J. E., Quinn, S. M., Zhang, B., Trojanowski, J. Q., & Lee, V. M.-Y. (2002). Neuronal alpha-synucleinopathy with severe movement disorder in mice expressing A53T human alpha-synuclein. Neuron, 34(4), 521–533.
Goldstein, J. L., & Brown, M. S. (1990). Regulation of the mevalonate pathway. Nature, 343(6257), 425–430.
Goldstein, J. L., Dana, S. E., & Brown, M. S. (1974). Esterification of Low-Density Lipoprotein Cholesterol in Human Fibroblasts and Its Absence in Homozygous Familial Hypercholesterolemia. Proc. Natl. Acad. Sci. U. S. A., 71(11), 4288–4292.

Goldstein, J. L., DeBose-Boyd, R. A., & Brown, M. S. (2006). Protein sensors for membrane sterols. Cell, 124(1), 35–46.
Gong, J.-S., Kobayashi, M., Hayashi, H., Zou, K., Sawamura, N., Fujita, S. C., … Michikawa, M. (2002). Apolipoprotein E (ApoE) Isoform-dependent Lipid Release from Astrocytes Prepared from Human ApoE3 and ApoE4 Knock-in Mice*. J. Biol. Chem., 277(33), 29919–29926.
Goritz, C., Mauch, D. H., & Pfrieger, F. W. (2005). Multiple mechanisms mediate cholesterol-induced synaptogenesis in a CNS neuron. Mol. Cell. Neurosci., 29(2), 190–201.
Govek, E.-E., Newey, S. E., & Aelst, L. Van. (2005). The role of the Rho GTPases in neuronal development. Genes Dev., 19(1), 1–49.
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. Mol. Cell, 30(2), 214–226.
Haar, E. Vander, Lee, S., Bandhakavi, S., Griffin, T. J., & Kim, D.-H. (2007). Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. Nat. Cell Biol., 9(3), 316–323.
Haas, R. H., Nasirian, F., Nakano, K., Ward, D., Pay, M., Hill, R., & Shults, C. W. (1995). Low Platelet Mitochondrial Complex I and Complex II/III Activity in Early Untreated Parkinson's Disease. Ann. Neurol., 37(6), 714–722.
Hall, A., & Lalli, G. (2010). Rho and Ras GTPases in Axon Growth, Guidance, and Branching. Cold Spring Harb. Perspect. Biol., 2(2), a001818.
Hardie, D. G., & Carling, D. (1997). The AMP-activated protein kinase. Fuel gauge of the mammalian cell? Eur. J. Biochem., 246(2), 259–273.
Hardie, D. G., Scott, J. W., Pan, D. A., & Hudson, E. R. (2003). Management of cellular energy by the AMP-activated protein kinase system. FEBS Lett., 546(1), 113–120.
Hawley, S. A., Davison, M., Woods, A., Davies, S. P., Beri, R. K., Carling, D., & Hardie, D. G. (1996). Characterization of the AMP-activated Protein Kinase Kinase from Rat Liver and Identification of Threonine 172 as the Major Site at Which It Phosphorylates AMP-activated Protein Kinase*. J. Biol. Chem., 271(44), 27879–27887.
Hawley, S. A., Pan, D. A., Mustard, K. J., Ross, L., Bain, J., Edelman, A. M., … Hardie, D. G. (2005). Calmodulin-dependent protein kinase kinase-β is an alternative upstream kinase for AMP-activated protein kinase. Cell Metab., 2(1), 9–19.
Higgins, M. E., Davies, J. P., Chen, F. W., & Ioannou, Y. A. (1999). Niemann-Pick C1 Is a Late Endosome-Resident Protein That Transiently Associates with Lysosomes and the Trans-Golgi Network. Mol. Genet. Metab., 68(1), 1–13.
Hirsch-Reinshagen, V., Zhou, S., Burgess, B. L., Bernier, L., McIsaac, S. A., Chan, J. Y., … Wellington, C. L. (2004). Deficiency of ABCA1 Impairs Apolipoprotein E Metabolism in Brain*. J. Biol. Chem., 279(39), 41197–41207.
Hoeffer, C. A., & Klann, E. (2010). mTOR signaling: At the crossroads of plasticity, memory and disease. Trends Neurosci., 33(2), 67–75.
Holstein, S. A., & Hohl, R. J. (2004). Isoprenoids: Remarkable Diversity of Form and Function. Lipids, 39(4), 293–309.
Hooff, G. P., Wood, W. G., Müller, W. E., & Eckert, G. P. (2010). Isoprenoids, small GTPases and Alzheimer's disease. Biochim. Biophys. Acta, 1801(8), 896–905.
Hoppel, C. L., Greenblatt, D., Kwok, H., Arora, P. K., Singh, M. P., & Sayre, L. M. (1987). Inhibition of mitochondrial respiration by analogs of 4-phenylpyridine and 1-methyl-4-phenylpyridinium cation (MPP+), the neurotoxic metabolite of MPTP. Biochem. Biophys. Res. Commun., 148(2), 684–693.
Horton, J. D., Goldstein, J. L., & Brown, M. S. (2002). SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J. Clin. Invest., 109(9), 1125–1131.
Horton, J. D., Shimomura, I., Brown, M. S., Hammer, R. E., Goldstein, J. L., & Shimano, H. (1998). Activation of cholesterol synthesis in preference to fatty acid synthesis in liver and adipose tissue of transgenic mice overproducing sterol regulatory element- binding protein-2. J. Clin. Invest., 101(11), 2331–2339.
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. Mol. Biol. Cell, 20(7), 1981–1991.
Hua, X., Nohturfft, A., Goldstein, J. L., & Brown, M. S. (1996). Sterol Resistance in CHO Cells Traced to Point Mutation in SREBP Cleavage-Activating Protein. Cell, 87(3), 415–426.
Hua, X., Sakai, J., Ho, Y. K., Goldstein, J. L., & Brown, M. S. (1995). Hairpin Orientation of Sterol Regulatory Element-binding Protein-2 in Cell Membranes as Determined by Protease Protection*. J. Biol. Chem., 270(49), 29422–29427.
Hua, X., Wu, J., Goldstein, J. L., Brown, M. S., & Hobbs, H. H. (1995). Structure of the Human Gene Encoding Sterol Regulatory Element Binding Protein-1 (SREBF1) and Localization of SREBF1 and SREBF2 to Chromosomes 17p11.2 and 22q13. Genomics, 25(3), 667–673.
Huang, X., Sterling, N. W., Du, G., Sun, D., Stetter, C., Kong, L., … Mailman, R. B. (2019). Brain Cholesterol Metabolism and Parkinson's Disease. Mov. Disord., 34(3), 386–395.
Hurley, R. L., Anderson, K. A., Franzone, J. M., Kemp, B. E., Means, A. R., & Witters, L. A. (2005). The Ca2+/Calmodulin-dependent Protein Kinase Kinases Are AMP-activated Protein Kinase Kinases*. J. Biol. Chem., 280(32), 29060–29066.
Ignatius, M. J., Skene, J. H. P., Muller, H. W., & Shooter, E. M. (1987). Examination of a Nerve Injury-Induced, 37 kDa Protein: Purification and Characterization*. Neurochem. Res., 12(10), 967–976.

Ikonen, E. (2008). Cellular cholesterol trafficking and compartmentalization. Mol. Cell, 9(2), 125–138.
Infante, R. E., Wang, M. L., Radhakrishnan, A., Kwon, H. J., Brown, M. S., & Goldstein, J. L. (2008). NPC2 facilitates bidirectional transfer of cholesterol between NPC1 and lipid bilayers, a step in cholesterol egress from lysosomes. Proc. Natl. Acad. Sci. U. S. A., 105(40), 15287–15292.
Infante, R. E., & Radhakrishnan, A. (2017). Continuous transport of a small fraction of plasma membrane cholesterol to endoplasmic reticulum regulates total cellular cholesterol. Elife, 6, e25466.
Inoki, K., Li, Y., Xu, T., & Guan, K.-L. (2003). Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev., 17(15), 1829–1834.
Inoki, K., Zhu, T., & Guan, K.-L. (2003). TSC2 Mediates Cellular Energy Response to Control Cell Growth and Survival. Cell, 115(5), 577–590.
Ivatt, R. M., Sanchez-Martinez, A., Godena, V. K., Brown, S., Ziviani, E., & Whitworth, A. J. (2014). Genome-wide RNAi screen identifies the Parkinson disease GWAS risk locus SREBF1 as a regulator of mitophagy. Proc. Natl. Acad. Sci. U. S. A., 111(23), 8494–8499.
Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Rüegg, M. A., Hall, A., & Hall, M. N. (2004). Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat. Cell Biol., 6(11), 1122–1128.
Jackson-Lewis, V., Jakowec, M., Burke, R. E., & Przedborski, S. (1995). Time course and morphology of dopaminergic neuronal death caused by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Neurodegeneration, 4(3), 257–269.
Jackson-Lewis, V., & Przedborski, S. (2007). Protocol for the MPTP mouse model of Parkinson's disease. Nat. Protoc., 2(1), 141–151.
Jakes, R., Spillantini, M. G., & Goedert, M. (1994). Identification of two distinct synucleins from human brain. FEBS Lett., 345(1), 27–32.
Jensen, P. H., Nielsen, M. S., Jakes, R., Dotti, C. G., & Goedert, M. (1998). Binding of α-Synuclein to Brain Vesicles Is Abolished by Familial Parkinson's Disease Mutation*. J. Biol. Chem., 273(41), 26292–26294.
Jensen, P. H., Hager, H., Nielsen, M. S., Højrup, P., Gliemann, J., & Jakes, R. (1999). α-Synuclein Binds to Tau and Stimulates the Protein Kinase A-catalyzed Tau Phosphorylation of Serine Residues 262 and 356*. J. Biol. Chem., 274(36), 25481–25489.
Josephs, K. A., Matsumoto, J. Y., & Lindor, N. M. (2004). Heterozygous Niemann-Pick disease type C presenting with tremor. Neurology, 63(11), 2189–2190.
Jovanovic-Tucovic, M., Harhaji-Trajkovic, L., Dulovic, M., Tovilovic-Kovacevic, G., Zogovic, N., Jeremic, M., … Markovic, I. (2019). AMP-activated protein kinase inhibits MPP+-induced oxidative stress and apoptotic death of SH-SY5Y cells through sequential stimulation of Akt and autophagy. Eur. J. Pharmacol., 863, 172677.
Jurevics, H., & Morell, P. (1995). Cholesterol for Synthesis of Myelin Is Made Locally, Not Imported into Brain. J. Neurochem., 64(2), 895–901.
Karasinska, J. M., & Hayden, M. R. (2011). Cholesterol metabolism in Huntington disease. Nat. Rev. Neurol., 7(10), 561–572.
Kaushik, S., & Cuervo, A. M. (2015). Proteostasis and aging. Nat. Med., 21(12), 1406–1415.
Kennedy, M. A., Barrera, G. C., Nakamura, K., Baldán, Á., Tarr, P., Fishbein, M. C., … Edwards, P. A. (2005). ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation. Cell Metab., 1(2), 121–131.
Kim, D.-H., Sarbassov, D. D., Ali, S. M., King, J. E., Latek, R. R., Erdjument-Bromage, H., … Sabatini, D. M. (2002). mTOR Interacts with Raptor to Form a Nutrient-Sensitive Complex that Signals to the Cell Growth Machinery. Cell, 110(2), 163–175.
Kim, J. B., Spotts, G. D., Halvorsen, Y.-D., Shih, H.-M., Ellenberger, T., Towle, H. C., & Spiegelman, B. M. (1995). Dual DNA Binding Specificity of ADD1/SREBP1 Controlled by a Single Amino Acid in the Basic Helix-Loop-Helix Domain. Mol. Cell. Biol., 15(5), 2582–2588.
Kim, J., Ee, S., Jittiwat, J., Ong, E., Farooqui, A., Jenner, A., & Ong, W. (2011). Increased expression of acyl-coenzyme A: cholesterol acyltransferase-1 and elevated cholesteryl esters in the hippocampus after excitotoxic injury. Neuroscience, 185, 125–134.
Kim, K., Stevens, M. V, Akter, M. H., Rusk, S. E., Huang, R. J., Cohen, A., … Sack, M. N. (2011). Parkin is a lipid-responsive regulator of fat uptake in mice and mutant human cells. J. Clin. Invest., 121(9), 3701–3712.
Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., … Shimizu, N. (1998). Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature, 392(6676), 605–608.
Klaidman, L. K., Adams Jr, J. D., Leung, A. C., Kim, S. S., & Cadenas, E. (1993). Redox cycling of MPP+: evidence for a new mechanism involving hydride transfer with xanthine oxidase, aldehyde dehydrogenase, and lipoamide dehydrogenase. Free Radic. Biol. Med., 15(2), 169–179.
Klein, A. D., & Mazzulli, J. R. (2018). Is Parkinson's disease a lysosomal disorder? Brain, 141(8), 2255–2262.
Kluenemann, H. H., Nutt, J. G., Davis, M. Y., & Bird, T. D. (2013). Parkinsonism syndrome in heterozygotes for Niemann-Pick C1. J. Neurol. Sci., 335(1–2), 219–220.
Koldamova, R. P., Lefterov, I. M., Ikonomovic, M. D., Skoko, J., Lefterov, P. I., Isanski, B. A., … Lazo, J. S. (2003). 22R-Hydroxycholesterol and 9-cis-Retinoic Acid Induce ATP-binding Cassette Transporter A1 Expression and Cholesterol Efflux in Brain Cells and Decrease Amyloid β Secretion*. J. Biol. Chem., 278(15), 13244–13256.
Kölsch, H., Heun, R., Kerksiek, A., Bergmann, K. v, Maier, W., & Lütjohann, D. (2004). Altered levels of plasma 24S- and 27-hydroxycholesterol in demented patients. Neurosci. Lett., 368(3), 303–308.

Koob, A. O., Ubhi, K., Paulsson, J. F., Kelly, J., Rockenstein, E., Mante, M., … Masliah, E. (2010). Lovastatin ameliorates alpha-synuclein accumulation and oxidation in transgenic mouse models of alpha-synucleinopathies. Exp. Neurol., 221(2), 267–274.
Kotti, T. J., Ramirez, D. M. O., Pfeiffer, B. E., Huber, K. M., & Russell, D. W. (2006). Brain cholesterol turnover required for geranylgeraniol production and learning in mice. Proc. Natl. Acad. Sci. U. S. A., 103(10), 3869–3874.
Kowal, R. C., Herz, J., Goldstein, J. L., Esser, V., & Brown, M. S. (1989). Low density lipoprotein receptor-related protein mediates uptake of cholesteryl esters derived from apoprotein E-enriched lipoproteins. Proc. Natl. Acad. Sci. U. S. A., 86(15), 5810–5814.
Kraytsberg, Y., Kudryavtseva, E., McKee, A. C., Geula, C., Kowall, N. W., & Khrapko, K. (2006). Mitochondrial DNA deletions are abundant and cause functional impairment in aged human substantia nigra neurons. Nat. Genet., 38(5), 2005–2007.
Kurdi, A., Martinet, W., & Meyer, G. R. Y. De. (2018). mTOR Inhibition and Cardiovascular Diseases: Dyslipidemia and Atherosclerosis. Transplantation, 102(2S-1), S44–S46.
Kwon, H. J., Abi-Mosleh, L., Wang, M. L., Deisenhofer, J., Goldstein, J. L., Brown, M. S., & Infante, R. E. (2009). Structure of N-Terminal Domain of NPC1 Reveals Distinct Subdomains for Binding and Transfer of Cholesterol. Cell, 137(7), 1213–1224.
Laffitte, B. A., Repa, J. J., Joseph, S. B., Wilpitz, D. C., Kast, H. R., Mangelsdorf, D. J., & Tontonoz, P. (2001). LXRs control lipid-inducible expression of the apolipoprotein E gene in macrophages and adipocytes. Proc. Natl. Acad. Sci. U. S. A., 98(2), 507–512.
Landgraf, R. R., Goswami, D., Rajamohan, F., Harris, M. S., Calabrese, M. F., Hoth, L. R., … Griffin, P. R. (2013). Activation of AMP-Activated Protein Kinase Revealed by Hydrogen/Deuterium Exchange Mass Spectrometry. Structure, 21(11), 1942–1953.
Lange, Y., Ye, J., & Chin, J. (1997). The Fate of Cholesterol Exiting Lysosomes*. J. Biol. Chem., 272(27), 17018–17022.
Lange, Y., Ye, J., Rigney, M., & Steck, T. L. (1999). Regulation of endoplasmic reticulum cholesterol by plasma membrane cholesterol. J. Lipid Res., 40(12), 2264–2270.
Laplante, M., & Sabatini, D. M. (2012). mTOR Signaling in Growth Control and Disease. Cell, 149(2), 274–293.
Lashuel, H. A., Petre, B. M., Wall, J., Simon, M., Nowak, R. J., Walz, T., & Lansbury, P. T. (2002). Alpha-synuclein, especially the Parkinson's disease-associated mutants, forms pore-like annular and tubular protofibrils. J. Mol. Biol., 322(5), 1089–1102.
Lawn, R. M., Wade, D. P., Garvin, M. R., Wang, X., Schwartz, K., Porter, J. G., … Oram, J. F. (1999). The Tangier disease gene product ABC1 controls the cellular apolipoprotein-mediated lipid removal pathway. J. Clin. Invest., 104(8), R25–R31.
Lee, F. J. S., Liu, F., Pristupa, Z. B., & Niznik, H. B. (2001). Direct binding and functional coupling of alpha-synuclein to the dopamine transporters accelerate dopamine-induced apoptosis. FASEB J., 15(6), 916–926.
Lee, J. W., Park, S., Takahashi, Y., & Wang, H.-G. (2010). The Association of AMPK with ULK1 Regulates Autophagy. PLoS One, 5(11), e15394.
Lee, M. K., Stirling, W., Xu, Y., Xu, X., Qui, D., Mandir, A. S., … Price, D. L. (2002). Human alpha-synuclein-harboring familial Parkinson's disease-linked Ala-53 --> Thr mutation causes neurodegenerative disease with alpha-synuclein aggregation in transgenic mice. Proc. Natl. Acad. Sci. U. S. A., 99(13), 8968–8973.
Lehmann, J., Kliewer, S. A., Moore, L. B., Smith-Oliver, T. A., Oliver, B. B., Su, J.-L., … Willson, T. M. (1997). Activation of the Nuclear Receptor LXR by Oxysterols Defines a New Hormone Response Pathway*. J. Biol. Chem., 272(6), 3137–3140.
Li, F., & Zhang, H. (2019). Lysosomal Acid Lipase in Lipid Metabolism and Beyond. Arterioscler. Thromb. Vasc. Biol., 39(5), 850–856.
Li, S., Brown, M. S., & Goldstein, J. L. (2010). Bifurcation of insulin signaling pathway in rat liver: mTORC1 required for stimulation of lipogenesis, but not inhibition of gluconeogenesis. Proc. Natl. Acad. Sci. U. S. A., 107(8), 3441–3446.
Li, Y., Inoki, K., & Guan, K.-L. (2004). Biochemical and Functional Characterizations of Small GTPase Rheb and TSC2 GAP Activity. Mol. Cell. Biol., 24(18), 7965–7975.
Li, Y., Xu, S., Mihaylova, M. M., Zheng, B., Hou, X., Jiang, B., … Zang, M. (2011). AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice. Cell Metab., 13(4), 376–388.
Liang, Y., Lin, S., Beyer, T. P., Zhang, Y., Wu, X., Bales, K. R., … Paul, S. M. (2004). A liver X receptor and retinoid X receptor heterodimer mediates apolipoprotein E expression, secretion and cholesterol homeostasis in astrocytes. J. Neurochem., 88(3), 623–634.
Linton, M. F., Gish, R., Hubi, S. T., Bütler, E., Esquivel, C., Bry, W. I., … Young, S. G. (1991). Phenotypes of apolipoprotein B and apolipoprotein E after liver transplantation. J. Clin. Invest., 88(1), 270–281.
Lipton, J. O., & Sahin, M. (2014). The Neurology of mTOR. Neuron, 84(2), 275–291.
Liscum, L., Ruggiero, R. M., & Faust, J. R. (1989). The Intracellular Transport of Low Density Lipoprotein-derived Cholesterol Is Defective in Niemann-Pick Type C Fibroblasts. J. Cell Biol., 108(5), 1625–1636.
Liu, Y., Roghani, A., & Edwards, R. H. (1992). Gene transfer of a reserpine-sensitive mechanism of resistance to N-methyl-4-phenylpyridinium. Proc. Natl. Acad. Sci. U. S. A., 89(19), 9074–9078.
Lund, E. G., Guileyardo, J. M., & Russell, D. W. (1999). cDNA cloning of cholesterol 24-hydroxylase, a mediator of cholesterol homeostasis in the brain. Proc. Natl. Acad. Sci. U. S. A., 96(13), 7238–7243.

Lund, E. G., Xie, C., Kotti, T., Turley, S. D., Dietschy, J. M., & Russell, D. W. (2003). Knockout of the Cholesterol 24-Hydroxylase Gene in Mice Reveals a Brain-specific Mechanism of Cholesterol Turnover*. J. Biol. Chem., 278(25), 22980–22988.
Lütjohann, D., Breuer, O., Ahlborg, G., Nennesmo, I., Sidén, Å., Diczfalusy, U., & Björkhem, I. (1996). Cholesterol homeostasis in human brain: Evidence for an age-dependent flux of 24S-hydroxycholesterol from the brain into the circulation. Proc. Natl. Acad. Sci. U. S. A., 93(18), 9799–9804.
Magalhaes, J., Gegg, M. E., Migdalska-Richards, A., Doherty, M. K., Whitfield, P. D., & Schapira, A. H. V. (2016). Autophagic lysosome reformation dysfunction in glucocerebrosidase deficient cells: relevance to Parkinson disease. Hum. Mol. Genet., 25(16), 3432–3445.
Mahley, R. W. (1988). Apolipoprotein E: Cholesterol Transport Protein with Expanding Role in Cell Biology. Science, 240(4852), 622–630.
Maioli, S., Båvner, A., Ali, Z., Heverin, M., Ismail, M.-A.-M., Puerta, E., … Björkhem, I. (2013). Is It Possible to Improve Memory Function by Upregulation of the Cholesterol 24S-Hydroxylase (CYP46A1) in the Brain? PLoS One, 8(7), e68534.
Maroteaux, L., Campanelli, J. T., & Scheller, R. H. (1988). Synuclein: A Neuron-Specific Protein Localized to the Nucleus and Presynaptic Nerve Terminal. J. Neurosci., 8(8), 2804–2815.
Mast, N., Lin, J. B., Anderson, K. W., Björkhem, I., & Pikuleva, I. A. (2017). Transcriptional and post-translational changes in the brain of mice deficient in cholesterol removal mediated by cytochrome P450 46A1 (CYP46A1). PLoS One, 12(10), e0187168.
Matsuda, A., Nagao, K., Matsuo, M., Kioka, N., & Ueda, K. (2013). 24(S)-hydroxycholesterol is actively eliminated from neuronal cells by ABCA1. J. Neurochem., 126(1), 93–101.
Mauch, D. H., Nägler, K., Schumacher, S., Göritz, C., Müller, E.-C., Otto, A., & Pfrieger, F. W. (2001). CNS Synaptogenesis Promoted by Glia-Derived Cholesterol. Science, 294(5545), 1354–1357.
Maxfield, F. R., & Wüstner, D. (2012). Analysis of cholesterol trafficking with fluorescent probes. Methods Cell Biol., 108, 367–393.
Meaney, S., Bodin, K., Diczfalusy, U., & Björkhem, I. (2002). On the rate of translocation in vitro and kinetics in vivo of the major oxysterols in human circulation: critical importance of the position of the oxygen function. J. Lipid Res., 43(12), 2130–2135.
Meredith, G. E., & Rademacher, D. J. (2011). MPTP mouse models of Parkinson's disease: an update. J. Parkinsons. Dis., 1(1), 19–33.
Milagre, I., Nunes, M. J., Gama, M. J., Silva, R. F., Pascussi, J. M., Lechner, M. C., & Rodrigues, E. (2008). Transcriptional regulation of the human CYP46A1 brain-specific expression by Sp transcription factors. J. Neurochem., 106(2), 835–849.
Momcilovic, M., Hong, S.-P., & Carlson, M. (2006). Mammalian TAK1 Activates Snf1 Protein Kinase in Yeast and Phosphorylates AMP-activated Protein Kinase in Vitro*. J. Biol. Chem., 281(35), 25336–25343.
Mori, F., Tanji, K., Yoshimoto, M., Takahashi, H., & Wakabayashi, K. (2002). Demonstration of alpha-synuclein immunoreactivity in neuronal and glial cytoplasm in normal human brain tissue using proteinase K and formic acid pretreatment. Exp. Neurol., 176(1), 98–104.
Moutinho, M., Nunes, M. J., Gomes, A. Q., Gama, M. J., Cedazo-Minguez, A., Rodrigues, C. M. P., … Rodrigues, E. (2015). Cholesterol 24S-Hydroxylase Overexpression Inhibits the Liver X Receptor (LXR) Pathway by Activating Small Guanosine Triphosphate-Binding Proteins (sGTPases) in Neuronal Cells. Mol. Neurobiol., 51(3), 1489–1503.
Moutinho, M., Nunes, M. J., & Rodrigues, E. (2017). The mevalonate pathway in neurons: It's not just about cholesterol. Exp. Cell Res., 360(1), 55–60.
Moutinho, M., Nunes, M. J., & Rodrigues, E. (2016). Cholesterol 24-hydroxylase: Brain cholesterol metabolism and beyond. Biochim. Biophys. Acta, 1861(12 PtA), 1911–1920.
Murphy, D. D., Rueter, S. M., Trojanowski, J. Q., & Lee, V. M. (2000). Synucleins are developmentally expressed, and alpha-synuclein regulates the size of the presynaptic vesicular pool in primary hippocampal neurons. J. Neurosci., 20(9), 3214–3220.
Musanti, R., Parati, E., Lamperti, E., & Ghiselli, G. (1993). Decreased cholesterol biosynthesis in fibroblasts from patients with Parkinson disease. Biochem. Med. Metab. Biol., 49(2), 133–142.
Mytilineou, C., Werner, P., Molinari, S., Di Rocco, A., Cohen, G., & Yahr, M. D. (1994). Impaired oxidative decarboxylation of pyruvate in fibroblasts from patients with Parkinson's disease. J. Neural Transm., 8(3), 223–228.
Nakanishi, M., Goldstein, J. L., & Brown, M. S. (1988). Multivalent Control of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase. J. Biol. Chem., 263(18), 8929–8937.
Ng, E. L., & Tang, B. L. (2008). Rab GTPases and their roles in brain neurons and glia. Brain Res. Rev., 58(1), 236–246.
Nicklas, W. J., Vyas, I., & Heikkila, R. E. (1985). Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenyl-pyridine, a metabolite of the neurotoxin, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. Life Sci., 36(26), 2503–2508.
Nieweg, K., Schaller, H., & Pfrieger, F. W. (2009). Marked differences in cholesterol synthesis between neurons and glial cells from postnatal rats. J. Neurochem., 109(1), 125–134.
Noguchi, N., Saito, Y., & Urano, Y. (2014). Diverse functions of 24(S)-hydroxycholesterol in the brain. Biochem. Biophys. Res. Commun., 446(3), 692–696.
Nohturfft, A., Brown, M. S., & Goldstein, J. L. (1998). Topology of SREBP Cleavage-activating Protein, a Polytopic Membrane Protein with a Sterol-sensing Domain*. J. Biol. Chem., 273(27), 17243–17250.

Nohturfft, A., DeBose-Boyd, R. A., Scheek, S., Goldstein, J. L., & Brown, M. S. (1999). Sterols regulate cycling of SREBP cleavage-activating protein (SCAP) between endoplasmic reticulum and Golgi. Proc. Natl. Acad. Sci. U. S. A., 96(20), 11235–11240.
Nunes, M. J., Milagre, I., Schnekenburger, M., Gama, M. J., Diederich, M., & Rodrigues, E. (2010). Sp proteins play a critical role in histone deacetylase inhibitor-mediated derepression of CYP46A1 gene transcription. J. Neurochem., 113(2), 418–431.
Nunes, M. J., Moutinho, M., Gama, M. J., Rodrigues, C. M. P., & Rodrigues, E. (2013). Histone deacetylase inhibition decreases cholesterol levels in neuronal cells by modulating key genes in cholesterol synthesis, uptake and efflux. PLoS, 8(1), e53394.
Ohyama, Y., Meaney, S., Heverin, M., Ekström, L., Brafman, A., Shafir, M., … Björkhem, I. (2006). Studies on the transcriptional regulation of cholesterol 24-hydroxylase (CYP46A1): marked insensitivity toward different regulatory axes. J. Biol. Chem., 281(7), 3810–3820.
Okabe, A., Urano, Y., Itoh, S., Suda, N., Kotani, R., Nishimura, Y., … Noguchi, N. (2013). Adaptive responses induced by 24S-hydroxycholesterol through liver X receptor pathway reduce 7-ketocholesterol-caused neuronal cell death. Redox Biol., 2, 28–35.
O'Sullivan, S. S., Williams, D. R., Gallagher, D. A., Massey, L. A., Silveira-Moriyama, L., & Lees, A. J. (2008). Nonmotor Symptoms as Presenting Complaints in Parkinson's Disease: A Clinicopathological Study. Mov. Disord., 23(1), 101–106.
Owen, J. L., Zhang, Y., Bae, S.-H., Farooqi, M. S., Liang, G., Hammer, R. E., … Brown, M. S. (2012). Insulin stimulation of SREBP-1c processing in transgenic rat hepatocytes requires p70 S6-kinase. Proc. Natl. Acad. Sci. U. S. A., 109(40), 16184–16189.
Pai, J., Guryev, O., Brown, M. S., & Goldstein, J. L. (1998). Differential Stimulation of Cholesterol and Unsaturated Fatty Acid Biosynthesis in Cells Expressing Individual Nuclear Sterol Regulatory Element-binding Proteins*. J. Biol. Chem., 273(40), 26138–26148.
Paisán-Ruiz, C., Jain, S., Evans, E. W., Gilks, W. P., Simón, J., van der Brug, M., … Singleton, A. B. (2004). Cloning of the Gene Containing Mutations that Cause PARK8-Linked Parkinson's Disease. Neuron, 44(4), 595–600.
Paul, R., Choudhury, A., Kumar, S., Giri, A., Sandhir, R., & Borah, A. (2017). Cholesterol contributes to dopamine-neuronal loss in MPTP mouse model of Parkinson's disease: Involvement of mitochondrial dysfunctions and oxidative stress. PLoS One, 12(2), e0171285.
Pentchev, P. G., Comly, M. E., Kruth, H. S., Tokoro, T., Butler, J., Sokol, J., … Brady, R. O. (1987). Group C Niemann-Pick disease: faulty regulation of low-density lipoprotein uptake and cholesterol storage in cultured fibroblasts. FASEB J., 1(1), 40–45.
Peterson, T. R., Sengupta, S. S., Harris, T. E., Carmack, A. E., Kang, S. A., Balderas, E., … Sabatini, D. M. (2011). mTOR Complex 1 Regulates Lipin 1 Localization to Control the SREBP Pathway. Cell, 146(3), 408–420.
Pfrieger, F. W. (2003). Cholesterol homeostasis and function in neurons of the central nervous system. Cell. Mol. Life Sci., 60(6), 1158–1171.
Pfrieger, F. W., & Ungerer, N. (2011). Cholesterol metabolism in neurons and astrocytes. Prog. Lipid Res., 50(4), 357–371.
Pitas, R. E., Boyles, J. K., Lee, S. H., Foss, D., & Mahley, R. W. (1987). Astrocytes synthesize apolipoprotein E and metabolize apolipoprotein E-containing lipoproteins. Biochim. Biophys. Acta, 917(1), 148–161.
Poels, J., Spasic, M. S., Callaerts, P., & Norga, K. K. (2009). Expanding roles for AMP-activated protein kinase in neuronal survival and autophagy. BioEssays, 31(9), 944–952.
Poewe, W., Seppi, K., Tanner, C. M., Halliday, G. M., Brundin, P., Volkmann, J., … Lang, A. E. (2017). Parkinson disease. Nat. Rev. Dis. Prim., 3, 17013.
Poirier, J. (1994). Apolipoprotein E in animal models of CNS injury and in Alzheimer's disease. Trends Neurosci., 17(12), 525–530.
Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., … Nussbaum, R. L. (1997). Mutation in the α-Synuclein Gene Identified in Families with Parkinson's Disease. Science, 276(5321), 2045–2047.
Przedborski, S., Jackson-Lewis, V., Naini, A. B., Jakowec, M., Petzinger, G., Miller, R., & Akram, M. (2001). The parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): a technical review of its utility and safety. J. Neurochem., 76(5), 1265–1274.
Quan, G., Xie, C., Dietschy, J. M., & Turley, S. D. (2003). Ontogenesis and regulation of cholesterol metabolism in the central nervous system of the mouse. Dev. Brain Res., 146(1–2), 87–98.
Radhakrishnan, A., Goldstein, J. L., Mcdonald, J. G., & Brown, M. S. (2008). Switch-like Control of SREBP-2 Transport Triggered by Small Changes in ER Cholesterol: A Delicate Balance. Cell Metab., 8(6), 512–521.
Radhakrishnan, A., Ikeda, Y., Kwon, H. J., Brown, M. S., & Goldstein, J. L. (2007). Sterol-regulated transport of SREBPs from endoplasmic reticulum to Golgi: Oxysterols block transport by binding to Insig. Proc. Natl. Acad. Sci. U. S. A., 104(16), 6511–6518.
Raju, A., Jaisankar, P., Borah, A., & Mohanakumar, K. P. (2017). 1-Methyl-4-Phenylpyridinium-Induced Death of Differentiated SH-SY5Y Neurons Is Potentiated by Cholesterol. Ann. Neurol., 24(4), 243–251.
Ramirez, D. M. O., Andersson, S., & Russell, D. W. (2008). Neuronal Expression and Subcellular Localization of Cholesterol 24- Hydroxylase in the Mouse Brain. J. Comp. Neurol., 507(5), 1676–1693.

Repa, J. J., Liang, G., Ou, J., Bashmakov, Y., Lobaccaro, J.-M. A., Shimomura, I., … Mangelsdorf, D. J. (2000). Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. Genes Dev., 14(22), 2819–2830.

Robak, L. A., Jansen, I. E., van Rooij, J., Uitterlinden, A. G., Kraaij, R., Jankovic, J., … Shulman, J. M. (2017). Excessive burden of lysosomal storage disorder gene variants in Parkinson's disease. Brain, 140(12), 3191–3203.

Robenek, H., Hofnagel, O., Buers, I., Robenek, M. J., Troyer, D., & Severs, N. J. (2006). Adipophilin-enriched domains in the ER membrane are sites of lipid droplet biogenesis. J. Cell Sci., 119(Pt 20), 4215–4224.

Rochet, J.-C., Conway, K. A., & Lansbury, P. T. (2000). Inhibition of fibrillization and accumulation of prefibrillar oligomers in mixtures of human and mouse alpha-synuclein. Biochemistry, 39(35), 10619–10626.

Roczniak-Ferguson, A., Petit, C. S., Froehlich, F., Qian, S., Ky, J., Angarola, B., … Ferguson, S. M. (2012). The Transcription Factor TFEB Links mTORC1 Signaling to Transcriptional Control of Lysosome Homeostasis. Sci. Signal., 5(228), ra42.

Rosa, A., Duarte-Silva, S., Silva-Fernandes, A., Nunes, M., Carvalho, A., Rodrigues, E., … Castro-Caldas, M. (2018). Tauroursodeoxycholic Acid Improves Motor Symptoms in a Mouse Model of Parkinson's Disease. Mol. Neurobiol., 55(12), 9139–9155.

Ross, F. A., MacKintosh, C., & Hardie, D. G. (2016). AMP-activated protein kinase: a cellular energy sensor that comes in 12 flavours. FEBS J., 283(16), 2987–3001.

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. Nat. Cell Biol., 15(7), 741–750.

Saher, G., Brügger, B., Lappe-Siefke, C., Möbius, W., Tozawa, R., Wehr, M. C., … Nave, K.-A. (2005). High cholesterol level is essential for myelin membrane growth. Nat. Neurosci., 8(4), 468–475.

Sakai, J., Duncan, E. A., Rawson, R. B., Hua, X., Brown, M. S., & Goldstein, J. L. (1996). Sterol-Regulated Release of SREBP-2 from Cell Membranes Requires Two Sequential Cleavages, One Within a Transmembrane Segment. Cell, 85(7), 1037–1046.

Sakai, J., Nohturfft, A., Cheng, D., Ho, Y. K., Brown, M. S., & Goldstein, J. L. (1997). Identification of Complexes between the COOH-terminal Domains of Sterol Regulatory Element-binding Proteins (SREBPs) and SREBP Cleavage-Activating Protein*. J. Biol. Chem., 272(32), 20213–20221.

Sakamoto, K., Göransson, O., Hardie, D. G., & Alessi, D. R. (2004). Activity of LKB1 and AMPK-related kinases in skeletal muscle: effects of contraction, phenformin, and AICAR. Am. J. Physiol. Endocrinol. Metab., 287(2), E310–E317.

Sakamoto, S., Miyara, M., Sanoh, S., Ohta, S., & Kotake, Y. (2017). Mild MPP+ exposure-induced glucose starvation enhances autophagosome synthesis and impairs its degradation. Sci. Rep., 7, 46668.

Sakashita, N., Miyazaki, A., Takeya, M., Horiuchi, S., Chang, C. C. Y., Chang, T.-Y., & Takahashi, K. (2000). Localization of Human Acyl-Coenzyme A:Cholesterol Acyltransferase-1 (ACAT-1) in Macrophages and in Various Tissues. Am. J. Pathol., 156(1), 227–236.

Sancak, Y., Thoreen, C. C., Peterson, T. R., Lindquist, R. A., Kang, S. A., Spooner, E., … Sabatini, D. M. (2007). PRAS40 Is an Insulin-Regulated Inhibitor of the mTORC1 Protein Kinase. Mol. Cell, 25(6), 903–915.

Sanchez, H. B., Yieh, L., & Osborne, T. F. (1995). Cooperation by Sterol Regulatory Element-binding Protein and Sp1 in Sterol Regulation of Low-Density Lipoprotein Receptor Gene*. J. Biol. Chem., 270(3), 1161–1169.

Sanders, M. J., Grondin, P. O., Hegarty, B. D., Snowden, M. A., & Carling, D. (2007). Investigating the mechanism for AMP activation of the AMP-activated protein kinase cascade. Biochem. J., 403(1), 139–148.

Saporito, M. S., Thomas, B. A., & Scott, R. W. (2000). MPTP activates c-Jun NH(2)-terminal kinase (JNK) and its upstream regulatory kinase MKK4 in nigrostriatal neurons in vivo. J. Neurochem., 75(3), 1200–1208.

Sarbassov, D. D., Ali, S. M., Kim, D.-H., Guertin, D. A., Latek, R. R., Erdjument-Bromage, H., … Sabatini, D. M. (2004). Rictor, a Novel Binding Partner of mTOR, Defines a Rapamycin-Insensitive and Raptor-Independent Pathway that Regulates the Cytoskeleton. Curr. Biol., 14(14), 1296–1302.

Sardiello, M., Palmieri, M., di Ronza, A., Medina, D. L., Valenza, M., Gennarino, V. A., … Ballabio, A. (2009). A Gene Network Regulating Lysosomal Biogenesis and Function. Science, 325(5939), 473–477.

Sato, R., Inoue, J., Kawabe, Y., Kodama, T., Takano, T., & Maeda, M. (1996). Sterol-dependent Transcriptional Regulation of Sterol Regulatory Element-binding Protein-2*. J. Biol. Chem., 271(43), 26461–26464.

Schapira, A. H. V, Cooper, J. M., Dexter, D., Clark, J. B., Jenner, P., & Marsden, C. D. (1990). Mitochondrial Complex I Deficiency in Parkinson's Disease. J. Neurochem., 54(3), 823–827.

Schlachetzki, J. C. M., Saliba, S. W., & Oliveira, A. C. P. de. (2013). Studying neurodegenerative diseases in culture models. Rev. Bras. Psiquiatr., 35, S92–S100.

Schmidt, N., & Ferger, B. (2001). Neurochemical findings in the MPTP model of Parkinson's disease. J. Neural Transm., 108(11), 1263–1282.

Schultz, J. R., Tu, H., Luk, A., Repa, J. J., Medina, J. C., Li, L., … Shan, B. (2000). Role of LXRs in control of lipogenesis. Genes Dev., 14(22), 2831–2838.

Seo, Y. K., Jeon, T.-I., Chong, H. K., Beisinger, J., Xie, X., & Osborne, T. F. (2011). Genome-wide localization of SREBP-2 in hepatic chromatin predicts a role in autophagy. Cell Metab., 13(4), 367–375.

Settembre, C., Malta, C. Di, Polito, V. A., Arencibia, M. G., Vetrini, F., Erdin, S., … Ballabio, A. (2011). TFEB Links Autophagy to Lysosomal Biogenesis. Science, 332(6036), 1429–1433.

Settembre, C., Zoncu, R., Medina, D. L., Vetrini, F., Erdin, S., Erdin, S. U., … Ballabio, A. (2012). A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB. EMBO J., 31(5), 1095–1108.

Sever, N., Lee, P. C. W., Song, B.-L., Rawson, R. B., & DeBose-Boyd, R. A. (2004). Isolation of Mutant Cells Lacking Insig-1 through Selection with SR-12813, an Agent That Stimulates Degradation of 3-Hydroxy-3-methylglutaryl-Coenzyme A Reductase*. J. Biol. Chem., 279(41), 43136–43147.
Shafaati, M., Olin, M., Båvner, A., Pettersson, H., Rozell, B., Meaney, S., … Björkhem, I. (2011). Enhanced production of 24S-hydroxycholesterol is not sufficient to drive liver X receptor target genes in vivo. J. Intern. Med., 270(4), 377–387.
Shaw, R. J., Kosmatka, M., Bardeesy, N., Hurley, R. L., Witters, L. A., DePinho, R. A., & Cantley, L. C. (2004). The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. Proc. Natl. Acad. Sci. U. S. A., 101(10), 3329–3335.
Shimano, H. (2000). Sterol Regulatory Element-binding Protein-1 as a Dominant Transcription Factor for Gene Regulation of Lipogenic Enzymes in the Liver. Trends Cardiovasc. Med., 10(7), 275–278.
Shimano, H. (2001). Sterol regulatory element-binding proteins (SREBPs): transcriptional regulators of lipid synthetic genes. Prog. Lipid Res., 40(6), 439–452.
Shobab, L. A., Hsiung, G.-Y. R., & Feldman, H. H. (2005). Cholesterol in Alzheimer' s disease. Lancet Neurol., 4(12), 841–852.
Sidransky, E., Nalls, M. A., Aasly, J. O., Aharon-Peretz, J., Annesi, G., Barbosa, E. R., … Ziegler, S. G. (2009). Multicenter analysis of glucocerebrosidase mutations in Parkinson's disease. N. Engl. J. Med., 361(17), 1651–1661.
Sleat, D. E., Wiseman, J. A., El-Banna, M., Price, S. M., Verot, L., Shen, M. M., … Lobel, P. (2004). Genetic evidence for nonredundant functional cooperativity between NPC1 and NPC2 in lipid transport. Proc. Natl. Acad. Sci. U. S. A., 101(16), 5886–5891.
Sobo, K., Blanc, I. Le, Luyet, P.-P., Fivaz, M., Ferguson, C., Parton, R. G., … van der Goot, F. G. (2007). Late Endosomal Cholesterol Accumulation Leads to Impaired Intra-Endosomal Trafficking. PLoS One, 2(9), e851.
Sodero, A. O., Vriens, J., Ghosh, D., Stegner, D., Brachet, A., Pallotto, M., … Dotti, C. G. (2012). Cholesterol loss during glutamate-mediated excitotoxicity. EMBO J., 31(7), 1764–1773.
Song, B.-L., Javitt, N. B., & DeBose-Boyd, R. A. (2005). Insig-mediated degradation of HMG CoA reductase stimulated by lanosterol, an intermediate in the synthesis of cholesterol. Cell Metab., 1(3), 179–189.
Sonsalla, P. K., & Heikkila, R. E. (1986). The influence of dose and dosing interval on MPTP-induced dopaminergic neurotoxicity in mice. Eur. J. Pharmacol., 129(3), 339–345.
Spillantini, M.-G., Crowther, R. A., Jakes, R., Hasegawa, M., & Goedert, M. (1998). alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. Proc. Natl. Acad. Sci. U. S. A., 95(11), 6469–6473.
Stoessl, A. J., Lehericy, S., & Strafella, A. P. (2014). Imaging insights into basal ganglia function, Parkinson's disease, and dystonia. Lancet, 384(9942), 532–544.
Sugii, S., Reid, P. C., Ohgami, N., Du, H., & Chang, T.-Y. (2003). Distinct Endosomal Compartments in Early Trafficking of Low Density Lipoprotein-derived Cholesterol*. J. Biol. Chem., 278(29), 27180–27189.
Sun, L.-P., Li, L., Goldstein, J. L., & Brown, M. S. (2005). Insig Required for Sterol-mediated Inhibition of Scap/SREBP Binding to COPII Proteins in Vitro*. J. Biol. Chem., 280(28), 26483–26490.
Tabas, I. (2002). Consequences of cellular cholesterol accumulation: basic concepts and physiological implications. J. Clin. Invest., 110(7), 905–911.
Tabernero, A., Bolaños, J. P., & Medina, J. M. (1993). Lipogenesis from lactate in rat neurons and astrocytes in primary culture. Biochem. J., 294, 635–638.
Takahashi, Y., Nada, S., Mori, S., Soma-Nagae, T., Oneyama, C., & Okada, M. (2012). The late endosome/lysosome-anchored p18-mTORC1 pathway controls terminal maturation of lysosomes. Biochem. Biophys. Res. Commun., 417(4), 1151–1157.
Tatton, N. A., & Kish, S. J. (1997). In situ detection of apoptotic nuclei in the substantia nigra compacta of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice using terminal deoxynucleotidyl transferase labelling and acridine orange staining. Neuroscience, 77(4), 1037–1048.
Tauchi-Sato, K., Ozeki, S., Houjou, T., Taguchi, R., & Fujimoto, T. (2002). The Surface of Lipid Droplets Is a Phospholipid Monolayer with a Unique Fatty Acid Composition*. J. Biol. Chem., 277(46), 44507–44512.
Threadgill, R., Bobb, K., & Ghosh, A. (1997). Regulation of Dendritic Growth and Remodeling by Rho, Rac, and Cdc42. Neuron, 19(3), 625–634.
Tian, J., Goldstein, J. L., & Brown, M. S. (2016). Insulin induction of SREBP-1c in rodent liver requires LXRα-C/EBPβ complex. Proc. Natl. Acad. Sci. U. S. A., 113(29), 8182–8187.
Valente, E. M., Abou-Sleiman, P. M., Caputo, V., Muqit, M. M., Harvey, K., Gispert, S., … Wood, N. W. (2004). Hereditary Early-Onset Parkinson's Disease Caused by Mutations in PINK1. Science, 304(5674), 1158–1160.
Vaughan, A. M., & Oram, J. F. (2006). ABCA1 and ABCG1 or ABCG4 act sequentially to remove cellular cholesterol and generate cholesterol-rich HDL. J. Lipid Res., 47(11), 2433–2443.
Wahrle, S. E., Jiang, H., Parsadanian, M., Legleiter, J., Han, X., Fryer, J. D., … Holtzman, D. M. (2004). ABCA1 Is Required for Normal Central Nervous System ApoE Levels and for Lipidation of Astrocyte-secreted apoE*. J. Biol. Chem., 279(39), 40987–40993.
Wang, C., Kang, X., Zhou, L., Chai, Z., Wu, Q., Huang, R., … Yue, Z. (2018). Synaptotagmin-11 is a critical mediator of parkin-linked neurotoxicity and Parkinson's disease-like pathology. Nat. Commun., 9(1), 81.

Wang, L., Schuster, G. U., Hultenby, K., Zhang, Q., Andersson, S., & Gustafsson, J.-Å. (2002). Liver X receptors in the central nervous system: From lipid homeostasis to neuronal degeneration. Proc. Natl. Acad. Sci. U. S. A., 99(21), 13878–13883.
Waterham, H. R. (2006). Defects of cholesterol biosynthesis. Fed. Eur. Biochem. Soc., 580(23), 5442–5449.
Weber, L. W., Boll, M., & Stampfl, A. (2004). Maintaining cholesterol homeostasis: Sterol regulatory element-binding proteins. World J. Gastroenterol., 10(21), 3081–3087.
Wheeler, S., & Sillence, D. J. (2019). Niemann-Pick type C disease: cellular pathology and pharmacotherapy. J. Neurochem.
Whitney, K. D., Watson, M. A., Collins, J. L., Benson, W. G., Stone, T. M., Numerick, M. J., … Kliewer, S. A. (2002). Regulation of Cholesterol Homeostasis by the Liver X Receptors in the Central Nervous System. Mol. Endocrinol., 16(6), 1378–1385.
Wojtanik, K. M., & Liscum, L. (2003). The Transport of Low Density Lipoprotein-derived Cholesterol to the Plasma Membrane Is Defective in NPC1 Cells*. J. Biol. Chem., 278(17), 14850–14856.
Wolf, B., Lopes, M. B. S., VandenBerg, S. R., & Gonias, S. L. (1992). Characterization and Immunohistochemical Localization of α2-Macroglobulin Receptor (Low-density Lipoprotein Receptor-related Protein) in Human Brain. Am. J. Pathol., 141(1), 37–42.
Wong, E., & Cuervo, A. M. (2010). Autophagy gone awry in neurodegenerative diseases. Nat. Publ. Gr., 13(7), 805–811.
Woods, A., Cheung, P. C. F., Smith, F. C., Davison, M. D., Scott, J., Beri, R. K., & Carling, D. (1996). Characterization of AMP-activated protein kinase beta and gamma subunits. Assembly of the heterotrimeric complex in vitro*. J. Biol. Chem., 271(17), 10282–10290.
Woods, A., Dickerson, K., Heath, R., Hong, S.-P., Momcilovic, M., Johnstone, S. R., … Carling, D. (2005). Ca 2+/calmodulin-dependent protein kinase kinase-β acts upstream of AMP-activated protein kinase in mammalian cells. Cell Metab., 2(1), 21–33.
Woods, A., Johnstone, S. R., Dickerson, K., Leiper, F. C., Fryer, L. G. D., Neumann, D., … Carling, D. (2003). LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. Curr. Biol., 13(22), 2004–2008.
Wüstner, D., Mondal, M., Tabas, I., & Maxfield, F. R. (2005). Direct Observation of Rapid Internalization and Intracellular Transport of Sterol by Macrophage Foam Cells. Traffic, 6(5), 396–412.
Xiao, B., Heath, R., Saiu, P., Leiper, F. C., Leone, P., Jing, C., … Gamblin, S. J. (2007). Structural basis for AMP binding to mammalian AMP-activated protein kinase. Nature, 449(7161), 496–500.
Xu, S., Benoff, B., Liou, H.-L., Lobel, P., & Stock, A. M. (2007). Structural Basis of Sterol Binding by NPC2, a Lysosomal Protein Deficient in Niemann-Pick Type C2 Disease*. J. Biol. Chem., 282(32), 23525–23531.
Yang, T., Espenshade, P. J., Wright, M. E., Yabe, D., Gong, Y., Aebersold, R., … Brown, M. S. (2002). Crucial Step in Cholesterol Homeostasis: Sterols Promote Binding of SCAP to INSIG-1, a Membrane Protein that Facilitates Retention of SREBPs in ER. Cell, 110(4), 489–500.
Zelcer, N., Hong, C., Boyadjian, R., & Tontonoz, P. (2009). LXR Regulates Cholesterol Uptake Through Idol-Dependent Ubiquitination of the LDL Receptor. Science, 325(5936), 100–104.
Zhang, F. L., & Casey, P. J. (1996). Protein Prenylation: Molecular Mechanisms and Functional Consequences. Annu. Rev. Biochem., 65, 241–269.
Zhang, J., & Liu, Q. (2015). Cholesterol metabolism and homeostasis in the brain. Protein Cell, 6(4), 254–264.
Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., … Moller, D. E. (2001). Role of AMP-activated protein kinase in mechanism of metformin action. J. Clin. Invest., 108(8), 1167–1174.
Zhou, Q., Liu, C., Liu, W., Zhang, H., Zhang, R., Liu, J., … Chen, L. (2015). Rotenone induction of hydrogen peroxide inhibits mTOR-mediated S6K1 and 4E-BP1/eIF4E pathways, leading to neuronal apoptosis. Toxilogical Sci., 143(1), 81–96.