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Characterisation of the ghrelinergic system in human models of neurodegenerative diseases

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Summary

Calorie restriction (CR) has well established neuroprotective properties across species and is known to prevent cognitive deficits in several mouse models and in aged humans. Our group and others have shown that CR beneficial effects are dependent on the circulating hormone acyl-ghrelin (AG), that promotes hippocampal memory function and protects against neurodegeneration in rodents. Indeed, ghrelin knockout (KO) mice show defects in memory task performance and plasma ghrelin levels are significantly reduced in aged humans. Because of its connection to memory and ageing, AG has been studied in mouse models of Alzheimer's disease (AD) and Parkinson's disease (PD), the two most common causes of dementia in the aged population. However, very few of these studies have been performed in human models.

This project aimed to determine the expression of key regulatory proteins of the ghrelin pathway in the young and aged healthy human brain, as well as brain of AD and PD patients, combining immunohistochemistry, RNA in situ hybridization, and molecular biology. My findings show that the key enzymes of the ghrelinergic axis – GHS-R1a, GOAT and APT1 – are highly expressed in the aged healthy human hippocampus, but their expression level is impaired in PD with dementia . Further studies are needed to determine a potential correlation between the activity of ghrelin in the brain and the level of cognitive impairment and dementia. On the contrary, very little difference is observed between early and late stage of AD, suggesting that the ghrelinergic system may not be impaired in these patients.

The second aim was to characterize the effects of AG on a human neuronal cell line treated with rotenone or amyloid-β oligomers (AβOs) to simulate oxidative stress from PD and AD, respectively. Using confocal microscopy and molecular biology techniques, I assessed neuronal cell death and mitochondrial function and reported significant mitochondrial toxicity in these models, that worsen by chronic pre-treatment with the inactive form of AG, unacylated ghrelin (UAG).

Ultimately, this project supports the ghrelinergic axis as a potential target for future studies aiming to identify treatments to limit the progression of dementia in humans.

Declaration

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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Statement 1

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Surely my first thanks will go to Jeff and Alwena for their outstanding support and guidance. Their kindness, positivity and infinite patience has made me a better person and a stronger scientist. I will be forever grateful to have found you in my path.

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"Above all, don't fear difficult moments. The best comes from them."

(Rita Levi Montalcini)

Abbreviations

6-OHDA	6-hydroxyl-dopamine
α7nChR	α7 nicotinic acetylcholine receptor
Aβ	Amyloid-β
AβOs	Aβ oligomers
AC	Adenylate cyclase
ACC	Acetyl-CoA carboxylase
Ach	Acetylcholine
Acsl1	Acyl-CoA synthetase family member 1
ACTH	Adreno-cortico-trophic hormone
AD	Alzheimer's disease
ADM	ReN cells differentiation media
AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride
AG	Acylated ghrelin
AG:UAG	Ratio between acylated and unacylated ghrelin
AGRP	Agouti-related peptide
AHN	Adult hippocampal neurogenesis
AICD	APP intracellular domain
Akt	Protein kinase B (PKB)
ALP	Macro-autophagy or autophagy-lysosome pathway
ALS	Amyotrophic lateral sclerosis
AMP	Adenosine 3',5'-monophosphate
AMPK	5' AMP-activated protein kinase
ANT	Adenine nucleotide translocases
Apaf1	Apoptotic protease activating factor 1
APM	ReN cells proliferation media
APP	Amyloid precursor protein
APT1/2	Acyl-protein thioesterase ½
ARC	Hypothalamic arcuate nucleus
AREs	Antioxidant response elements
ATP	Adenosine triphosphate
β2M	β2-microglobulin

BACE1	b-site APP-cleaving enzyme 1
BAK	BCL2-homologous antagonist/killer
BAX	BCL-associated X protein
BBB	Blood-brain-barrier
BChE	Butyl-cholinesterase
BCL2	B cell lymphoma 2 family
BCSFB	Blood-cerebrospinal fluid barrier
BDR	Brains for Dementia Research
BLAST	Basic local alignment search tool (NCBI)
BMCP1	Brain mitochondrial carrier protein 1
CA	Cornu Ammonis
CALERIES	Comprehensive Assessment of the Long-term Effects of Reducing Intake of Energy
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase type II
cAMP	3',5'-cyclic adenosine 5'-monophosphate
CCCP	Uncoupler carbonyl cyanide m-chlorophenyl hydrazone
CDK1	Cyclin-dependent kinases 1
CNS	Central Nervous System
Co-IP	Co-immunoprecipitation assay
CoQ	Ubiquinone
CP	Choroid plexus
CR	Calorie restriction
CRON	Calorie Restriction with Optimal Nutrition
CSF	Cerebrospinal fluid
ctr	control
CVO	Circumventricular organs
CytC	Cytochrome c
DA	Dopamine
DAB	3,3'-diaminobenzidine
DAG	Diacylglycerol
DBH	Dopamine-β-hydroxylase
DG	Dentate gyrus of the hippocampus
DIV	Days *in vitro*
DJ-1	Deglycase 1

DMSO	Dimethyl-sulfoxide
DRP1	Dynamin-related protein 1
EC50	Receptor's signaling capacity
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
ERK1/2	Pro-autophagic extracellular regulated protein kinases 1/2
ETC	Mitochondrial electron transport chain
ETV5	ERM transcription factor variant 5
FAs	Fatty acids
FASN	FA synthase
bFGF/FGF2	Fibroblast growth factor 2
FFPE	Formalin-fixed paraffine-embedded
fMRI	Functional Magnetic Resonance Imaging
FOXO	Forkhead box proteins
GABA	Gamma-aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBA1	β-glucocerebrosidase
GCL	Granule cell layer of the DG
GDP	Guanosine diphosphate
GFAP	Glial fibrillary acidic protein
GH	Growth Hormone
GHRL	Ghrelin gene
GHRP-6	GH releasing peptide-6
GHS-R	Growth hormone secretagogue receptor
GOAT	Ghrelin O-acyl transferase
GPCR	G-protein coupled receptor
GSK3β	Glycogen synthase kinase 3β
GTP	Guanosine triphosphate
HAT1	histone acetyltransferase 1
HDL	High density lipoproteins
HO-1	heme oxygenase 1
HFIP	Hexafluoroisopropanol
HHAT	Hedgehog acyltransferase

IFN-I	Pro-inflammatory cytokine interferon 1
IHC	Immunohistochemistry
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
IP_3	Inositol-triphosphate
KMCP1	Kidney mitochondrial carrier protein 1
KO	Knock-out
LARS2	Leucyl-tRNA synthetase
LC3	Light chain 3
LCFA	Long-chain fatty acids
LDL	Low density lipoproteins
L-DOPA	3,4-dihydroxy-L-phenylalanine
LEAP2	Liver-expressed antimicrobial peptide 2
LRRK2	Leucine-rich repeat kinase 2
LTP	Long-term potentiation
LYPLA1/2	Lysophospholipase I/II (gene name for APT1/2)
MAP2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein kinase
MATP	Mutation in the Tau gene
MBOAT4	Membrane-bound O-acyltransferase 4
MC3	Melanocortin3 receptor
MCFA	Medium-length chains fatty acids
MDCs	Mitochondrial-derived compartments
MDVs	Mitochondria-derived vesicles
ME	Median eminence
Mfn1/2	Mitofusin 1 and 2
MMP	Mitochondrial membrane potential
MoCA	Montreal Cognitive Assessment
MPP	Mitochondrial processing peptidase
mPTP	Mitochondrial permeability transition pores
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mtDNA	Mitochondrial DNA
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin

NA	Noradrenalin
Nestin	Neuroepithelial stem cell protein
NeuN	Neuronal nuclei
NF-kβ	Nuclear factor k-light-chain-enhancer of activated B cells
NFTs	Neurofibrillary tangles
NMDA	N-methyl-D-aspartate
NMS	Non-motor symptoms
NPY	Neuropeptide Y
NRF1/2	Nuclear factor erythroid 2-related factors 1/2
OCR	Oxygen consumption rate
OMM	Outer mitochondrial membrane
OPA1	OPA1 Mitochondrial Dynamin Like GTPase
OTR	Oxytocin receptor
OXPHOS	Oxidative phosphorylation
PBS	Phosphate-buffered saline
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PAI-1	Plasminogen activator inhibitor type 1
PARK2	Parkin gene
Parkin	Cytoplasmic E3 ubiquitin ligase
PARL	Presenilin-associated rhomboid-like
PC1/3	Prohormone convertase 1 and 3
PD	Parkinson's disease
PDD	Parkinson disease with dementia
PGC1α	Proliferator-activated receptor coactivator 1α
PI3K	Phosphoinositide 3-OH kinase
PINK1	Induced kinase 1
PKA	Protein kinase A
PLC	Second messenger phospholipase C
pmi	*Post-mortem* interval
Porc	Porcupine
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PPIB	Peptidylpropyl isomerase B
PSEN1/2	Presenilin 1/2
p-Tau	Hyper-phosphorylated tau protein

PTEN	Phosphatase and tensin homolog
PVN	Paraventricular nucleus
Raptor	Regulator-associated protein of the mTOR
RNA-ISH	RNA in situ hybridisation
RNA-seq	RNA sequencing
ROS	Reactive oxygen species
RT	Room temperature
RT-qPCR	Quantitative reverse transcriptase polymerase chain reaction
SCFA	Short-length chains fatty acids
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SIRT1	Sirtuin1
SH-SY5Y	Human neuroblastoma cell line
SN	Substantia nigra
SN4741	Mouse dopaminergic cell line
SNCA	α-synuclein gene
SOD	Superoxide dismutases
SUMO	Small ubiquitin-like modifiers
TFAM	Mitochondrial transcriptional factor A
TH	Tyrosine hydroxylase
TIM23	Translocase of the internal membrane
TNF-α	Tumour necrosis factor α
TOM	Translocase of the outer membrane
UAG	Unacylated ghrelin
UCP	Uncoupling proteins
ULK1	Unc-51 like autophagy-activating kinase 1
UPR	Unfolded protein stress response
UPS	Ubiquitin-proteasome system
VGluT2	Vesicular glutamate transporter 2
VM	Ventral mesencephalon
VTA	Ventrotegmental area
WB	Western Blot

Measurement units

bp	base pair
^{o}C	degrees Celsius
Da	Dalton
l	litres
M	molar
mol	moles

Metric prefixes

k	kilo; 10^{3}
c	centi; 10^{-2}
m	milli; 10^{-3}
µ	micro; 10^{-6}
n	nano; 10^{-9}
p	pico; 10^{-12}
f	fempto; 10^{-15}

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Chapter 1

Introduction

1.1 Calorie Restriction

1.1.1 CR effects on health and lifespan

The term 'Calorie Restriction' (CR) refers to a dietary intervention that consists of reducing the daily food intake by 20-40% without causing malnutrition. CR is the most robust non-pharmacological intervention known for extending lifespan and quality of life for several species, from yeast (Lin et al. 2000) to primates (Mattison et al. 2017), including fruit flies (Pletcher et al. 2002), worms (Houthoofd et al. 2002), rodents (McCay et al. 1935; Omodei and Fontana 2011) and dogs (Kealy et al. 2002). The first evidence of the beneficial effects of a dietary intervention dates back 85 years (McCay et al. 1935). Since then, numerous studies performed in rodents reported that life-long CR extends lifespan by up to 50% (Weindruch and Walford 1982b; Byung Pal Yu et al. 1985; Pugh et al. 1999; Lee et al. 2002a; Dhahbi et al. 2004), reduces the severity of auto-immune diseases (Jolly et al. 2001) and prevents the onset of several disorders, including diabetes (Ohneda et al. 1995; Baumeier et al. 2015), and cancer (Rous 1911; Weindruch and Walford 1982a; Pugh et al. 1999; Dhahbi et al. 2004; Shelton et al. 2010; Lashinger et al. 2016).

Since the 1980s, three 30 year-long CR studies have taken place on rhesus monkeys (Bodkin et al. 2003; Colman et al. 2009; Colman et al. 2014), a species that shares 93% of the human genome and shows similar ageing patterns (Gibbs et al. 2007). The three studies adopted a 30% reduction dietary regimen, however they produced conflicting results on the animals' survival rate. Indeed, there were numerous differences in the experimental design, for instance one study used prevalently female monkeys and included diabetic and healthy animals (Bodkin et al. 2003), the other two used only healthy animals, both male and female, but the feeding patterns and macronutrient intake were very different.

Despite the discrepancies, all three studies showed significant reduction of age-related conditions, such as osteoporosis and arthritis, cancer, cardiovascular diseases and diabetes (Mattison et al. 2017). Consistently, CR monkeys also had a reduction in markers of inflammation (Willette et al. 2010), oxidative stress (Zainal et al. 2000) and brain astrogliosis (Sridharan et al. 2013), all hallmarks of ageing and cognitive decline. Another major feature of ageing is brain atrophy. Unfortunately, this trait is rarely visible in small animals, making it difficult to reproduce in most models of ageing. Notably, CR rhesus monkeys showed significantly higher grey matter volume in subcortical brain areas involved in motor and executive functions, compared to ad-libitum fed animals (Colman et al. 2009), although the exact consequences of these findings are unknown – since no behavioural testing was reported for these animals.

Interestingly, CR in non-human primates was more effective if started during adulthood (Mattison et al. 2017). This is contrary to studies carried out in mice which mostly suggest that better results are obtained when the dietary regime is initiated early in life (Goodrick et al. 1990; Forster et al. 2003). Considering the great potential of CR as a therapeutic agent, especially for age-related disorders, further studies will be needed to determine the cellular mechanisms that are involved in different species and to clarify the optimal timings for therapeutic strategy.

In humans, the study of long-term CR is quite challenging due to scarce adherence to the dietary regime and difficulties in following-up with the subjects through many decades. However, there have been occasions in history when such dietary conditions have been required. Two cases were documented in Scandinavian countries, where the government forced their populations to undertake a CR regime with balanced nutrients: for 2 years in Denmark during world war I (Hindhede 1920), and 4 years in Norway during world war II (Strom and Jensen 1951). As a consequence, a significant drop in the death rate was observed in the two populations, 30 and 34% respectively. In particular, the authors noticed that the nutrient-balanced diet imposed by the government was lower in fat compared to the usual diet of the general population, suggesting a correlation with the significant reduction of cardio-vascular diseases, like atherosclerosis, observed in the 10-year period since the beginning of the war (Hindhede 1920).

In 1994 the “Calorie Restriction Society” was founded in North Carolina, USA, whose members self-impose a 30% CRON diet (Calorie Restriction with Optimal Nutrition) in order to extend their lifespan and health. On average, this diet has been maintained for 15 years so far, and it is the only documented long-term CR practice ever performed in humans. Preliminary data on participant general health showed improved heart rate, reduction of cardio-metabolic risk factors and reduction of molecular damage markers, like antioxidants and enzymes associated to autophagy and protein misfolding, to an extent that resemble those of individuals 10-20 years younger (Fontana et al. 2004; Stein et al. 2012; Mercken et al. 2013).

Sadly, one of the founders of the society, Roy Walford, died at the age of 79 due to ALS, a neurological disease with metabolic impairment. Indeed, it has been hypothesised that negative energy balance may negatively affect motor neurones, although there are only indirect evidences of this in humans (Mattson et al. 2007) and conflicting evidence in mice (Patel et al. 2010; Bhattacharya et al. 2012). Despite the few negative reports, the increasing number of references indicating the beneficial effects of CR on health and lifespan induced the US National Institute of Aging in 2002, to coordinate a series of CR randomised clinical trials on non-obese healthy individuals, named CALERIES (Comprehensive Assessment of the Long-term Effects of Reducing Intake of Energy). A total of six studies were performed, divided in two phases of different length – between 6 months and 3 years – and CR between 16% and 30%, aiming to identify the best CR approach and its feasibility and safety in humans (Heilbronn et al. 2006; Racette et al. 2006; Das et al. 2007; Rickman et al. 2011; Rochon et al. 2011; Martin et al. 2016). All studies showed many positive outcomes: a decline in inflammatory markers; reduced insulin resistance; a decrease in total cholesterol and low density lipoprotein (LDL) versus an increase in high density lipoproteins (HDL); increase of growth hormone (GH); increase of pro-autophagic proteins and molecular chaperons like the 5' AMP-activated protein kinase (AMPK) and the sirtuins, that regulate DNA repair, stress resistance and stem cell function (Fontana et al. 2015; Das et al. 2017; Most et al. 2017).

One of the positive hallmarks of CR in all species is the improvement of mitochondrial efficiency, defined as the ratio between oxygen consumption and reactive oxygen species (ROS) production (Martin-Montalvo and De Cabo 2013). Skeletal muscle biopsies were collected from the subjects in the CALERIE studies, revealing that CR subjects have 35% more mitochondrial mass than controls. Moreover, several specific mitochondrial transcription factors are increased, resulting in an overall improved mitochondrial function and ultimately reduced ROS and ROS-derived damage (Civitarese et al. 2007). Overall, the modulation of these factors contributes to general health, increases lifespan and most importantly, prevent the onset of age-related disorders.

1.1.2 CR effects on brain and cognition

One of the main medical concerns about dieting is the occurrence of negative behaviours associated with eating disorders, like binge eating and guilt (Fuhrman et al. 1951). However, several studies in rodents showed that CR improves mood and reduces anxiety and depression-like behaviour (Inoue et al. 2004; Parikh et al. 2016). Studies performed in humans undergoing CR also reported improved morale, reduced food inhibition, reduced tension and improved perception of general health (Redman and Ravussin 2011; Hussin et al. 2013; Martin et al. 2016). In rodents, CR exerts numerous positive effects on the brain: it increases recovery after brain injury (Sandu et al. 2018), improves synaptic plasticity (Fontán-Lozano et al. 2008), preserves neuronal stem cell function (Apple et al. 2019), and increases neurogenesis, memory and learning in the adult brain (Hornsby et al. 2016; Morgan et al. 2017). CR is also neuroprotective in mouse models of ageing (Singh et al. 2018), Alzheimer's disease (AD) (Van Cauwenberghe et al. 2016) and Parkinson's disease (PD) (Maswood et al. 2004; Bayliss et al. 2016a).

In humans, results from the first phase of the CALERIE studies, did not show any significant effect, neither positive or negative, on memory or attention after 6 months of CR (Martin et al. 2016), suggesting CR is safe, but not necessarily beneficial. Interestingly, a study from the second phase, performed over a longer period of time (2 years) reported a significant increase in working memory performance in dieting individuals compared to controls (Leclerc et al. 2020).

An independent research performed on healthy elderly volunteers outside of the CALERIE studies, showed highly improved memory in CR subjects, as early as 3 months into the CR regimen (Witte et al. 2009), suggesting that differences in experimental strategies, testing techniques and possibly other factors like an individual's social background, and more, may heavily influenced the outcome of these studies. Although numerous evidences reported the beneficial effect of CR on memory and cognition, more studies will be needed to define the exact terms and conditions to get the most benefit out of the CR regime.

1.1.3 General considerations about CR feasibility

The last 20 years have seen increased interest in the CR paradigm in the context of age-related cognitive disorders, since most studies indicate that CR positively affects brain function. However, there are still concerns regarding applying these studies to humans. For instance, there are conflicting views about: 1) by what extent should they undergo CR; 2) for how long 3) at what age should CR be carried out to maximise benefit; and 4) whether subjects' metabolic state may limit CR effects. Indeed, some evidence suggest that CR may not be suitable for all individuals. A stand-alone CR study reported adverse effects in elderly humans, including changes in bone density and structure (Villareal et al. 2016), discouraging its use in the aged, most vulnerable population. Moreover, a study performed on 41 of the most common wild type strains of rats used in research, showed that the CR response is heavily affected by their genotype: in half of the strains, dieting shortened the lifespan or induced no significant change (Liao et al. 2010). A deeper analysis showed that the beneficial effects positively correlated with the strain's efficiency in using dietary resources and maintaining adiposity (Rikke et al. 2010; Liao et al. 2011), suggesting that CR effects may be reliant on the individual's own metabolic state.

In the literature, the overall effects of CR are still unclear. The organism's response to CR is a very complex, well conserved, mechanism of defence from starvation and energy imbalance. To study a phenomenon like this, with so many variables, yet maintaining scientific accuracy, is challenging. More studies are needed to clarify the actual feasibility in humans. Research should focus on obtaining a deeper knowledge on the 'hormonal signature' and the molecular mediators of CR, to plan a more tailored therapeutic approach for age-related disorders.

1.2 Ghrelin

1.2.1 Discovery and functions

Ghrelin is a 28 amino-acid hormone mainly produced within the oxyntic glands of the stomach by X/A-like cells in rats (Date et al. 2000) and P/D1 cells in humans (Rindi et al. 2002). An increasing number of studies support the hypothesis that ghrelin is the key mediator of the anti-ageing (Fujitsuka et al. 2016) and neuroprotective effects of CR (reviewed in Bayliss and Andrews 2016). Indeed, it has been shown to significantly increase during CR (Cummings et al. 2002), and also to affect the central nervous system in most species (Malik et al. 2008; Reimer et al. 2010).

Ghrelin was first discovered in 1999 as the endogenous ligand of the GH secretagogue receptor (GHS-R), able to stimulate the release of GH from the pituitary gland in rats (Kojima et al. 1999). Ghrelin is an orexigenic hormone, meaning it is able to induce food intake and regulate energy homeostasis (Tschöp et al. 2000). Indeed, its level in the bloodstream increases before meal times (Cummings et al. 2001) and decreases after feeding (Tschöp et al. 2001). Its release is dependent on the dietary supply, hence ghrelinergic cells in the stomach exhibit chemosensory capacity and express gustatory proteins and various fatty acid (FA) receptors (Müller et al. 2015). Moreover, the vagus nerve modulates secretion through a feedback mechanism based on caloric load (Callahan et al. 2004), diet composition (Monteleone et al. 2003), insulin (Murdolo et al. 2003) and glucose levels (Sakata et al. 2012). Once in the circulation, ghrelin stimulates the sense of hunger by acting on neuropeptide Y (NPY) and agouti-related peptide (AGRP) neurones in the hypothalamic arcuate nucleus (ARC) in the brain; this effect led to the nickname “the hunger hormone” (Nakazato et al. 2001; Cowley et al. 2003). Indeed, its primary function is to induce a shift from negative to positive energy balance by acting on digestion and metabolism.

Ghrelin injections in healthy individuals stimulates hunger (Levin et al. 2006), gastric motility and secretion (Ang et al. 2009), and regulates insulin and circulating glucose levels (Tong et al. 2010). In mice, ghrelin modulates adiposity and energy utilization (Theander-Carrillo et al. 2006). Some studies in rodents reported that male animals seem to be more sensitive to ghrelin-induced appetite

compared to female, unless the female animals have been ovariectomised (Clegg et al. 2007; Butera 2010; Asarian and Geary 2013), suggesting that ghrelin may interact with circulating estrogens, and this interaction may affect its function. Notably, studies conducted in humans reported that plasma ghrelin levels are higher in woman than in men (Barkan et al. 2003; Makovey et al. 2007), while other studies reported no sex differences (Serra-Prat et al. 2015), although the technical variability between the experiments may have influenced the outcome.

Interestingly, in rodents ghrelin has been shown to modulate taste sensation (Cai et al. 2013) and reward seeking behaviour (Jerlhag et al. 2006). Indeed, an increasing number of studies show that ghrelin does not just affect metabolism, but also it exerts a plethora of effects, influencing most organs in the body, including the brain (Stievenard et al. 2017) (figure 1.1).

In rodents, ghrelin-mediated neuroprotective effects were first reported in 2002, when Frago and colleagues injected a GHS-R agonist, GH releasing peptide-6 (GHRP-6), in rats and observed increased neuronal survival and reduction of apoptosis via inhibition of caspase 3 and 9 (Frago et al. 2002). Moreover, ghrelin has been shown to mediate CR-induced anxiolytic and antidepressant behaviours (Lutter et al. 2008), and to stimulate synapse formation in primary cortical neurones (Stoyanova et al. 2013). It also exhibits neuroprotective properties in various animal and cellular models: mouse models of brain ischemia (Miao et al. 2007), traumatic injury (Lopez et al. 2012), and neurodegenerative disorders (V. dos Santos et al. 2013); cellular models of oxidative damage (Chung et al. 2007) and glutamate excitotoxicity (Lee et al. 2012); rat models of glaucoma (Can et al. 2015) and multiple sclerosis (Liu et al. 2019).

In the hippocampus, an area of the brain implicated in memory and learning, ghrelin affects synaptic density, induces spine remodelling and increases long-term potentiation (LTP) (Carlini et al. 2002; Diano et al. 2006; Carlini et al. 2010; Perea Vega et al. 2021; Ribeiro et al. 2021). Moreover, it promotes neural plasticity, via adult hippocampal neurogenesis (AHN) (Hornsby et al. 2016; Buntwal et al. 2019; Huang et al. 2019a) making it a versatile and highly promising therapeutic tool.

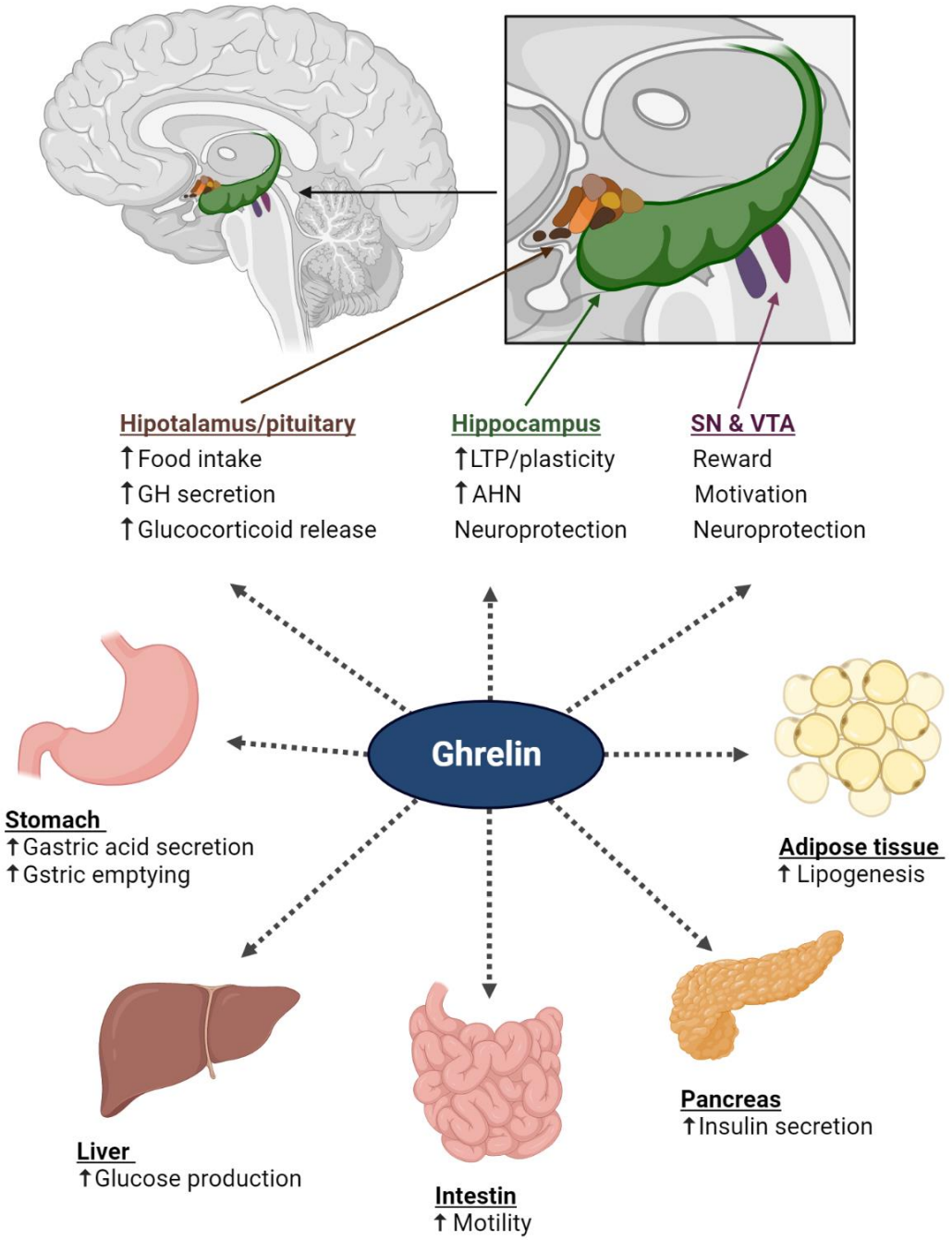


Figure 1.1 Ghrelin activity engages the whole organism

Ghrelin mediates the beneficial effects of CR on metabolism and health-span, affecting all organs and systems of the body, including the brain (*created with BioRender.com*).

1.2.2 Ghrelin effects on human metabolism and brain function

Following the promising data collected in rodents, numerous researches have been performed in humans to determine the effect of ghrelin administration. In 2013, a study thoroughly screened the available literature via database searches, in order to delineate a comprehensive picture of ghrelin research in humans (Garin et al. 2013). The authors selected 121 studies where ghrelin was either injected or ingested, for a total of more than 2000 patients, with doses ranging from 0.003 to 1.33μg/kg·min via iv infusions, and from 0.03 to 10.0μg/kg via ingestion.

One of these studies reported that injecting a single dose of ghrelin on healthy subjects, either 1 or 5μg/kg, quickly increase plasma concentration from the average baseline of 168.6fmol/mL, to 1058.7 and 6598.9fmol/mL respectively.

Moreover, the half-life of the injected hormone in the plasma was between 27 and 31 minutes, while the estimated half-life of the endogenous peptide is 9-13 minutes (Akamizu et al. 2004). Another group administered 10μg/kg to healthy volunteers, and after 1 minute measured an average of 150ng/mL of circulating ghrelin (corresponding to 50550fmol/mL), 61-fold higher than the baseline of the subjects before the injections. Despite the higher dosage, this study reported that ghrelin half-life was only 10 minutes (Nagaya et al. 2001). The broad difference between the studies is attributable to differences in the experimental settings, but it may also be a consequence of technical difficulties in isolating the hormone. Indeed, ghrelin mostly circulates associated to larger plasma proteins to prevent degradation, including albumin and HDL (Beaumont et al. 2003; De Vriese et al. 2007; Lufrano et al. 2016), making it difficult to precisely separate and quantify the active molecule in plasma. Despite the discrepancies, all the studies analysed by Garin and colleagues reported only mild adverse effect after ghrelin administration, such as gastric rumbles, in about 20% of the participant and no severe effects (Garin et al. 2013). Ultimately, the study reported that short-term ghrelin administration is safe and well tolerated for clinical use.

Most of the ghrelin studies performed in humans in the past decades, focus on appetite and energy intake, confirming ghrelin pivotal role in regulating glucose metabolism in healthy (Broglio et al. 2001; Arosio et al. 2003; Akamizu et al. 2004; Broglio et al. 2004) and obese subjects (Tassone et al. 2003; Guido et al. 2007). However, numerous other beneficial effects have been reported, like improved

cardiovascular functions in patients with heart failure (Nagaya et al. 2004), and increased respiratory performance and muscle strength in patients with respiratory diseases (Nagaya et al. 2005). Interestingly, ghrelin administration has also been shown to stimulate the secretion of GH, cortisol and the adreno-cortico-trophic hormone (ACTH), all involved in the regulation of the circadian rhythm in humans (Frieboes et al. 1995; Weikel et al. 2003). Indeed, when injected before sleep, ghrelin improves its duration and quality, even after sleep deprivation or in subjects affected with insomnia, and the effect is more evident with short-term treatments (Morin et al. 2018).

Overall, these data confirm the widespread idea that ghrelin exerts beneficial effects on metabolism and general healthspan. However, in contrast to most research performed in rodents, data focusing on mood and stress response in humans, have been quite inconsistent. Several groups measured endogenous ghrelin levels in patients with major depressive disorder, finding it to be either reduced (Barim et al. 2009), increased (Gecici et al. 2005; Kurt et al. 2007; Ozsoy et al. 2014), or unchanged (Schanze et al. 2008; Kluge et al. 2009; Matsuo et al. 2012). Ghrelin level was higher during severe and treatment-resistant depression (Ishitobi et al. 2012; Algul and Ozcelik 2018) while anti-depressant medications were found to either reduce circulating ghrelin (Barim et al. 2009; Ishitobi et al. 2012) or increase it (Tunçel et al. 2016). Finally, when used as a therapeutic agent, ghrelin administration reduced depressive symptoms in men but not women (Kluge et al. 2011; Steiger et al. 2011), while ghrelin injection in 9 healthy subjects showed mood improvement in only 3 of them, independently from their gender (Schmid et al. 2005). The great variance in these studies is most likely due to the multiplicity of the experimental parameters adopted by the different studies, like the small sample number and different testing and analysis strategies, but also variables such as patients' gender and metabolic state, severity of the depressive condition and type of the therapy. Moreover, chronic stress and depression often lead to altered feeding behaviours like binge eating, which will have an impact on ghrelin production (Schellekens et al. 2012), making it difficult to study its anti-depressant activity independently from its influence on food seeking behaviours.

Studying memory and cognitive functions in humans is also quite difficult as numerous biological, social and environmental factors may influence the outcome. One study performed on healthy young males, showed increased memory processing of food-related pictures after ghrelin administration, compared to unrelated images (Malik et al. 2008). Functional Magnetic Resonance Imaging (fMRI) scans performed at the same time, detected activation of areas involved in the visual and emotional response to food (amygdala, orbitofrontal cortex, thalamus), the dopaminergic areas of the reward system (substantia nigra/ventral tegmental area), and also areas involved in attention and memory, like the hippocampus (Malik et al. 2008).

In 2016, Kunath and colleagues reported a similar study on healthy male subjects, in which the stimulus was presented in form of food names, rather than images. Surprisingly, the authors could not detect any changes on the participant's performance, nor in any of the parameters measured, including fMRI scans (Kunath et al. 2016). This discrepancy could be explained by the fact that different types of memory, like visual or auditory memory, activate specific brain circuits (Butters et al. 1970) with different recall efficiency (Cohen et al. 2009b). Therefore, it is possible that ghrelin's effect on memory function only affect certain brain pathways (such as the visual circuit), but not the others. Investigating ghrelin's role may be fundamental to shed some light on its effect on memory and attention and its potential in clinical therapy.

1.2.3 The "survival" hormone

In rodents, ghrelin improves performance on different memory tasks, like fear conditioning, object recognition and spatial memory retention (Carlini et al. 2002; Diano et al. 2006; Kent et al. 2015). To explain these results, Kunath and Dresler used the analogy of a wild squirrel awakening from hibernation, whose only way to survive from starvation is to remember exactly where it hid the food before winter (Kunath and Dresler 2014). In this example, memory retention is intended as a survival mechanism to cope with metabolic changes like hunger, of which ghrelin represents part of the molecular signature. Higher attention and cognitive performances during hunger, would account for increased capability of food-hunting compared to competitors, and this could also justify how such a complex mechanism has been so well conserved among distant species.

The same reasoning could be applied to any type of energy deficit, including CR, which induces the increase of circulating ghrelin in all tested species (Cummings et al. 2002; Reimer et al. 2010). Indeed, both CR (Thanos et al. 2008) and ghrelin (Malik et al. 2008) have independently been shown to modulate the activation of the dopaminergic reward system that modulates mood, anxiety, and motivation.
All evidences collected so far, suggest that ghrelin is not just the "hunger" signature, but rather acts as a "survival" hormone during low energy conditions, protecting both body and mind, alleviating depression, stress, and improving cognitive functions, ultimately increasing the chances of survival and the endurance of the species.

1.2.4 Ghrelin gene and protein processing

The ghrelin gene (GHRL) is a single-copy gene whose sequence is highly conserved among species, with 100% identity on the first 10 amino acids at the N-terminal region, between all the species tested so far (Tomasetto et al. 2001; Angeloni et al. 2004; Seim et al. 2007; Kaiya et al. 2008). Indeed, this region is known to be essential for ghrelin binding with its receptor (section 1.2.8).
In humans, GHRL is located on the short arm of chromosome 3 and comprises 4 exons encoding a 117 amino acid long polypeptides precursor named pre-proghrelin (Wajnrajch et al. 2000). The precursor undergoes multiple processing steps and may generate multiple products which are then secreted (figure 1.2). After a first cleavage of the N-terminal signal peptide, the remaining 94 amino acid peptide named proghrelin is modified in the endoplasmic reticulum (ER) by the possible addition of octanoic acid (Kojima et al. 1999), by the enzyme ghrelin O-acyl transferase (GOAT), as will be better described in section 1.2.5. Either octanoylated or non-octanoylated forms of proghrelin can then be cleaved and the main enzymes involved are prohormone convertase 1 and 3 (PC1/3) (Zhu et al. 2006), although other enzymes, such as PC2 and furin, may also be involved (Takahashi et al. 2009). This cleavage process occurs in the Golgi, generating either the N-terminal 28 amino acid ghrelin peptide (octanoylated or non-octanoylated) ('mature ghrelin') (Zhu et al. 2006) or the C-terminal peptide (C-ghrelin), that is further processed to generate the hormone obestatin, which is reported to have independent functions in comparison to ghrelin (Zhang et al. 2005; Seim et al. 2011).

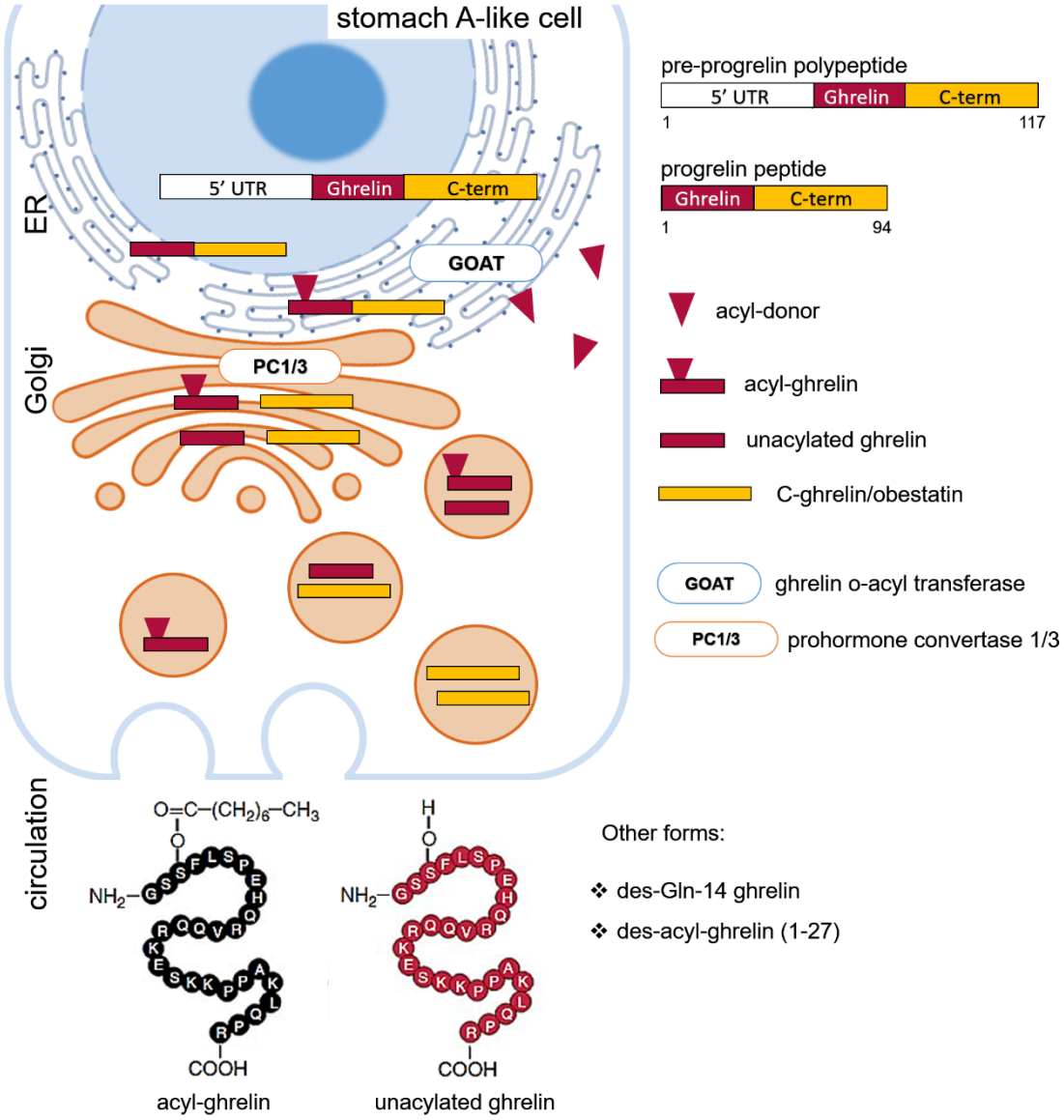


Figure 1.2 Processing of the human ghrelin peptide

Pre-proghrelin polypeptide precursor undergoes post-translational modifications in the ER and Golgi. Several products can be generated in the process, including acyl-ghrelin and unacylated-ghrelin, and ultimately secreted in the circulation (5'UTR = untranslated region) (*created with BioRender.com*).

Since not all the proghrelin localised in the ER is acylated before the final cleavage, both acylated (AG) and unacylated (UAG) ghrelin are released in the bloodstream, through secretory granules (Zhu et al. 2006) in a calcium dependent manner (Mani et al. 2014b). Moreover, several shorter ghrelin variants may also be released in the circulation. For instance, the des-Gln^{24}-C-ghrelin, is a C-ghrelin peptide lacking the exon 2 at the N-terminal and a glutamine residue in position 24 (Seim et al. 2007). des-Gln^{24}-C-ghrelin has been shown to be expressed in human cancer tissue (Jeffery et al. 2005; Yeh et al. 2005) and to bind GHS-R *in vitro* (Hosoda et al. 2000b) although its exact function is still unclear. These evidences, suggest a complicated tissue- and/or stage-dependent regulation of GHRL splicing process (Tanaka et al. 2001).

Lastly, both acyl-ghrelin and unacylated-ghrelin can be phosphorylated while transiting the Golgi by protein kinase C (PKC) at the Serine 18 in the C-terminal region. This process affects their secondary structure and in particular their ability to bind membranes and transport vesicles (Dehlin et al. 2008). It has been hypothesised that this modification could influence their release and transport in the circulation, their cellular uptake and subcellular localisation, although it is not clear whether and how this could affect acyl-ghrelin binding with GHS-R.
Overall, these studies show that GHRL is a complex gene, with a multitude of products, whose fine regulation may be essential in understanding ghrelin's activity in the body.

1.2.5 GOAT and ghrelin acylation

Since its discovery, it was clear that ghrelin's acylation is necessary for its function. In 2008, two separate studies identified the membrane-bound O-acyltransferase 4 (MBOAT4) - commonly referred to as ghrelin O-acyltransferase (GOAT) - as the catalysing enzyme in mice (Yang et al. 2008a) and humans (Gutierrez et al. 2008). There are 11 known MBOAT sequences in the human genome, from which 16 different enzymes originate as a result of alternative splicing (Yang et al. 2008a). All members of the MBOAT superfamily are integral highly conserved membrane enzymes, known to catalyse the addition of FA onto hydroxyl groups of membrane-embedded targets, mostly lipids or lipopolysaccharides (Hofmann 2000). They also

participate in several biological pathways including membrane biosynthesis/remodelling and lipid-signaling (Chang et al. 2011).
Other than GOAT, only two other members of the family are known to transfer FA to proteins. First, hedgehog acyltransferase (HHAT), also known as Rasp, that palmitoylates a cysteine residue in several substrates like Hedgehog proteins (Chamoun et al. 2001) and the Epidermal Growth Factor Receptor ligand, Spitz (Miura et al. 2006). Second, Porcupine (Porc), that transfer long-chain fatty acids (LCFA, 16 carbons or more) on both a cysteine and a serine in Wingless proteins, like Wnt3 (Zhai et al. 2004). In both cases, acylation is essential for the proteins' function and activity. However, neither of those enzymes are able to acylate ghrelin (Yang et al. 2008a).

The MBOAT4 gene is located on chromosome 8p12 and its sequence is highly conserved from prokaryotes to humans, with 74% gene identity and 98% protein similarity between humans, mice, and rats (Stengel et al. 2010). Indeed, extracts of the enzyme from mouse, rat and even zebrafish, have been shown to successfully catalyse acylation of the human ghrelin peptide (Gutierrez et al. 2008).
GOAT is a 450 amino acids protein, organised in 11 transmembrane helices, mostly localised in the ER compartment – although a recent study reported that it can shuffle between the ER and the plasma membrane, via small lipidic vesicles (Hopkins et al. 2017). In the ER, GOAT transfers an octanoyl group onto the third serine residue (S3) of either the proghrelin peptide just before this is transferred to the Golgi, or directly onto the mature unacylated ghrelin (Taylor et al. 2013). The exact mechanism of this reaction has been recently reported, revealing that GOAT catalyses the reaction inside an internal channel: the acyl donor accesses the channel from the cytoplasm, while proghrelin comes from the lumen of the ER. After the reaction, octanoylated ghrelin dissociates from the channel and transits through GOAT to reach the Golgi (Campaña et al. 2019). Interestingly, it has been shown that GOAT is not able to acylate any peptides longer than proghrelin: the *in vitro* addiction of just two extra amino acids at the N-terminus of proghrelin, significantly reduces the peptide ability to accept an octanoyl group, suggesting that GOAT would not be able to acylate pre-proghrelin either (Yang et al. 2008b).

Interestingly, two studies have shown that GOAT is the only enzyme involved in ghrelin acylation. Using an embryonal cell line, that does not produce ghrelin nor express GOAT, Gutierrez and colleagues performed co-transfections with the human ghrelin sequence and the cDNA from each one of the MBOAT family members: once exposed to octanoic acid, acyl-ghrelin production was detected only in the cells that received both ghrelin and MBOAT4 cDNA (Gutierrez et al. 2008). Shortly after, a second study, performed *in vivo*, reported complete absence of circulating acylated ghrelin in MBOAT4 knockout (KO) mice (Kirchner et al. 2009), therefore confirming that GOAT is the only enzyme able to acylate ghrelin.
On the other end, Darling and colleagues in 2015 showed that ghrelin is the only substrate of GOAT. In the attempt of identifying other potential substrates, they used ghrelin mimetics to characterise the chemical properties that are either essential, favoured or tolerated for the activity of the human GOAT enzyme. Substitutions at ghrelin N-terminal glycine and serine (G1 and S2) totally abolish octanoylation, while peptides that are 10 or 18 amino acids shorter in the C-terminal sequence exhibit a markedly reduced binding affinity with GOAT. Researchers then compared these sequence requirements with bioinformatics simulations and protein databases, showing that ghrelin is the only possible GOAT substrate within the human proteome (Darling et al. 2015).

This exclusive relationship is unusual in many ways. Numerous proteins are known to be modified by the addition of a FA, however, it is uncommon for a protein to be esterified on a serine residue, like ghrelin, since most of the known acylation processes occur either on a cysteine (S-acylation) or via an amide bond at the N-terminal residue (N-acylation) (Zeidman et al. 2009). Moreover, ghrelin is the only known peptide to receive an octanoic acid (Hougland 2019), although traces of ghrelin acylated with FAs of different lengths have been found in human stomach and plasma (Hosoda et al. 2003).
While the other MBOAT family members mostly transfer LCFA (Chang et al. 2011), GOAT *in vitro* shows an unusual predilection for the short-length chains (SCFA, 6 carbons or less) and medium-length chains fatty acids (MCFA, between 8 and 16 carbons). In particular GOAT binds FA with length between C2 and C16, with a preference for C8-C12 (Gutierrez et al. 2008). However, it has been shown that either the six-carbon (Ohgusu et al. 2009) or the eight-carbon (Yang et al. 2008c;

Taylor et al. 2013) acyl chains, have the best chance to be incorporated into the ghrelin structure. Indeed, when GOAT binds to different acyl chains, ghrelin does not easily bind the enzyme, suggesting that longer FA may constrict the GOAT active site, preventing ghrelin access (Darling et al. 2015). Nevertheless, it has been shown that octanoylated ghrelin is the most prevalent circulating form in human plasma (Hosoda et al. 2003), although it is not clear why octanoic, and not hexanoic acid – for which GOAT shows higher affinity (Ohgusu et al. 2009) – is the primary acyl donor for ghrelin acylation.

As for the source of the acyl donor, it is now clear that diet composition affects the length of the attached carbon chain. In particular, LCFA are found in plant-based oils, such as palm or seeds, and in animal fat, while MCFA are found in dry fruit, fish, and dairy (reviewed in Jala and Ganesh Kumar 2018). Before use, LCFA need to undergo a fragmentation process called β-oxidation, that produces MCFA, SCFA, and energy (Reddy and Hashimoto 2001). Interestingly, pharmacological inhibition of the β-oxidation, suppresses acyl-ghrelin production in cells, without affecting total protein or mRNA levels (Ikenoya et al. 2018), confirming the importance of this process for ghrelin acylation.

MCFAs are also supplied from the diet, and can be directly utilized for the acylation process, without having to be further processed (Nishi et al. 2005; Kirchner et al. 2009; Lemarié et al. 2018). Notably, digestive cells can be of two type, “open”, which can take uptake nutrients directly from the stomach and intestine, or “closed”, that do not make contact with the digestive lumen (Solcia et al. 2000). In the rat digestive tract, both types are ghrelin-secreting cells, however the “open” ones are the most abundant in the duodenum, where most of the diet lipids’ absorbance occurs (Sakata et al. 2002). This suggests that MCFA could be taken up by ghrelinergic cells during digestion, and directly used for ghrelin synthesis. However, this hypothesis does not match with the fact that acyl-ghrelin is notably reduced in the plasma after meals. Indeed, more studies are needed to determine the timings of ghrelin synthesis and whether the FA may be stored inside the ghrelinergic cells for a certain amount of time.

Interestingly, the nutrient composition of the diet can also affect ghrelin synthesis and secretion (Lee et al. 2002b) and high-fat diets have been shown to significantly reduce circulating acyl-ghrelin (Moesgaard et al. 2004; Handjieva-Darlenska and Boyadjieva 2009), suggesting that the sensing and/or uptake of FA in the digestive track may rather operate a negative feedback on acyl-ghrelin production.
Taken together, these evidences confirm the importance of a well-balanced dietary regimen for the correct functioning of the ghrelinergic system.

Although FA are largely introduced through diet, our body is also able to synthetise them in liver (Dorn et al. 2010), adipose tissue (Mayas et al. 2010), and brain (Knobloch et al. 2013). The process, known as lipogenesis, occurs when FA are not supplied by the diet in sufficient amounts, and is catalysed by the multienzyme system of the FA synthase (FASN) that produces LCFA by consecutively adding two-carbon acyl donors, in the form of acetyl-coenzyme A (acetyl-CoA), to the three-carbons malonyl-CoA substrate (Maier et al. 2006). A study performed in a murine ghrelin-producing cell line, showed that pharmacological inhibition of one of the enzyme involved in LCFA synthesis, the acyl-CoA synthetase family member 1 (Acsl1), significantly decreases ghrelin acylation (Bando et al. 2016), suggesting *de novo* FA synthesis is important for this process.
Interestingly, it has been shown that FASN conditional KO in mice neuronal precursor cells, exhibit impaired adult neurogenesis (Knobloch et al. 2013), while a specific FASN point mutation, that alters FA biosynthesis and the lipidic composition of cellular membranes, has been recently correlated to cognitive impairment in mice and humans (Bowers et al. 2020). Since acyl-ghrelin has been shown to increase AHN (Kent et al. 2015), and a reduction of its acylation significantly correlates with cognitive decline in humans (Hornsby et al. 2020) (as will be better described in chapter 3), it could be hypothesised that the detrimental effects of FASN KO in rodents, may be mediated by a decrease in ghrelin acylation. More studies are needed to confirm this hypothesis, however, it has been long known that FA biosynthesis is not favoured in physiological conditions, and solely occurs when dietary supply is insufficient (Weiss et al. 1986), hence this process may not be the preferential source of acyl donors for ghrelin acylation.

Thirdly, the gut microbiota is a highly complex microbial community that populates mammals digestive system and is known to produce SCFA, that can be absorbed and utilised for numerous scopes (Miller and Wolin 1996). Interestingly, microbiome-derived SCFAs have been shown to act as precursor for hepatic synthesis of MCFA and LCFA (Kindt et al. 2018), pointing to a potential – direct or indirect – role for the gut microbiota SCFA in the generation of acyl- ghrelin.

Ultimately, it has been hypothesised that all three aforementioned mechanisms – diet, *de novo* synthesis and gut microbiota – may contribute in generating acyl donors to be used for ghrelin acylation, suggesting the existence of a collaborative/redundant mechanism (Ikenoya et al. 2018).

1.2.6 APT1 and ghrelin de-acylation

Once de-acylated, proteins usually lose their biological functions and are quickly degraded in the lysosomes. However, in some cases, acylation is reversible, and the unacylated forms can circulate and be re-acylated locally, allowing a fine on-site modulation of their activity (Zeidman et al. 2009). Notably, the most abundant form of circulating ghrelin is unacylated, accounting for 90% of total ghrelin in the blood and 67% in the stomach of rats (Hosoda et al. 2000a). Indeed, ghrelin degradation by N-terminal proteolysis – that causes total loss of function – does not occur when unacylated ghrelin is in the circulation; but rather it requires that the hormone is in the stomach, liver, or kidney, as shown by a study that measured total ghrelin levels *in vitro*, after ghrelin was incubated with different tissue lysates (De Vriese et al. 2004), suggesting a localization-specific degradation.

Contrary to ghrelin acylation with GOAT, the deacylation of acyl-ghrelin is catalysed by a combination of enzymes, some of which are still unknown. In 2004, a study showed that the half-life of acyl-ghrelin in plasma varies notably between human (240 minutes) and rat (30 minutes), and the authors hypothesized that different enzymes may be involved in deacylating acyl-ghrelin in the two species: serine proteases and carboxylesterase in rodents, and other unknown esterases in humans (De Vriese et al. 2004). In rodents, acyl-protein thioesterase 1 (APT1) has been shown to deacylate ghrelin *in vivo* (Shanado et al. 2004) and *in vitro* (Satou et al. 2010), and it is currently considered to be the main enzyme involved in the deacylation of circulating acyl-ghrelin (Satou et al. 2011).

APT1 was originally isolated from rat liver in 1996 and called lysophospholipase I (LYPLA1), but later renamed due to its high affinity to thioester-containing proteins (Sugimoto et al. 1996; Duncan and Gilman 1998). A BLAST (*basic local alignment search tool*, *NCBI*) search of its sequence reveals homologues in a wide variety of species, with 91.74% sequence identity between human and mouse. APT1 is a 230 amino acids long protein and a member of the α/β serine hydrolase family. It primarily resides in the cytoplasm in a dimeric form, although dissociation is required to expose the active site for substrate binding (Devedjiev et al. 2000). Its crystalised structure shows a cysteine at the second amino acid position, and a highly conserved catalytic trio (Ser114, His203, Asp169); moreover, adjacent to the active site, there is a distinctive β5-α2 loop, that is not found in any other hydrolase lipases of the family (Devedjiev et al. 2000). This region forms a large and flexible hydrophobic channel that accommodates the whole acyl-chain of the substrate, allowing its correct positioning for the hydrolysis – albeit without contributing to substrate selectivity (Won et al. 2016).

APT1 has been long been considered the primary depalmitoylase enzyme in cells (Won et al. 2017); indeed, it typically catalyses the removal of long acyl chains, like palmitic acid, but the high adaptability of its channel would potentially allow the enzyme to interact with much shorter or longer carbon chains alike. Consistently, APT1 has been shown to have a very broad range of targets, from lysophospholipids to a variety of esters and thioester metabolites (Lin and Conibear 2015). Interestingly, APT1 does not seem to have a defined substrate recognition sequence, and the list of its substrates includes structurally diverse proteins: from small soluble intracellular proteins, like acyl-ghrelin and the G-protein Ras (Dekker et al. 2010), to larger enzymes like the endothelial nitric-oxide synthase (Yeh et al. 1999). To comply with the different sub-cellular localization of its substrate, APT1 can S-palmitoylate itself and the attached palmitate serves as a hydrophobic tag, transiently driving the enzyme near the membranes, where it can interact with a wider range of substrates (Kong et al. 2013; Vartak et al. 2014; Won et al. 2018). Nevertheless, APT1 does not deacylate any protein indiscriminately and the catalytic efficiency can also vary, depending on the type, location or activation status of the substrates (Zeidman et al. 2009).

Interestingly, the analysis of APT1 sequence let to the identification of a homolog with 64% sequence identity, lysophospholipase II (LYPLA2) or APT2 (Toyoda et al. 1999). Like its homolog, APT2 is also able to hydrolyse a variety of substrates, although it is still unclear whether their activity is mutually exclusive or redundant (Tomatis et al. 2010; Kong et al. 2013; Adachi et al. 2016; Vujic et al. 2016; Abrami et al. 2017; Won et al. 2018). For instance, a study showed that double APT1/APT2 pharmacological inhibition significantly reduces the hydrolysis rate of one of their substrates, the G-protein Ras, compared to the single APT1 knockdown (Dekker et al. 2010). However, this study could not exclude a compensatory mechanism from other unidentified hydrolases. Certainly, the two enzymes are diverse enough to allow isoform-selective inhibition (Won et al. 2016).

Satou and colleagues showed that both APT1 and APT2 are able to deacylate ghrelin in ghrelin-expressing murine cell lines (Satou et al. 2010). Interestingly, cell culture media from murine hepatocytes HepG2 cells, contained detectable levels of APT1, but not APT2; moreover, the secreted APT1 was fully functional, as it was able to deacylate synthetic acyl-ghrelin *in vitro*. The authors suggested that APT1 may also be released in the bloodstream, where it may exert a major role in deacylating circulating ghrelin, while APT2 activity may be only restricted to the intracellular space (Satou et al. 2010), although this hypothesis has not been confirmed *in vivo* yet (see chapter 6).

1.2.7 Unacylated Ghrelin

The unacylated form of ghrelin (sometimes referred to as des-acyl ghrelin, DAG), has been considered for a long time nothing more than an inactive form of acyl-ghrelin (Kojima et al. 1999). One study even reported that all circulating ghrelin in human plasma is actually acylated, and its unacylated form may be an artifact of sample handling (Blatnik et al. 2012). However, two studies in 2004 showed that unacylated ghrelin is able to inhibit the effects of its acylated counterpart on glucose metabolism and insulin sensitivity, when injected at the same time as acyl-ghrelin in human healthy subjects (Broglio et al. 2004; Gauna et al. 2004). Moreover, intravenous unacylated ghrelin administration overnight, reduces hyperglycaemia, inhibits lipolysis and increases post-prandial insulin levels, either in normal weight and obese diabetic subjects (Benso et al. 2012; Özcan et al. 2014).

Similar effects, that are opposite to that of acyl-ghrelin, were also shown in rodents (Inhoff et al. 2008; Fernandez et al. 2016; Dallak 2018) and other species (Gauna et al. 2005; Matsuda et al. 2006). Furthermore, our group recently reported the first evidence that unacylated ghrelin is able to counteract the pro-cognitive effects of acyl-ghrelin in mice (Hornsby et al. 2020). To do so, we used GOAT KO mice, that are not able to convert unacylated ghrelin to acyl-ghrelin therefore causing an accumulation of the unacylated ghrelin in the circulation. These mice display significant memory impairment in a "spatial memory" task, and show reduced markers of AHN and cell function, like c-FOS, Ki67 and dendritic spine arborisation. Interestingly, a 7-day treatment with acyl-ghrelin fully rescued the behavioural performance and the recovery persisted as long as 21 days after the last injection, suggesting that acyl-ghrelin effects on memory may result in long-term promotion of hippocampal functions (Hornsby et al. 2020). Collectively, this evidence suggests that unacylated ghrelin is more than an inactive product, and it should be investigated in parallel with its acylated form.

Kumar et al. showed that the antagonistic effects of unacylated ghrelin were dose-dependent in rodents, suggesting that unacylated ghrelin may be an endogenous competitive inhibitor of acyl-ghrelin (Kumar et al. 2010). However, no receptor for unacylated ghrelin has been identified thus far, and most literature agrees that unacylated ghrelin does not bind the acyl-ghrelin receptor (Gauna et al. 2005; Kumar et al. 2010; Lear et al. 2010). Indeed, a recent study showing GHS-R crystalised structure, demonstrated that unacylated ghrelin is not able to bind nor activate the receptor *in vitro* (Shiimura et al. 2020). Consistently, unacylated ghrelin administration has been shown to affect insulin signaling in GHS-R KO mice (Delhanty et al. 2010). This study, together with the knowledge that unacylated ghrelin is the major circulating form of ghrelin (Hosoda et al. 2000a), suggests that unacylated ghrelin should be considered a hormone distinct from acyl-ghrelin, that it may bind to an endogenous unidentified receptor with discrete functions (Gauna et al. 2005; Kumar et al. 2010; Lear et al. 2010) and that does not bind GHS-R.
However, not all studies agree on this matter. Some suggest that acyl- and unacylated ghrelin may act synergically in their effects, as have been shown for inhibition of cancer cell proliferation (Cassoni et al. 2004), induction of adipogenesis *in vivo* (Thompson et al. 2004), and inhibition of apoptosis in

cardiomyocytes (Lear et al. 2010), endothelial cells (Baldanzi et al. 2002), and pancreatic β-cells (Granata et al. 2007). Interestingly, all those effects were prevented by inhibiting or deleting GHS-R, suggesting that unacylated ghrelin may indeed bind the receptor. This contrast may be explained in several ways. Firstly, it is possible that the administered dose of unacylated ghrelin was too high and non-physiological in some of these experiments. Indeed, two studies performed on human cell lines reported that unacylated ghrelin is able to stimulate GHS-R when administered between high nanomolar to micromolar range – approximately 1,000 to 100,000 fold higher than unacylated ghrelin concentration in the plasma (Gauna et al. 2007; Heppner et al. 2014). Secondly, it has been recently described that GOAT is able to re-acylate unacylated ghrelin to acyl-ghrelin locally in several tissues, as will be better described in section 1.4.4. Since this is a relatively recent finding, most of the previous literature – affirming that unacylated ghrelin acts as an agonist of GHS-R – did not take in consideration the possibility of in-situ re-acylation, nor did they quantify GOAT expression and activity in their samples. Therefore, it cannot be excluded that in those experiments where unacylated ghrelin was added to the cells, it was acting as a precursor, rather than an agonist, enriching the existing pool of the "active" acyl-ghrelin. This hypothesis would also explain why some researchers found a strong positive correlation between unacylated ghrelin administration and GHS-R activity. Thirdly, different methods of stabilizing acyl-ghrelin and unacylated ghrelin in samples may have affected the outcomes. It has been shown that acyl-chain degrades quickly, once the hormone is extracted from blood, suggesting that its stabilization with serine protease inhibitors, like AEBSF, is essential to determine the correct ratio between acyl-ghrelin and unacylated ghrelin in samples (Blatnik and Soderstrom 2011; Delhanty et al. 2015). Ultimately, whilst the unacylated ghrelin receptor is unknown, it cannot be excluded that unacylated ghrelin may bind GHS-R in an allosteric modulatory site or may influence its activity in a different way, either by competitive inhibition or indirectly via a different receptor. Indeed, GHS-R has been shown to dimerise with several other known and unknown proteins on cell membranes, as will be better described in section 1.2.9.

1.2.8 GHS-R

Growth hormone secretagogue receptor (GHS-R) was first identified in 1996, as the mediator of growth hormone response in the hypothalamus and pituitary gland (Howard et al. 1996). Indeed, GHS-R has been detected in the brain, as it will be better described in section 1.4.4.

The GHS-R gene is highly conserved in mammals, with 90% sequence homology between human, rat, pig, and sheep (Palyha et al. 2000), and consist of two exons and one intron that undergo alternative splicing, so that two distinct forms of the receptors have been identified (figure 1.3). The full mRNA transcript codifies for GHS-R1a, a 366 amino acid G-protein coupled receptor (GPCR), comprising seven transmembrane domains (TM1 to TM7), three intracellular and three extracellular loops (Howard et al. 1996; Holst et al. 2003). GHS-R1a crystal structure has been recently resolved (Shiimura et al. 2020), showing two main structural differences compared to other GPCR receptors. Firstly, the ligand-binding compartment is split into two pockets, cavity I and II. Authors suggested that ghrelin enters both cavities, although selective mutations of the amino acid residues inside the pockets showed that cavity I is essential for recognition of the ghrelin peptide sequence. Interestingly, mutations in cavity II do not affect ghrelin's binding, but rather inhibit GHS-R1a constitutive activation (Shiimura et al. 2020), although it is not clear whether this process is dependent on ghrelin access/binding to cavity II. Second, the two transmembrane segments, TM6 and 7, that surround the ligand cavity, are unusually sloped and the gap between them is occupied by large hydrophobic chains, essential for recognition of the ghrelin acyl moiety (Shiimura et al. 2020). Interestingly, TM6 and 7 have been previously shown to mediate GPCRs conformational change to their active form (Zhou et al. 2019), therefore authors suggested that the interaction between ghrelin's acyl chain and this region may contribute to the receptor transition to its ligand-activated conformation (Shiimura et al. 2020), as hypothesised in a previous study (Ferré et al. 2019).

Differently, the GHS-R1b isoform is generated when the splicing site between exon 1 and 2 is skipped and a short 24 amino acids sequence is included in the final transcripts, due to an intronic stop codon (Howard et al. 1996). This results in a truncated receptor, with 289 amino acids, of which the first 265 are shared with the 1a isoform, and only five transmembrane domains (Howard et al. 1996).

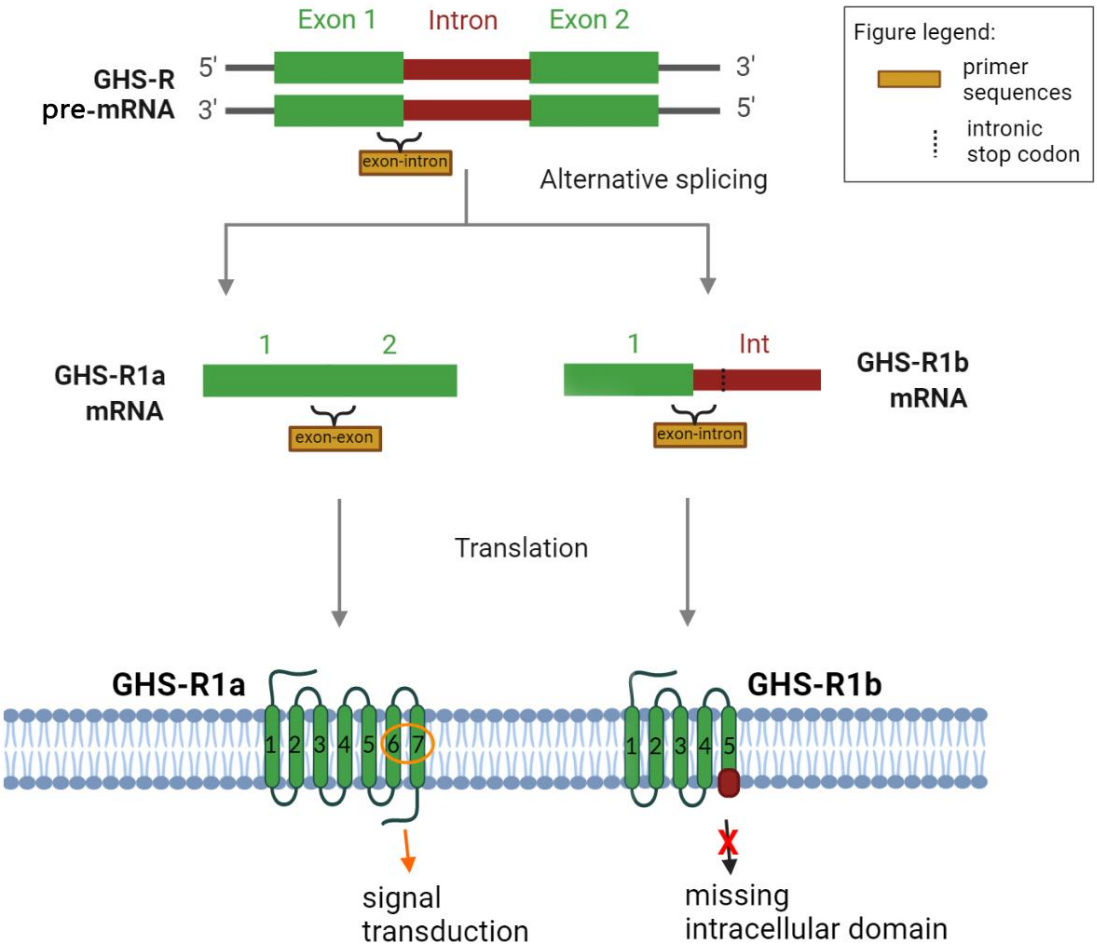


Figure 1.3 GHS-R gene alternative splicing

GHS-R gene consists of two exons and one intron and can produce two products by alternative splicing. Upon receptor activation, the 1a isoform is able to produce an intracellular signal thanks to the transmembrane domains TM6 and TM7, which are not present in the 1b isoform. Yellow boxes show possible primers/probes recognition sequences: exon-exon for GHSR-1a and exon-intron for GHS-R1b (overlapping with GHS-R precursor mRNA) (*created with BioRender.com*).

Because of this alternative splicing, designing specific probes/primers for GHS-R1b isoform (figure 1.3, yellow boxes) is challenging, due to sequence overlapping with GHS-R pre-mRNA, therefore GHS-R1b mRNA expression has been poorly investigated in literature so far, as will be better described in section 6.
Interestingly, while GHS-R1a receptor is mostly located in the cellular membrane, GHS-R1b protein – detected using an antibody specific for the short intronic sequence – has been largely found in the ER (Chow et al. 2012) and in the nucleus (Smith et al. 2005), suggesting that its activity on the plasma membrane may be dependent on the regulation of its translocation. Since GHS-R1b is lacking the 13 amino acids that form TM6 and TM7, the two domains involved in signal transduction (Zhou et al. 2019), GHS-R1b is not able to induce any of the GHS-R1a known intracellular pathways.

All GPCRs are able to form homo- or heterodimers and, although not required for their function, dimerisation plays an important regulatory function (Kasai and Kusumi 2014). GHS-R1a mostly functions as a homodimer (see section 1.2.9) (Holst et al. 2005), however it can also heterodimerise with GHS-R1b in transfected human cells, and this association reduces GHS-R1a trafficking to the cell membrane (Chow et al. 2012), prevents ghrelin-dependent conformational changes of the GHS-R1a subunit and decreases its constitutive activity (Leung et al. 2007; Mary et al. 2013; Navarro et al. 2016), therefore suggesting that GHS-R1b acts as dominant negative regulator of GHS-R1a (Leung et al. 2007). Interestingly, the heterodimerisation has been shown to occur with the same efficiency as the homodimerization (Leung et al. 2007) and the two forms of the receptor have been found to be co-expressed in healthy tissues (Gnanapavan et al. 2002; Carraro et al. 2004), as well as several cancerous tissues and cell lines (Korbonits et al. 2001; Jeffery et al. 2002; Cassoni et al. 2004; Barzon et al. 2005), suggesting a tissue- or cell type-dependent differential expression.

Since its discovery, it was clear that the acylation of ghrelin is essential for the binding with its receptor. In particular, it has been shown that as little as 10 picomoles of octanoylated acyl-ghrelin is enough to activate GHS-R1a signaling in GHS-R-transfected murine cells, with 2.5 nanomolar inducing 50% of the receptor's signaling capacity (EC50) (Kojima et al. 1999). Another study performed

in silico using synthetic human ghrelin peptides and synthetic human GHS-R1a receptor, showed an EC50 of 32 nanomolar (Bednarek et al. 2000), suggesting that GHS-R1a activation may be somehow more efficient in a living system. Interestingly, shorter ghrelin peptides are also able to activate the receptor, as long as the first 4 (Matsumoto et al. 2001) or 5 (Bednarek et al. 2000) amino acids at the N-terminal (Gly1-Ser2-Ser3-Phe4-Leu5-NH2) are present, suggesting they are essential for GHS-R1a activation. Indeed, this sequence is highly conserved among most species (Tine et al. 2016). As for the acylation itself, it has been reported that GHS-R1a does not discriminate between Ser2 or Ser3 being acylated (Bednarek et al. 2000), although the L-configuration of the Ser3 seems to be necessary for the receptor's function (Matsumoto et al. 2001). Moreover, other bonds, like the thioether *in lieu* of the O-ester, retain GHS-R1a activity *in vitro*, and conveniently increase the stability of the acyl chain, suggesting these modifications may be adapted for the generation of acyl-ghrelin-based drugs with longer stability (Bednarek et al. 2000; Matsumoto et al. 2001). Ghrelin acylated with shorter or longer carbon chains, is also able to activate GHS-R1a, although the canonical eight-carbon chain is the most efficient compared to longer chains (C11 to C16), both *in vitro* (Matsumoto et al. 2001) and *in vivo* (Heppner et al. 2012).

GHS-R1a has been shown to have strong, ligand-independent, constitutive activity. When GHS-R1a is expressed in a human cell line that does not express ghrelin, approximately 50% of its maximum activity can be detected – measured as the increase in intracellular calcium induced by the activation of the receptor (Holst et al. 2003). It is generally believed that ghrelin production is not produced in the brain, as will be better described in section 14.4. Since accessing the brain from the blood is generally a highly restricted process, as described in section 1.4.1, it has been hypothesised that ghrelin may not be able to reach its receptor in the deepest regions of the brain, such as the hippocampus. If that were the case, GHS-R1a activation in those areas would be mediated only by its own constitutive activity (Wellman and Abizaid 2015) or by the activation of its dimerisation partners (see section 1.2.9) (Kern et al. 2015). However, this theory is in contrast with studies where ghrelin administration in rodents was seen to specifically increase GHS-R1a activity in deep brain structures, such as the hippocampus (Diano et al. 2006) and the substantia nigra (SN) (Bayliss et al. 2016a).

Overall, these evidences highlight that GHS-R1a kinetics is very complex, and further studies are needed to clarify the exact dynamic of its interaction with acyl-ghrelin *in vivo*.

1.2.9 GHS-R1a dimerisation and signaling

GHS-R1a belongs to the rhodopsin family, or class A, of GPCRs, membrane proteins that are involved in a variety of intricate signaling networks, mediated by the small intracellular G proteins. GPCRs dimerisation is often the first step for induction of intracellular signals after ligand binding (Weiss and Schlessinger 1998; Kasai et al. 2011). As with most GPCRs, GHS-R1a can form homo- and heterodimers that have been predicted to be essential for its function (reviewed in Wellman and Abizaid 2015). As a homodimer, it has been hypothesised that the two GHS-R1a subunits have negative co-operativity: one molecule of acyl-ghrelin binds to the first subunit, then a conformational change in the receptor occurs preventing another molecule from binding on the other subunit (Holst et al. 2005). Without the ligand, GPCR monomers are associated in their intracellular portion to the heterotrimer Gα/Gβ/Gγ, with Gα bound to guanosine diphosphate (GDP). After the dimerisation and binding of the ligand, the receptor undergoes conformational changes that induce the exchange of GDP with guanosine triphosphate (GTP), followed by disassociation of two subunits, Gα-GTP and Gβ/Gγ, that activate specific signaling pathways (second messengers) (recently reviewed in Kontou et al. 2021).

Several G proteins have been identified, of which at least 16 Gα, 5 Gβ, and 11 Gγ subunits in humans, whose sequence is highly conserved among mammals (Hurowitz et al. 2000). In the hypothalamus, GHS-R1a homodimers regulate hunger and food intake, primarily through the $G\alpha_{q/11}$ subunit and the second messenger phospholipase C (PLC) (figure 1.4, point 1) (Howard et al. 1996). PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce inositol-triphosphate (IP_3) and diacylglycerol (DAG), then IP_3 reaches its receptor in the ER, causing the mobilization of intracellular Ca^{2+} from stores (figure 1.4, point 2). Ca^{2+} also acts as a second messenger, engaging several enzymes such as the Ca^{2+}/calmodulin-dependent protein kinase type II (CaMKII).

CaMKII phosphorylates a variety of intracellular substrates involved in memory and learning (Halt et al. 2012; Park et al. 2016) including the AMP-activated kinase (AMPK) (Anderson et al. 2008; Andrews et al. 2008). Interestingly, Sirtuin1 (SIRT1), an enzyme activated during CR and involved in glucose and lipid metabolism (Rodgers and Puigserver 2007; Cohen et al. 2009a), is an important regulator of AMPK phosphorylation in the hypothalamus, via the p53 transcription factor (Velásquez et al. 2011); in turn, pAMPK enhances SIRT1 activity in promoting mitochondria activity (Cantó et al. 2009) (figure 1.4, point 3).

pAMPK acts as energy sensor in the hypothalamus (Minokoshi et al. 2004; López et al. 2010) and its expression significantly increases shortly after ghrelin treatment (López et al. 2008). Moreover, exogenous pAMPK administration in mice increases food intake (Andersson et al. 2004) and mediates the beneficial effects of ghrelin in the brain (Andrews et al. 2009; Bayliss et al. 2016a; Huang et al. 2019b), heart (Zhang et al. 2013) and liver (Qin et al. 2014). Once activated, pAMPK is involved in a complex signaling circuit. First, it phosphorylates the regulator-associated protein of the mammalian target of rapamycin (Raptor), which binds to the mammalian target of rapamycin (mTOR) complex – whose main function is to favour cell growth by inducing protein synthesis in response to high energetic status – ultimately resulting in mTOR sequestration and inhibition (Gwinn et al. 2008). Second, pAMPK phosphorylates the Unc-51 like autophagy-activating kinase 1 (ULK1), favouring autophagy (Lee et al. 2010) and promoting the recycling of damaged proteins and organelles in response to low energetic status (Kuma and Mizushima 2010). Moreover, in its function as energy sensor, pAMPK also regulates mitochondrial homeostasis, as it will better described in section 1.3.6.

After PIP_2 cleavage, DAG remains anchored to the plasma membrane and recruits the phosphoinositide 3-OH kinase (PI3K) complex that phosphorylate a series of proteins, including the protein kinase B, also known as Akt, and the pro-autophagic extracellular regulated protein kinases 1 and 2 (ERK1/2) (Camiña et al. 2007). Akt phosphorylates the mitogen-activated protein kinase (MAPK) that regulates cell differentiation and survival (Peltier et al. 2007), and activates the mTOR complex, ultimately favouring cell growth (figure 1.4, point 4) (Kumar et al. 2005; Zeng and Zhou 2008), while ERK1/2 (Kim et al. 2014) contributes to LC3 cleavage and

initiate the autophagic machinery (figure 1.4, point 5). Interestingly, AMPK and ERK pathways, have been shown to antagonise each other in several cell types (Hwang et al. 2013; Kawashima et al. 2015), suggesting that the balance between the pro-autophagic state (mTOR OFF) and the protein synthesis (mTOR ON) after ghrelin-induced activation of GHS-R1a, is a finely regulated process. The exact mechanism has not yet been clarified; however, it is possible that the configuration of GHS-R1a determines the shift between the two pathways, or that each signaling cascade is upregulated in specific cell types.

GHS-R1a signaling has also been shown to be mediated by other G proteins. For instance, when the receptor binds to $G\alpha_s$, its activation leads to the mobilisation of the enzyme adenylate cyclase (AC), and the formation of second messenger 3',5'-cyclic adenosine 5'-monophosphate (cAMP) and its downstream effector, protein kinase A (PKA) (Kohno et al. 2003). Other subunits that have been associated to GHS-R1a include $G\alpha_i$, that inhibits AC, reducing the formation of the cAMP and resulting in modulation of GABA release from the hypothalamic neurones, to regulate of food intake (Soto et al. 2015), and $G\alpha_{12/13}$, involved in cytoskeleton remodelling and cell migration (Lin et al. 2009). Lastly, GHS-R1a, when bound to acyl-ghrelin, can interact with the small intracellular protein β-arrestin that prevent coupling with G proteins, "arresting" its activation (desensitization), and recruiting the clathrin complex that promotes receptor internalization for recycling or degradation (Holliday et al. 2007).

It is not clear how the receptor regulates its binding to different G proteins. Interestingly, GHS-R1a signaling seems to be functionally biased, meaning that there is an intrinsic mechanism of ligand selection – where the receptor selectively prefers one ligand over another in certain conditions – and preferentially activates a specific signaling pathway. Although the exact mechanism is not clear, it has been hypothesized that it may be dependent on cell type, maturation stage, or ligand availability (Sivertsen et al. 2011; Damian et al. 2015; M'Kadmi et al. 2015; Ramirez et al. 2019).

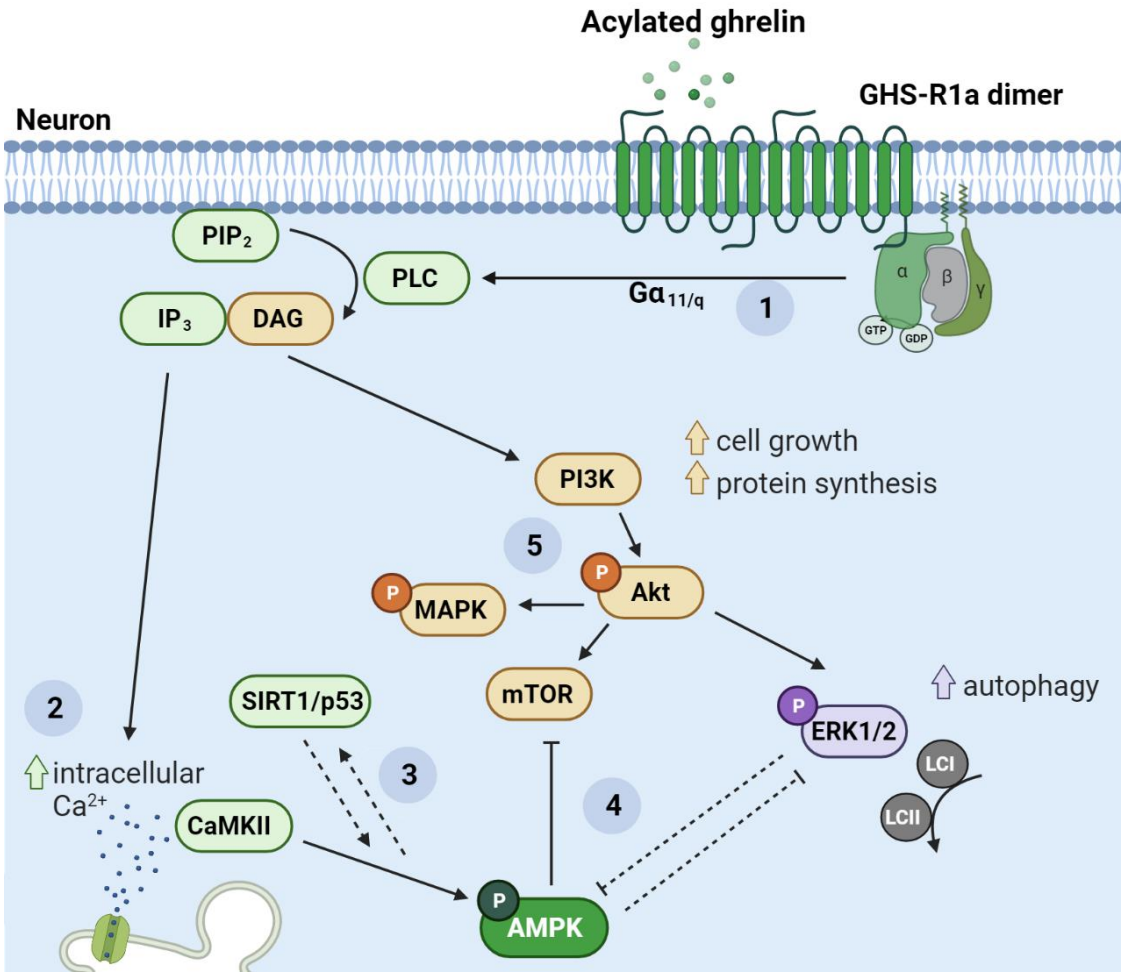


Figure 1.4 GHS-R1a neuronal signaling pathways

(**1**) Upon GHS-R1a dimerisation, $G\alpha_{q/11}$-GTP subunit activates PLC, that process PIP_2 into IP_3 and DAG. (**2**) PIP_2 induces the release of Ca^{2+} from the ER, activating Ca^{2+}-sensitive kinases, such as CAMKII. (**3**) One of CAMKII main targets is pAMPK, whose activation is favoured by the SIRT1 pathway (and vice versa). (**4**) pAMPK is involved in an intricated signaling pathway that ultimately inhibits mTOR signaling and stimulates autophagy via ERK1 and ERK2. (**5**) However, another pathway may be initiated via DAG, which stimulated a kinases cascade mediated by PI3K and Akt, ultimately leading to the activation of mitogenic signals such as MAPK and mTOR. (**5**) Since PI3K activation is also able to mediate autophagy via phosphorylation of ERK1/2, the balance between mTOR activation/inactivation may be crucial to determine the outcome (*created with BioRender.com*).

It has been shown that GHS-R1a dimerise with the dopamine receptor D1R (sometimes referred to with the name of its gene, DRD1), and this interaction is very important, since dopaminergic signaling is associated with food reward (Hernandez and Hoebel 1988; Wise 2006) and memory formation (Packard and White 1989; Centonze et al. 2001). In particular, acyl-ghrelin has been shown to increase dopaminergic signaling in a human neuroblastoma cell line, once dimerisation with D1R has induced a shift in the G protein coupled to GHS-R1a, from $G\alpha_{q/11}$ to $G\alpha_{i/o}$ (Jiang et al. 2006). Another study showed that, upon dimerisation with GHS-R1a, D1R shifts from $G\alpha_s$ to $G\alpha_q$, in mice hippocampal primary cells, allowing dopamine to activate Ca^{2+} signaling in the absence of ghrelin (Kern et al. 2015). Interestingly, the authors also reported improved spatial memory functions in mice after injection with a D1R agonist, effect that is abrogated in GHS-R1a KO mice. Moreover, they showed co-localisation of D1R and GHS-R1a receptors in the mouse brain, most abundant in the dentate gyrus (DG) of the hippocampus (Kern et al. 2015), the main area involved in neurogenesis and memory formation, as it will be better described in section 1.4.3. GHS-R1a/D1R dimers have been detected in several other brain areas, including: substantia nigra and VTA, that regulate motor activity and whose impairment has been associated to Parkinson's disease; and prefrontal cortex and hippocampus, associated with cognition and learning, notably involved in Alzheimer's disease (Jiang et al. 2006). Moreover, GHS-R1a dimerises with a second dopamine receptor, D2R, resulting in PLC activation via the $G\beta\gamma$ subunit and mobilization of intracellular Ca^{2+} Interestingly this induces an anti-orexigenic effect, that has been shown to occur only when GHS-R1a is not bound to ghrelin, therefore mediating dopamine response to food intake (Kern et al. 2012).

The dimerisation of GHS-R1a with the 5-hydroxytryptamine (serotonin) 5-HT2C receptor has been shown to significantly reduce ghrelin signaling, suggesting that serotonin may act as a negative regulator of food intake (Schellekens et al. 2013; Schellekens et al. 2015). Interestingly, GHS-R1 deficient mice exhibit increased serotoninergic activity in the ARC, but decreased in the paraventricular nucleus (PVN), both areas associated to feeding and stress response (Patterson et al. 2010). Moreover, ghrelin central administration to wild type mice increases mRNA expression of serotonin receptors in the amygdala (Hansson et al. 2014), suggesting a region-specific modulation of the serotoninergic signalling.

GHS-R1a also dimerises with other GPCRs, such as the melanocortin3 receptor (MC3), known for its hypothalamic orexigenic effect that are amplified by its dimerisation with GHS-R1a (Rediger et al. 2011); and the oxytocin receptor (OTR), that regulates mood and socialisation, to whom surprisingly the GHS-R1a receptor has been shown to act as a negative regulator, by increasing its internalisation (Wallace Fitzsimons et al. 2019).

Lastly, it has been recently shown that the liver-expressed antimicrobial peptide 2 (LEAP2) is a competitive inverse agonist of GHS-R1a, and acts by stabilizing the inactive conformation of the receptor, therefore preventing acyl-ghrelin binding (Wang et al. 2019; Li et al. 2020). Interestingly, peripheral ghrelin administration in mice reduced LEAP2 mRNA in the liver as well as the circulating protein (Ge et al. 2018), suggesting the existence of a feedback regulation between the two hormones. Consistently, peripheral LEAP2 administration in mice blocks acyl-ghrelin effects on general metabolism, such as food intake and GH release (Ge et al. 2018), while intra-cranial administration in rats completely abolishes hypothalamic neurone activation and also prevent ghrelin-mediated regulation of blood glucose and body temperature (Islam et al. 2020). Thereby, LEAP2 has been suggested for clinical use as an anti-obesity drug; indeed, obese subjects display high LEAP2 and low acyl-ghrelin in plasma after fasting (Mani et al. 2019). However studies performed in mice using synthetic GHS-R1a inhibitors, report contradictory results and suggest that high LEAP2 levels not only prevents ghrelin-induced food intake, but may also cause a form of ghrelin resistance (reviewed in Andrews 2019), hence discouraging its use in patients. On the contrary, LEAP2-neutralizing antibodies increase endogenous acyl-ghrelin activity and GH release (Ge et al. 2018), thus representing a potential therapeutic strategy to increase ghrelinergic tenor in the brain.

Overall, these studies highlight the complexity of GHS-R1a signaling, which depends on a combination of factors: circulating ligands, the receptor's constitutive activity, and the dimerisation with other GPCRs, ultimately activating dozens of different second messengers and intracellular pathways. The complexity of GHS-R1a signaling explains the multitude of functions in which ghrelin is involved: stem cell proliferation (Chung et al. 2013), cell differentiation (Gong et al. 2020); autophagy (Ferreira-Marques et al. 2016); gene expression and protein synthesis (Goto et al. 2006).

1.3 Mitochondrial network and homeostasis

1.3.1 Mitochondria network remodelling

Mitochondria are double membraned intracellular organelles, that originated one billion years ago when the aerobic bacterium SAR11 *clade Rickettstiales*, initiated an endosymbiotic relationship with an ancestral anaerobic eukaryotic cell. The bacterium slowly evolved into the organelle known as mitochondrion, while the host cell acquired the bacteria's ability to autonomously use oxygen for energy supply (Sagan 1967; Thrash et al. 2011). Nowadays, each eukaryotic cell contains thousands of mitochondria that form a highly dynamic network, constantly changing in number, morphology, connectivity, and subcellular localization, adapting to the energetic need of the cell. In neurones, the size and shape of these networks depends on highly regulated processes (recently reviewed in Fenton et al. 2021): fission/fusion, axonal transport, biogenesis and mitophagy.

Mitochondria fission and fusion are modulated by a large family of GTPases, known as dynamin-related proteins. One of them, dynamin-related protein 1 (DRP1) is a ubiquitous cytoplasmic protein (Imoto et al. 1998) whose activation induces a shift toward mitochondria fission (Shin et al. 1997; De Vos et al. 2005; Fonseca et al. 2019). DRP1 interacts with microtubules components such as tubulin polymers (Strack et al. 2013) and actin (Ji et al. 2015) that are assembled around the organelles, more specifically at the mitochondria-ER contact site (Friedman et al. 2011). With their guidance, DRP1 translocates on the outer mitochondrial membrane (OMM) where it binds to specific proteins (Ji et al. 2017) and lipids on the surface of the mitochondria, like cardiolipin (Stepanyants et al. 2015), that act as markers to flag the position of the fission site. Then DRP1 polymerises into ring-like structures around the organelles (Smirnova et al. 1998) and its GTPase domain cleaves the lipidic layer of the OMM, initiating mitochondrial fission (Kamerkar et al. 2018), while the proteins intervening in the fission of the IMM are less known. DRP1 requires specific post-translational modifications to activate the fission process. Notably, phosphorylation at serine residue 616 (s616) by cellular kinases such ask ERK1/2 results in activation of its GTPase activity (Kashatus et al. 2015), while phosphorylation at s637 by cAMP/PKA signaling inhibits it, suppressing

mitochondrial fission (Chang and Blackstone 2007). Many other, less studied, post-translational modifications have been identified. Phosphorylation at s40/44 by glycogen synthase kinase 3β (GSK3β) promotes fission and has been associated with oxidative stress vulnerability in AD (Yan et al. 2015); GSK3β can also phosphorylate s693, with the opposite effect of reducing mitochondrial fragmentation and increasing cell survival after oxidative stress (Chou et al. 2012); phosphorylation at s656 by cAMP/PKA inhibits fission, similarly to s637 (Cribbs and Strack 2007); lastly, phosphorylation at s585 by cyclin-dependent kinases 1 (CDK1)/cyclin B complex, mediates mitochondria division during mitosis *in vitro* (Taguchi et al. 2007). Ultimately, DRP1 can also be bound to small ubiquitin-like proteins or SUMO (small ubiquitin-like modifiers), affecting DRP1 localization and membrane translocation (Figueroa-Romero et al. 2009). All these processes are tightly regulated depending on cell type and developmental stage, further highlighting the complexity of DRP1 signaling and the sophistication of mitochondrial network remodelling and functioning.

Opposite to fission, the pathways that lead to mitochondrial fusion are less understood. The main enzymes involved are the dynamin family GTPases mitofusin 1 and 2 (Mfn1/2) anchored to the OMM, and OPA1 on the inner mitochondrial membrane (IMM). First, parental mitochondria are directed in proximity to the ER membranes, where the reaction occurs (Abrisch et al. 2020), and the OMMs are tethered, probably via Mfn1 (Ishihara et al. 2004). Indeed, in Mfn1-deficient cells, mitochondria are fragmented and fail to bind each other (Chen et al. 2003). Interestingly, Mfn2 is believed to have distinct functions, since depletion of Mfn2 in HeLa cells causes the formation of larger mitochondrial fragments (Eura et al. 2003). Once the OMMs are fused together, OPA1 mediates the fusion of the IMMs (Ban et al. 2017), and the mitochondrial content of the parental organelles are mixed, completing the fusion process. Interestingly, OPA1 is also required for the formation and stability of the IMM lipidic invaginations, named *cristae*, suggesting its role in structurally supporting the internal membranes and the overall structure of the organelles (Olichon et al. 2003).

Microtubules play a fundamental role in mitochondrial dynamics, guiding the organelles towards and away from the mitochondria-ER contact site where fission and fusion occur. Moreover, fission generates smaller mitochondria that can be more easily delocalised at further sites inside the cells (e.g., synapses and dendrites) or transported to lysosomes for degradation. Inside the neurones, mitochondria are transported along the axon, in a process known as "mitochondrial axonal transport", by the "motor proteins" kinesin, with direction from the soma to the axon, and dynein, that moves in the opposite direction (Pilling et al. 2006). The motor proteins bind the mitochondria membranes thanks to a family of adaptor proteins: TRAK1 and TRAK2 (sometimes referred to as their *drosophila* ortholog Milton), bind the OMM directly (van Spronsen et al. 2013), while Miro1 and Miro2 link TRAKs to the motor proteins, in a Ca^{2+} dependent way (López-Doménech et al. 2018). Moreover, actin is essential for anchoring the mitochondria at the synapses and dendrites (Gutnick et al. 2019), where a very intense ATP production occurs, in order to sustain the – energetically very costly – neuronal electrical activity (Howarth et al. 2012).

The processes described so far regulate size, content, and shape of the mitochondrial network, therefore remodelling the existing pool of organelles. On the contrary, biogenesis is a process that generates new mitochondria, and involves numerous enzymes, whose exact signaling pathway has not yet been entirely clarified. Firstly, the new mitochondrial proteins are either recruited from the pre-existing mitochondria, that redistribute their content via fission, or synthetised in the cytoplasm, from mRNA transcripts that are transcribed in the nucleus, and later transported inside the organelles (Miller and Hamilton 2012). Important players of this pathway are large transmembrane complexes of the mitochondrial import machinery, such as the translocase of the outer membrane (TOM) and the translocase of the internal membrane (TIM23). In particular, the TOM complex consists of seven components, three of which – TOM20, TOM22 and TOM70 – face the cytosolic side of the OMM (Ahting et al. 1999) and are therefore often used as markers to study mitochondrial morphology or number (Gelmetti et al. 2017; Fonseca et al. 2019). TOM and TIM enzymes mediate the internalisation of thousands of newly synthetised proteins from the cytosol into the mitochondrial matrix (Yamamoto et al. 2009; Waegemann et al. 2015), where the new proteins

are further processed and sorted into the relevant mitochondrial compartment. Interestingly a small number of proteins is produced within the mitochondrion by local transcription and replication of mitochondrial DNA-encoded genes. Indeed, each mitochondrion contains thousands of copies of mitochondrial DNA (mtDNA) (Wiesner et al. 1992), exclusively inherited from the maternal oocyte (Giles et al. 1980), that also need to be replicated during biogenesis. Each molecule consists of a double strand DNA, free of introns, that encodes 37 of the over 1300 genes that are necessary to the mitochondria (Anderson et al. 1981). MtDNA is loosely packed in large DNA-protein complexes, highly conserved among species, called nucleoids (Satoh and Kuroiwa 1991). During biogenesis, nucleoids are disassembled and a dedicated mitochondrial replication complex mediates mtDNA replication in the mitochondrial matrix, independently from the cell cycle, in either proliferating and post-mitotic cells (Magnusson et al. 2003).

Protein redistribution, synthesis and mtDNA replication, are precisely timed and finely controlled processes and their main regulator is the peroxisome proliferator-activated receptor coactivator 1α (PGC1α) (Puigserver et al. 1998). Cytosolic PGC1α is activated by either phosphorylation via pAMPK (Zong et al. 2002) or deacylation via SIRT1 (Nemoto et al. 2005), then it translocate to the nucleus where it binds to the nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR-γ) (Puigserver et al. 1998). Furthermore, AMPK phosphorylates and activates the histone acetyltransferase 1 (HAT1), creating a more relaxed chromatin-DNA structure (Marin et al. 2017) that favours the transcription of several transcription factors. Two of these, nuclear factor erythroid 2-related factors 1/2 (NRF1/2), activated by PPAR-γ, induce the expression of several nuclear encoded mitochondrial proteins, including the transcription factor A (TFAM) (Virbasius et al. 1993; Wu et al. 1999), one of the main components of mitochondrial nucleoids, that is involved in the final steps of mtDNA replication (Kaufman et al. 2007). Another important target of the PGC1α/PPAR-γ complex are the mitochondrial uncoupling proteins (UCP) (Kelly et al. 1998), a superfamily of enzymes inserted in the IMM that exert a variety of functions, from thermogenesis to oxidative stress reduction (see section 1.3.3).

Ultimately, mitochondrial biogenesis increases the energetic production of the cell, but also reduces oxidative stress by replacing malfunctioning mitochondria that have been degraded. Indeed, mitochondrial protein synthesis (Williams et al. 1987), biogenesis (Irrcher et al. 2003), and PGC1α expression (Pilegaard et al. 2003) are significantly increased in the muscle during physical exercise. Contrarily, impaired mitochondrial biogenesis has been associated to numerous diseases, including neurodegeneration (Golpich et al. 2017), while PGC1α signalling has been shown to be neuroprotective against oxidative stress and cell death (St-Pierre et al. 2006), further establishing the importance of these organelles in regulating cell homeostasis, especially in neurones.

1.3.2 Electron transport chain and ROS

Mitochondria are involved in numerous processes, such as calcium homeostasis (Lee et al. 2018b), lipid metabolism and myelin stability (Viader et al. 2013), apoptotic signaling (Joza et al. 2001), axonal transport (Chamberlain and Sheng 2019), and synaptic transmission (Lee et al. 2018a). However, their principal role is the maintenance of the bioenergetics of the cell by coupling redox catabolic processes, such as Krebs cycle and glycolysis – that collects the energy produced from glucose and lipid metabolism – with molecular oxygen acquired through respiration, in a process known as oxidative phosphorylation (OXPHOS), that produces over 90% of the total ATP in the cell (Keilin 1925; Chance and Williams 1956). This process occurs in the IMM, in a large enzymatic complex known as the electron transport chain (ETC) (figure 1.5) (Mitchell 1961), comprising four transmembrane proteins (complex I to IV) and two small electron transporters, ubiquinone (CoQ) and cytochrome c (cytC). These enzymes can alternatively accept and donate electrons (figure 1.5, blue arrows) to generate a proton (H^+) gradient (figure 1.5, green arrows), that increases the difference of electric potential ($\Delta\Psi_m$) (figure 1.5, orange arrow) between the inside and outside of the IMM - also known as mitochondrial membrane potential (MMP). An elevated MMP, activates the ATP synthase (sometimes referred to as complex V), a large rotating ion channel, sensitive to H^+ concentrations. When the channel is open, the H^+ freely transit through the IMM following a concentration gradient and releasing a high amount of energy, that is used to generate ATP (Chance and Williams 1956; Elston et al. 1998).

Interestingly, the lipidic *cristae* of the IMM form small pockets that significantly increase its area, so that the IMM is approximately 5 times wider that the OMM (Sjöstrand 1977; Perkins et al. 1997). Cells with more energetic demands, such as muscle cells and neurones, show even more *cristae*, significantly increasing the number of ETC complexes and, consequently, ATP production (Varanita et al. 2015). With such intense activity, the number of redox reactions occurring inside the cell is very high, resulting in the formation of reactive oxidative species (ROS), commonly known as free radicals, produced when an oxygen molecule (O_2) is reduced from a single electron "leaking" from the respiratory chain, and becomes the ROS precursor superoxide ion ($\cdot O_2^-$) (figure 1.5) (reviewed in Turrens 2003).
This process is highly dependent on the MMP: the higher the MMP, the higher the number of leaking electrons from the ETC (Nobes et al. 1990). Interestingly, although this process was originally thought to be random, there are now evidences showing that only 5% of the leak occurs through the lipidic layer (Brookes et al. 1997), while the remaining leak is controlled by a class of integral enzymes, the adenine nucleotide translocases (ANT) (Brand et al. 2005). Indeed, the electron transport process is not 100% efficient and, under physiological conditions, between 0.2 and 2% of the electrons in the ETC are used to generate ROS, more specifically at the CI and CIII (Brand 2010) (figure 1.5). ROS produced in this process act as second messenger, taking part in signaling pathways such as cell growth (Simon et al. 1998), proliferation and differentiation (Feng et al. 2019b), in numerous cell types including neurones (Tsatmali et al. 2006; Huo et al. 2009), where they also regulate synaptic plasticity (Betzen et al. 2009) and Ca^{2+}-mediated LTP in the hippocampus (Huddleston et al. 2008). Among other things, ROS have been shown to activate the AMPK pathway during hypoxia, resulting in activation of protective mechanisms, such as autophagy, and inhibition of mTOR (Emerling et al. 2009).
The amount of ROS generated during mitochondrial respiration determines which of these pathways is activated; however, high amount of ROS are dangerous for the cells since they are able to oxidize cellular lipids and proteins, resulting in inflammation and cell toxicity (figure 1.5) (Gargalovic et al. 2006; Kagan et al. 2017). Therefore, their intracellular concentration must be carefully monitored.

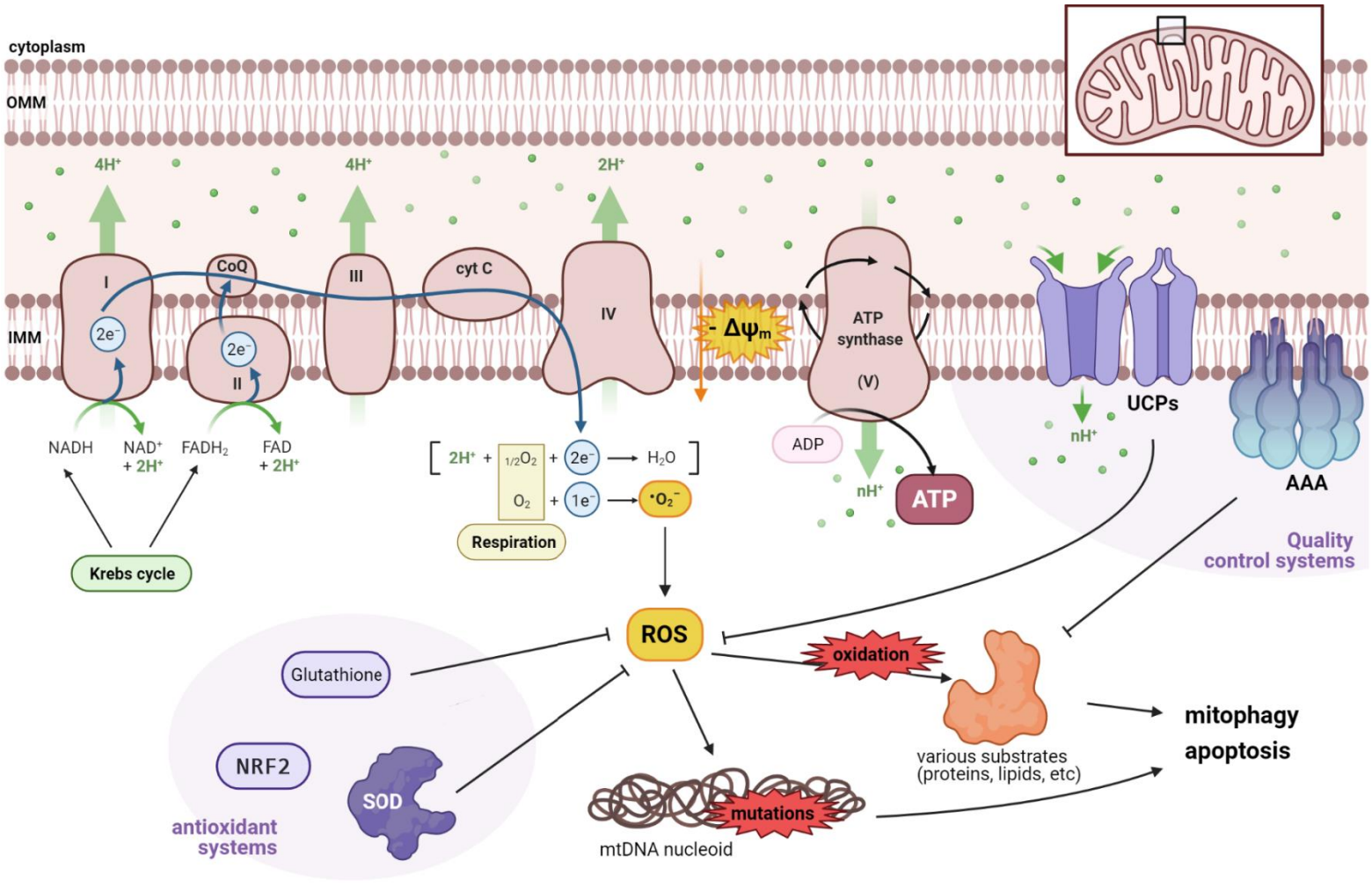


Figure 1.5 Mitochondrial ETC and ROS production

(Full legend in the next page)

Figure 1.5 Mitochondrial ETC and ROS production (full legend)

Mitochondrial ETC consists of four transmembrane complexes (I to IV), two electron transporters (CoQ and cytC), and the ATP synthase (V). Hydrogen ions (H^+, green arrows) are actively pushed through the IMM, producing a gradient that generates a variation in the MMP ($\Delta\Psi_m$, orange arrow). The electrons (blue arrows) move through the complexes and reduce oxygen to produce water. ROS are produced when a single electron 'escapes' the ETC, forming the superoxide ion. Oxidation of biological substrates and mtDNA mutations may induce mitophagy and, ultimately, cell death via apoptosis. Endogenous antioxidants and quality control systems may counteract the damage and reduce ROS production (*created with BioRender.com*).

1.3.3 Mitochondrial quality control

Antioxidant molecules are strongly present in nature, therefore they could be introduced with the diet, such as the case of vitamin E (Mattill 1927). Moreover, cells are equipped with diverse antioxidant systems that dispose of the ROS produced in excess (figure 1.5, bottom left). Most of these systems are modulated by the nuclear factor erythroid 2-related factor 2 (NRF2): upon oxidative stress NRF2 – physiologically present in its inactive form in the cytoplasm – is phosphorylated and translocates to the nucleus, where it binds to antioxidant response elements (AREs) in the DNA (Murphy and Park 2017). NRF2 increases the expression of numerous antioxidant proteins such as superoxide dismutases (SOD) (McCord and Fridovich 1969), enzymes involved in glutathione synthesis (Meister and Anderson 1983) and the heme oxygenase 1 (HO-1) (Alam et al. 1999). Moreover, NRF2 induces the expression of the genes involved in mitochondrial biogenesis such as PGC1α (Gureev et al. 2019), the mitochondrial transcriptional factor A (TFAM) and NRF1, further contributing to reducing oxidative species in the cell (Athale et al. 2012; Gureev et al. 2019).

If endogenous production exceeds the antioxidant capability of the cell, ROS may accumulate ultimately leading to cell damage.

In the cells, ROS are mostly responsible for mtDNA mutations (Aruoma et al. 1989; Kowaltowski and Vercesi 1999; Hollensworth et al. 2000). As previously mentioned, the number of mitochondria per cell can vary considerably, however, the number of mtDNA copies per cell is maintained relatively constant – within a certain range – in each cell type. Mostly cell types in culture have between 1000 and 5000 copies, while cells extracted from human biopsies show quite some variability, with the highest copy number – 400,000 – being found in oocytes – whose mitochondria are transmitted entirely to the progeny and therefore need to sustain the development process of the embryo (Wai et al. 2010). Because of the large number of copies, random mutations during mtDNA replication are very frequent (Gorman et al. 2015), ultimately leading to mitochondria carrying different version of the genome in the same cell. This phenomenon, called heteroplasmy, is still largely unknown and has been shown to be silently present in most of the global population (Irwin et al. 2009; Payne et al. 2013; Ye et al. 2014), whilst it can seldom be associated to mitochondrial diseases (Ye et al. 2014). Although mtDNA undergo mutations more frequently than nuclear DNA, mitochondrial function is not affected as much – probably due to a redundancy effect, where healthy mtDNA copies compensate for the defective ones (Wallace and Chalkia 2013). However, in the long term, the accumulation of ROS-induced mtDNA mutations can reduce mitochondrial efficiency and lead to cellular toxicity (Taylor et al. 2003) and even more ROS production (Vives-Bauza et al. 2006; Indo et al. 2007; Park et al. 2009), initiating a vitious cycle of oxidative stress and mitochondrial impairment.

To rescue mitochondrial damage after ROS overproduction, cells activate various quality control processes. For instance, mixing the existing pool of mitochondria via fusion/fission, results in redistribution of the mutated mtDNA copies among hundreds of organelles (Mouli et al. 2009), so that ultimately each mitochondrion would contain mostly wild type mtDNA copies. Moreover, interleaved in the IMM there are several proteases of the AAA superfamily (ATPases Associated with a variety of cellular Activities) (figure 1.5), whose function is to mediate proteolytic degradation of unfolded, oxidated or defective proteins of the ETC (Langer et al. 2001), while the cytoplasmic proteasomal machinery removes defective proteins in the OMM (Karbowski and Youle 2011).

An important protection mechanism is mediated by the UCP family. When the MMP raises significantly, it also increases the chance of proton leak and, consequently, the production of ROS (Korshunov et al. 1997). In this situation, a molecule or pathway able to induce a membrane depolarisation (reduction of the MMP), should significantly reduce proton leaking, preventing ROS from being produced.

UCPs are ion transporters whose main function is to dissipate the proton gradient generated during mitochondrial respiration, by providing an alternative route for H^+ to pass through the IMM (reviewed in Hass and Barnstable 2021). This could result in diverse outcomes, depending on the cell type and the UCP isoform.

The first uncoupler to be discovered, UCP1, was cloned from rodents in 1985 and is expressed exclusively in brown adipocytes (Bouillaud et al. 1985; Jacobsson et al. 1985). It was originally named thermogenin, since its function is to convert the proton gradient into heat (instead of ATP) and to regulate body temperature (Klaus et al. 1991). Interestingly, its homologues in plants, StUCP (Laloi et al. 1997) and AtUCP (Nogueira et al. 2011), are also involved in thermogenesis and stress response in cold weather, suggesting a high degree of conservation across species and kingdoms.

Identified in rodents a decade later, UCP2 (Fleury et al. 1997; Gimeno et al. 1997) is present at low levels in most tissues (Pecqueur et al. 2001), but is especially abundant in proliferating cells that rely on glycolysis as a main energetic source, such as stem cells (Zhang et al. 2011), and activated T cells (Rupprecht et al. 2012). Contrary to UCP1, UCP2 does not have a thermogenic function; instead, it has been shown to be particularly responsive to ROS, and to exert a protective function against oxidative damage in several cell types. In PC12 neuronal cells, overexpression of UCP2 significantly reduces cell death after H_2O_2-induced oxidative stress (Horvath et al. 2003). Similarly, retinal cells of transgenic mice expressing human UCP2 showed much greater survival rate, after injection in the eye of the excitotoxic kainic acid (that overstimulates neurones resulting in death) compared to wild type animals, and even more compared to UCP2 KO models (Barnstable et al. 2016). Interestingly, UCP2 has been shown to increase mitochondrial fission and degradation after severe stress, such as hypoxia in lung cells (Haslip et al. 2015) or ischemia in kidney tissue (Qin et al. 2019), further supporting its ubiquitous role in counteracting oxidative insult.

Other, less characterised members of the human UCP family are: UCP3 (Vidal-Puig et al. 1997), expressed in the heart and skeletal muscle, where it facilitates FA transport inside mitochondria during biogenesis (Hilse et al. 2018); UCP4 (Mao et al. 1999), almost exclusively expressed in the brain and associated to neuronal development (Smorodchenko et al. 2009) and neuroprotection (Cho et al. 2017); UCP5, also known as BMCP1 (Sanchis et al. 1998), expressed in several organs, including the brain, where it has been found to be neuroprotective (Kwok et al. 2010); and UCP6, or KMCP1, first identified in the kidney, that show a strong sequence homology to UCP5, although it has not been reported to have any uncoupling activity (Haguenauer et al. 2005).
Ultimately, these data show that mitochondria uncoupling may represent a strong, cell-specific, protection strategy against oxidative stress and related damage.

However, if the oxidative stress is prolonged, cells activate one last protective mechanism called mitophagy, a combined effect of fusion, fission and lysosomal-mediated autophagy that specifically isolate and degrade impaired mitochondria (Twig et al. 2008), in order to prevent the spread of oxidised proteins and lipids within the organelle network. Several mitophagic pathways have been recently identified in mammals (Di Rita et al. 2018; Yamada et al. 2019). The best characterised process – described only ten years ago (Jin et al. 2010; Narendra et al. 2010) – is mediated by the phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1) and the cytoplasmic E3 ubiquitin ligase, Parkin. In physiological conditions, PINK1 is mostly located in the cytosol. However, its protein sequence contains a mitochondrial transport region (MTS) that allows its relocation at the OMM (Silvestri et al. 2005; Lin and Kang 2008), where the TOM complex mediate its import and the TIM23 anchors PINK1 to the IMM (Lazarou et al. 2012). There, PINK1 is cleaved sequentially by the mitochondrial processing peptidase (MPP) (Greene et al. 2012) and the presenilin-associated rhomboid-like (PARL) (Deas et al. 2011) and the resulting shorter peptide is quickly degraded via proteasome (Yamano and Youle 2013). Indeed, using the most common laboratory techniques, PINK1 is barely detectable inside the healthy cells, unless it is stabilized or overexpressed (Lin and Kang 2008; Yamano and Youle 2013).

The aforementioned process requires intact mitochondria. Indeed, ROS and heteroplasmy may cause severe mitochondrial damage, resulting in the reduction of the MMP (Chinopoulos et al. 1999; Ye et al. 2014), that initiate the mitophagic pathway. More specifically, when the membrane is depolarized PINK1 is not cleaved and accumulates at the OMM where it is stabilized with the kinase domain accessible to cytosolic substrates (Zhou et al. 2008). Once activated by auto-phosphorylation (Aerts et al. 2015), uncleaved PINK1 recruits cytosolic Parkin at the OMM (Matsuda et al. 2010; Vives-Bauza et al. 2010). Parkin is a cytoplasmic E3-ubiquitine ligase, ubiquitously expressed, whose function is to ligate a chain of poly-ubiquitin on the surface of defective proteins, targeting them to the ubiquitin-proteasome system (UPS) for degradation (Imai et al. 2000; Shimura et al. 2000; Zhang et al. 2000). Parkin ligase activity relies on its ubiquitin-like domain, that is usually kept inactivated by intramolecular interactions (Chaugule et al. 2011; Ham et al. 2016). After it is recruited to the mitochondrion, full length (but not cleaved) PINK1 phosphorylates Parkin's ubiquitin-like domain (Kondapalli et al. 2012; Shiba-Fukushima et al. 2012; Kazlauskaite et al. 2014), freeing it from its auto-inhibition (Gladkova et al. 2018). At the same time, PINK1 phosphorylates free ubiquitin molecules (Kane et al. 2014; Koyano et al. 2014), making them accessible to Parkin's catalytic domain. Once activated, Parkin transfers small chains of poly-phospho-ubiquitin to numerous substrates in the OMM, forming transitory thioester bonds with the substrates (Iguchi et al. 2013; Zheng and Hunter 2013). Noteworthy targets of Parkin are: Mfn1/2, whose ubiquitin-induced degradation results in the inhibition mitochondrial fusion and induction of fission, concurring to separate the damaged mitochondrial components from the rest of the network (Gegg et al. 2010); and Miro, whose degradation causes the arrest of mitochondrial axonal transport (Wang et al. 2011). Moreover, poly-ubiquitinated substrates mobilise ubiquitin-binding autophagic receptors, such as p62 that binds the microtubule-associated-protein 1 light chain 3 (LC3) on the autophagosome surface, causing the two organelles to merge (Geisler et al. 2010). Lastly, LC3 is cleaved at the C-terminus, forming LC3-II (Kabeya et al. 2004), the mitochondrion/autophagosome combined content is released inside the lysosome and the damaged molecules are degraded by lysosomal hydrolases (Narendra et al. 2008).

Interestingly, this process may also involve smaller mitochondrial portions. Indeed, mitophagy has been shown to be preceded by extensive DRP1-mediated mitochondrial fission (Gomes and Scorrano 2008) that generates uneven subpopulations of organelles. This occurs when damaged proteins or mutated mtDNA nucleosomes are separated by the rest of the organelles and embedded in smaller mitochondrial-derived compartments (MDCs), some of which exhibits low MMP and are selectively targeted for degradation (Twig et al. 2008). A second independent mechanism is a membrane-budding process that generate mitochondria-derived vesicles (MDVs) that could also be transported to the lysosomes for selective mitophagy (Soubannier et al. 2012). However, this specific pathway has been shown to be independent of DRP1 and to also mediates communication among organelles (Sugiura et al. 2014), suggesting it may also be implicated in mitochondrial quality control.
Ultimately, mitophagy is an essential process for maintaining mitochondrial health and cell survival. Indeed, mutations in key proteins of the mitophagic pathway are associated to familiar PD and it will be better described in chapter 4.

When all the quality control pathways fail to repristinate mitochondrial function, cells trigger the apoptotic signalling mediated by enzymes of the B cell lymphoma 2 (BCL2) family. In particular, BCL-associated X protein (BAX) is incorporated in the OMM and oligomerises with BCL2-homologous antagonist/killer (BAK) (Chen et al. 2005; Kuwana et al. 2005) in specific sites, stabilising DRP1 on the OMM and inducing apoptotic mitochondrial fragmentation (Wasiak et al. 2007). BAX/BAK oligomerisation also results in abnormal increase in mitochondrial permeability, MMP dependent (Gottlieb et al. 2003), and OPA-1 mediated *cristae* remodelling that provokes the extrusion of damaged materials from the organelles (Scorrano et al. 2002; Yamaguchi et al. 2008). The electron transporter cytC is released from the IMM and binds the cytoplasmic apoptotic protease activating factor 1 (Apaf1) to form a large structure called apoptosome (Zou et al. 1997), ultimately resulting in the activation of the caspase-9/caspase-3 apoptotic pathway (Li et al. 1997).
In a separate process, severe mitochondrial damage causes high levels of Ca^{2+} in the mitochondrial matrix, triggering the opening of membrane-spanning mitochondrial permeability transition pores (mPTP) on the IMM. mPTP act as a release valve to reduce Ca^{2+} overload (Elrod et al. 2010) and induce *cristae*

remodelling (Bernardi et al. 2015) via a not yet well identified mechanism that may occur within the same BAX/BAK pathway (Scorrano et al. 2002).

Ultimately, the release of mtDNA fragments – from either BAX/BAK pores or mPTP – may induce severe inflammation via translocation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kβ) (Giampazolias et al. 2017) and activation of the pro-inflammatory cytokine interferon 1 (IFN-I) response (Saito et al. 2019), ultimately leading to cell death in several organs (Oka et al. 2012; Ding et al. 2014), including the brain (Patrushev et al. 2006; Kalani et al. 2018).

Notably, the pharmacological inhibition of mPTP with cyclosporin A has been shown to mitigate mitochondrial dysfunction (Bernardi et al. 2015). Similarly, cells lacking both BAX and BAK genes are resistant to pro-apoptotic compounds and mitochondrial toxins (Wei et al. 2001).

Indeed, targeting mitochondrial-mediated cell death has been pointed out as a prospective clinical strategy against several age-related neurodegenerative disorders (Wu et al. 2019; Dey et al. 2020; Shevtsova et al. 2021).

1.3.4 Free radical theory of ageing

Ageing is traditionally defined as the progressive deterioration of physiological capacities – or rather the accumulation, with time, of cellular and molecular changes associated to diminished responsiveness (resilience) to environmental stresses and increased vulnerability to diseases.

The molecular mechanisms of ageing are still highly debated among scientists, although one of the theories with most consensus is the mitochondrial theory of ageing, that points to ROS accumulation as the major contributor to the process. This theory, originally known as the "free radical theory of ageing", was formulated in 1956, after the first scientific evidences showed that the concentration of highly active oxidative species – physiologically produced in every living systems – increase with increasing metabolic activity and correlates with reduced lifespan, similarly to what happens after long exposure to radiations (Harman 1956).

Although originally contested from the scientific community, this theory was soon confirmed by numerous studies. Oxidative damage – measured as the amount of oxidized nucleosides – has been shown to significantly increase in several areas of the human brain, in both nuclear DNA and mtDNA (Mecocci et al. 1993), while

extensive mtDNA mutations have been detected by single cell qPCR in the SN of aged healthy humans (Kraytsberg et al. 2006).

Combining a serious of indirect evidences, this theory – later renamed "mitochondrial theory of ageing" – largely anticipated the role of oxidative stress and DNA damage in ageing and cancer, pointing at mitochondria's ROS as the major cause of ageing and age-related disorders (Harman 1972; Barja 2019).

Interestingly, the increment in life expectancy resulting from the CR regime is frequently suggested as opposing the ageing process attributed to the mitochondrial free radical theory of ageing (Barja 2007; Afanas'ev 2010). Indeed, a significantly diminished mitochondrial ROS production, per unit of O_2 consumed, seems to occur during CR, without impairing ATP production (López-Lluch et al. 2006). This evidence suggests that O_2 consumption and ROS production are independently regulated and that a CR regimen – or CR mimetics such as acyl-ghrelin – may prevent oxidative stress without affecting cellular metabolism, therefore representing a valid therapeutic strategy against mitochondrial ageing.

On the contrary, some studies show that CR may actually increase ROS production and that pharmacological treatments with anti-oxidant compounds may have detrimental effects in the long-term, such as pro-tumorigenesis and reduced lifespan (reviewed in Ristow and Zarse 2010).

It has been hypothesised that CR-mediated increase in ROS – although subtle – would induce the cells to activate an adaptive response to oxidative stress, ultimately leading to increased resilience in the long term. Opposite to Harman's theory, this hypothesis named "mitochondrial hormesis" or just "mitohormesis", suggests that a defined amount of intracellular ROS is required at all times, in order to promote cell health and prevent age-related disorders (Tapia 2006).

This theory does not completely overcome Harman's, rather it complements it by highlighting the fragility of all the biological systems. Indeed, ageing – just like any other disease and biological process – is just the result of an imbalance between the numerous factors constantly stimulating our systems, pulling in different directions. Hence, improving our understanding of this balance, is the key to finding solutions at improving life quality and preventing age-related diseases.

1.3.5 *In vitro* models of oxidative damage and neurodegeneration

The neural processing of information is metabolically very expensive: the brain uses approximately 20% of the whole body oxygen consumption, despite being only the 2% of the total weight (Kety 1957), therefore the OXPHOS process is particularly important in neurones. However, ROS production exponentially increases with age and since neurones are post-mitotic cells, hence survive many decades with little renewal, ROS will accumulate with time, ultimately resulting in neuronal cells being more susceptible to oxidative stress than other cell types.
Complex V impairment, with consequential loss of efficiency of the ETC system and ROS hyperproduction, have been associated with accelerated ageing (Warnsmann et al. 2021), and mtDNA mutations were found to accumulate in the brain of aged subjects with brain diseases (Bender et al. 2006). Moreover, ROS are considered to be key components in the aetiogenesis of several age-related neurodegenerative diseases, such as PD (Floor and Wetzel 1998; Yamamoto et al. 2007; Ikawa et al. 2011; Burbulla et al. 2017) and AD (Smith et al. 1998; Padurariu et al. 2010; Angelova et al. 2015; Mota et al. 2015). Interestingly, specific neuronal sub-populations show increased susceptibility to oxidative stress. This is the case of dopaminergic neurones in PD, and the hippocampal neurones of the CA1 in AD, as it will be better described in chapter 4 and 5, respectively.

The maintenance of a healthy population of mitochondria is essential for cell survival and to prevent age-related neuronal disorders. However, studying mitochondrial health directly in the brain of the patients is challenging, and animal models cannot fully recapitulate the complexity, length (several decades) and pathogenesis of human cognitive disorders. In the last twenty years, researchers have been focusing on *in vitro* systems such as human stem cells-derived neurones. These neurones originate from human pluripotent stem cells that can be guided towards maturation using either chemicals or genetic engineering. Hundreds of protocols are currently available for differentiation into specific cell types such as dopaminergic neurones (Zhang et al. 2014), cortical neurones (Hirabayashi et al. 2004), astrocytes (Krencik and Zhang 2011), retina cells (Mandai et al. 2017) and many others specialised cell populations that are otherwise difficult to obtain from patients.

In practice, stem cell-derived neurones currently represent one of the best models of human brain maturation – much closer, genetically and functionally, than animal models. However, in order to recapitulate specific disease-relevant phenotypes, it is often necessary to treat the cells with a toxin or drug that would induce such phenotype – sometimes even in cells that are already carrying disease-relevant mutations (Mertens et al. 2018) – therefore removing the "slow accumulation" element of neurodegenerative diseases from the system.

Several toxins can be used to induce mitochondrial damage and to simulate *in vitro* the accumulation of intracellular stress naturally occurring during ageing. For instance, long exposures to mitochondrial toxins, like the uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP) that causes membrane depolarization (reduction of MMP), induce severe mitochondrial damage, as can be determined by measuring specific mitochondrial markers, such as TOM20, cytC, and OPA1 (Geisler et al. 2010). Similarly, drugs such as rotenone could be used to induce PD-like symptoms in mice and PD-like molecular changes in cells, as it will be better described in chapter 4.

In these models, MMP can be taken in consideration as a measure of mitochondrial health. Several MMP probes, such as the MitoTracker™ Orange and many others, have been developed for research purposes based on the principle that positively charged fluorescent molecules that are able to cross the OMM (lipophilic), would accumulate in the intermembrane space (IMS), proportionally to the amount of its charge (the extent of the depolarisation) (Smith et al. 2003; Perry et al. 2011b; Logan et al. 2016). Most of these probes act as molecular sensor of oxidative stress: once in the IMS, they are oxidised by the ROS produced by actively respiring cells and emit a specific fluorescent signal. Once fixed, the intensity and localisation of the stain inside the cells allows indirect measure of mitochondria oxidative activity – the stronger the fluorescence, the higher the MMP – while preserving the cellular 3D structure (Agnello et al. 2008).

Following oxidative stress, disruption of the MMP can also induce changes in mitochondrial gene expression and protein synthesis (Guantes et al. 2015), ultimately reducing or inhibiting physiological processes such as mitochondria fission/fusion and biogenesis. Hence, molecular markers such as phosphorylated DRP1 (Cereghetti et al. 2008; Xu et al. 2016) or PGC1α (Carré et al. 2010; Davinelli et al. 2013), could also be taken in consideration to evaluate mitochondrial health.

Moreover, several protocols and commercial kits are available to analyse other aspects of mitochondrial health, both *in vitro* and *in vivo*, such as ATP production (Wibom et al. 1990; Shepherd et al. 2006; Salin et al. 2016) and oxidative stress (Sano et al. 1998; Roberts and Morrow 2000; Valgimigli et al. 2001; Giustarini et al. 2009; Marrocco et al. 2017). Lastly, a common way to quantify mitochondria activity consists of monitoring the oxygen concentration in the media of cultured cells or isolated mitochondria using specific oximeters, such as the Seahorse FX analyser (Wu et al. 2007).

In sum, each of the aforementioned protocols give helpful insights into mitochondrial health and function; however, due the complexity of the system and the variability of the models, a combination of diverse methodology is required in order to trace a comprehensive picture of mitochondria health *in vitro*.

1.3.6 Ghrelin regulates mitochondrial health via pAMPK

Since the discovery of its involvement in memory and brain function, an increasing number of studies have shown that acyl-ghrelin is also a key-mediator of mitochondrial health in several tissues. Acyl-ghrelin binds to GHS-R1a and induces AMPK phosphorylation via Ca^{2+}-sensitive kinases, as shown in section 1.2.9. Indeed, acyl-ghrelin increases AMPK phosphorylation – quantified as the ratio between pAMPK and total AMPK – in the SN of intraperitoneal-injected mice, as well as in cultured dopaminergic cell line SN4741 (Bayliss et al. 2016a).

As previously mentioned, in the hypothalamus, AMPK serves as a cellular energy sensor and its phosphorylation is regulated by a group of metabolic hormones, including ghrelin, leptin and insulin (Andersson et al. 2004; Minokoshi et al. 2004). Once activated, pAMPK phosphorylates a series of targets involved in cell metabolism, such as the acetyl-CoA carboxylase (ACC), whose main function is to regulate lipid oxidation and provide mitochondria with FA during biogenesis (Hu et al. 2003) (figure 1.6, point 1).

pAMPK also phosphorylates DRP1 at different sites, to modulate the remodelling of mitochondrial networks (figure 1.6, point 2): in case of an energy shortage, pAMPK would favour DRP1 phosphorylation at the anti-fission site s637, resulting in the organelles fusing in a large tubular network in order to maximize energy production (Wikstrom et al. 2013); on the other side, a more severe oxidative

stress would preferentially trigger mitochondria fission, with pDPR1 s616 interacting with Mfn1/2, resulting in the containment of damaged mitochondria and their consequential degradation (Zheng et al. 2018). Notably, two studies performed in mice reported that either a life-long 40% CR regime (Khraiwesh et al. 2013), or treatment with a CR mimetic compound (Liu et al. 2015) increases total DRP1 expression as well as the number, size and surface area of mitochondria, in the liver and brain respectively. These data suggest that CR affects DRP1 by favouring mitochondrial fission (phosphorylation at s616), however more studies are needed to determine if this effect is mediated by acyl-ghrelin. Preliminary studies from our group, showed that acyl-ghrelin treatment of SN4741 mouse neuronal cells significantly increase s616 phosphorylation (unpublished). Interestingly, it has been recently shown that activation of the ERK1/2 pathway results in preferential DRP1 phosphorylation at s616 and reduced MFN1 oligomerisation, overall increasing mitochondrial fission (Pyakurel et al. 2015; Prieto et al. 2016). Since acyl-ghrelin is a strong activator of the ERK pathway, via pAMPK, in several tissues and models (Zhang et al. 2007; Liang et al. 2012; Wang et al. 2012b; Waseem et al. 2014; Wang et al. 2020), it is reasonable to hypothesise that acyl-ghrelin is indeed the mediator of CR-induced DPR1 phosphorylation at s616. However, more studies need to be performed to investigate the role of the GHS-R/pAMPK/ERK pathway in mitochondrial fission and fusion balance.

As mentioned in section 1.2.9, pAMPK works along with SIRT1 to mediate mitochondrial health (Cantó et al. 2009). One of the key target of pAMPK/SIRT1 pathway, is PGC1α that then translocates to the nucleus and induces the expression of numerous enzymes of mitochondria biogenesis, like the nuclear respiratory factor 1 (NRF1) involved in mtDNA replication and cell growth (Bergeron et al. 2001) (figure 1.6, point 3). Accordingly, acyl-ghrelin injection in rodents, promotes mitochondrial respiration and increases mitochondrial number in NPY neurones in the hypothalamus, and this effect is abrogated in AMPK KO mice (Andrews et al. 2008). Interestingly, CR has also been shown to increase mtDNA copy number and to stimulate mitochondrial biogenesis via PGC1α; moreover using an *in vitro* model of CR, in which cell nutrients were reduced although maintained balanced, MMP and ROS generation were both significantly reduced, without negatively affecting ATP production (López-Lluch et al. 2006).

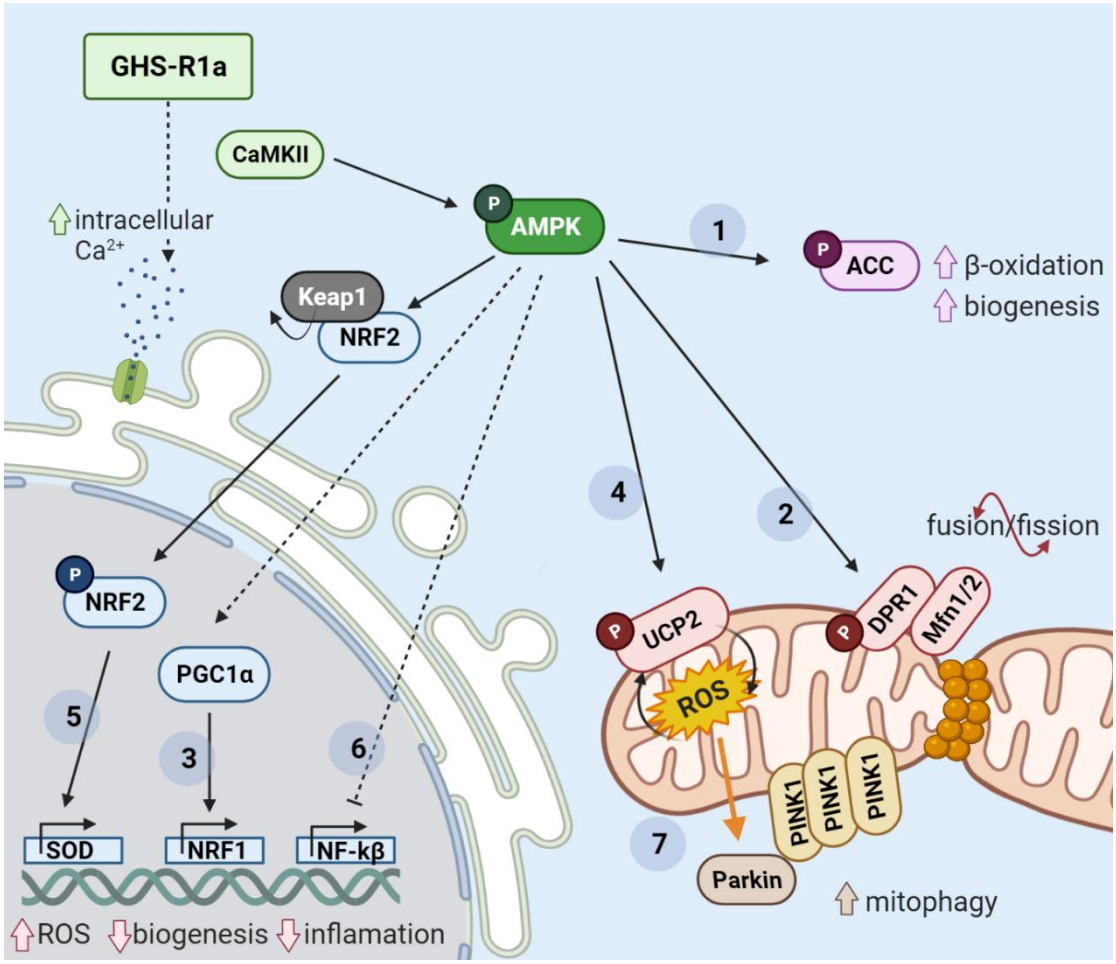


Figure 1.6 AMPK-mediated ghrelin effects on mitochondria homeostasis

Acyl-ghrelin binds to GHS-R1a resulting in phosphorylation of AMPK and activation of numerous downstream pathways involved in mitochondrial health. Three significant targets of pAMPK are: (**1**) ACC, involved in lipid metabolism; (**2**) DRP1, whose phosphorylation state determine the balance between fission and fusion; (**3**) PGC1α, resulting in increased mitochondria biogenesis; (**4**) UCP2 and (**5**) NRF2, resulting in reduced oxidative stress. (**6**) Moreover, pAMPK pathway also results in repression of pro-inflammatory factors. (**7**) Lastly, if the oxidative stress persists, acyl-ghrelin favours PINK1/Parkin mediated autophagy (*created with BioRender.com*).

In rodents, peripheral injected acyl-ghrelin increases UCP2 mRNA levels in NPY neurones in the hypothalamus of wild type animals, while ghrelin-mediated increase in mitochondrial number is abrogated in UCP2 KO animals (Andrews et al. 2008) (figure 1.6, point 4), suggesting that UCP2-mediated MMP uncoupling may also represent a potential molecular mechanism behind ghrelin-mediated neuroprotection.

The acyl-ghrelin/pAMPK pathway has also been shown *in vitro* to phosphorylate NRF2, which separates from its cytoplasmic inhibitor Keap1, translocates to the nucleus and increases gene expression of anti-oxidant enzymes (Wang et al. 2020) (figure 1.6, point 5), and prevents activation of immune cells, as reported from the reduction of the microglial marker IBA1 and the astrocytic GFAP (Bayliss et al. 2016a). Moreover acyl-ghrelin reduces the expression of pro-inflammatory cytokines, such as the plasminogen activator inhibitor type 1 (PAI-1) and the tumour necrosis factor α (TNF-α), by inhibiting the inflammatory pathway mediated by the nuclear factor kappa-light-chain-enhancer of activated β-cells (NF-kβ) (Ding et al. 2008; Moon et al. 2009) (figure 1.6, point 6).

Lastly, acyl-ghrelin preserves cellular health, by inducing PINK1/Parkin- mediated degradation of damaged mitochondria in conditions of extreme oxidative stress, via the AMPK/PGC1α pathway (Wang et al. 2021), although the exact conditions that determine the shift between a pro-survival response and induction of mitophagy, have not been entirely clarified yet (figure 1.6, point 7).

In summary, CR and acyl-ghrelin act as cellular regulators of mitochondrial homeostasis, aiming at both preserving/increasing them and degrading them when they are dysfunctional. Moreover, these evidences strongly point to pAMPK as being the key mediator of the ghrelinergic axis activity on mitochondrial function in the brain. Since mitochondria are involved in every possible mechanism in the body, more studies are needed to further characterise the role of the ghrelinergic axis in mitochondrial health, and to determine whether it is possible to target mitochondria via the ghrelinergic system, to restore cell health in brain diseases.

1.4 The ghrelinergic system in the human brain

1.4.1 Brain accessibility

Ghrelin mRNA is undetectable in the brain, therefore most, if not all, of the ghrelin in the brain is transported from the stomach via the circulation. In order to interact with GHS-R in the brain, circulating peptides need to cross the blood-brain-barrier (BBB) (figure 1.7 A), a highly stringent structure formed by three elements: endothelial cells from the capillary side, with numerous tight junctions that determine its selectivity; "end-feet" astrocytes that surround the external wall of the capillary, which are involved in the formation and maintenance of the barrier; and pericytes that provide structural support (Ballabh et al. 2004). Banks and colleagues showed that intracranial-injected radio-labelled human acyl-ghrelin is able to cross the BBB in mice, in both blood-to-brain and brain-to-blood directions, through a saturable system – most likely an active transport – while human unacylated ghrelin enters the brain by non-saturable transmembrane diffusion (Banks et al. 2002). These data were later supported by other studies (Diano et al. 2006; Pan et al. 2006), suggesting that the BBB plays a major role in determining ghrelin concentration in the brain.

Another source of circulating factors in the brain is the cerebrospinal fluid (CSF), a colourless liquid that fill the five ventricle cavities in the brain parenchyma, and surround its surface providing mechanical protection and nurture (Cushing 1914). The CSF is separated from the stroma by a distinct semi-permeable barrier, the blood-cerebrospinal fluid barrier (BCSFB), which consists of two distinct elements. First, the choroid plexus (CP) is a layer of cube-shaped epithelial cells adjacent to the lateral ventricles (figure 1.7 B) (Scott et al. 1974). CP cells produce the CSF, which is constantly filtered bidirectionally from and to the blood via "fenestrated" capillaries, that contain small pores to facilitate the exchange of molecules (Brown et al. 2004). Interestingly, the ependymal cells of the CP express tight junctions that prevent molecules to cross freely from and to the blood, therefore making the ependymal cells of the CP the main regulators of the CSF content (Dandy 1918; Davson 1966; Brown et al. 2004). Second, the circumventricular organs (CVO) are specialised structures in the third and fourth ventricles, containing an exclusive type of glial cells, the tanycytes (figure 1.7 C) (Nakai 1971).

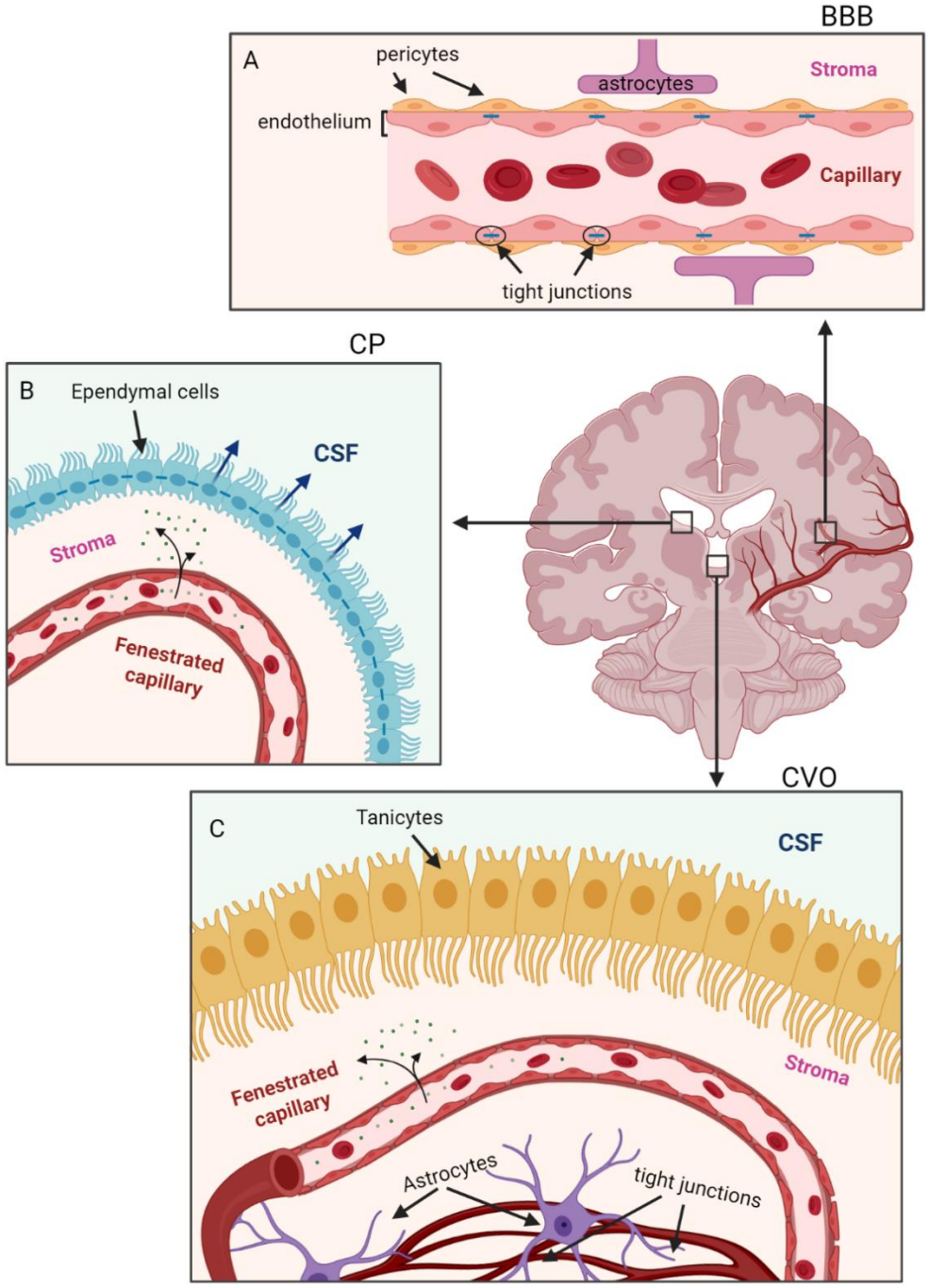


Figure 1.7 Anatomy of the cerebrovascular barriers

(**A**) The blood brain barrier (BBB) regulates transport of substances from the brain capillaries, to specialised astrocytes surrounding the endothelium. (**B**) Adjacent to the ventricles, the choroid plexus (CP) mediates transport from fenestrated capillary and the CSF, produced by the ependymal cells. (**C**) Lastly, the circumventricular organs (CVO), are specialised structures that share characteristics of both the BBB (tight junctions, specialised astrocytes) and the CP, (fenestrated capillaries, transport to the CSF) (*created with BioRender.com*).

Opposite to the CP, the blood vessels of the CVO are fenestrated only for a short tract, while for the rest of it they resemble a traditional BBB, with astrocytes creating a barrier around the vases (Morita et al. 2016). The CVOs come into contact with several areas of the brain, including the inferior portion of the hypothalamus, the median eminence (ME), where the BCSFB is highly permissive to allow hormones and peptides produced there to be secreted directly into the circulation (Morita et al. 2016). A recent study showed that a fluorescent analogue of acyl-ghrelin peripherally injected in mice, is internalised by the ependymal cells of the CP and by a specific subset of hypothalamic tanycytes (Uriarte et al. 2019). The authors synthesised several fluorescent variants of the hormone – truncated or mutated peptides that simulate the high variability of GHRL products – then measured their uptake efficiency. Their data show that both the tanycytes and the ependymal cells of the CP have very low-selectivity for the different variants of the fluorescent hormone, granting the access to all ghrelin molecules whose N-terminal sequence was intact (Uriarte et al. 2019). These data suggest that the BCSFB may also play an important role in controlling ghrelin access to the brain.

There are two main differences between the BBB and the BCSFB. First, the BCSFB is more permeable than the BBB, due to the fenestrated capillaries; second, while the CSF only flows between the ventricles and on the surface of the brain, the microcirculation – filtered by the BBB – goes deeply into the brain parenchyma reaching areas not directly accessible to the CSF (reviewed in Pardridge 2016; Mastorakos and McGavern 2019). Interestingly, different doses of circulating acyl-ghrelin have been shown to affect its brain localisation: a low dose of fluorescent acyl-ghrelin, peripherally injected in mice at rest, showed higher preference for the hypothalamus and was barely detectable in the CSF. Conversely, either a centrally injected or a peripherally injected high dose of fluorescent acyl-ghrelin was able to reach a wider number of regions and was measurable in the CSF (Cabral et al. 2014; Uriarte et al. 2019). Indeed, a new model has been proposed (Perello et al. 2019) to describe ghrelin accessibility to the brain: a small increase in circulating ghrelin, such as pre-prandially, would allow a quick, direct, and loosely selective access to the hypothalamus and the ARC – via the tanycytes of the ME – where ghrelin would exert its main orexigenic effects. On the contrary, a higher and steady increase of acyl-ghrelin in the blood, like during prolonged CR or pharmacological

intervention, would increase uptake from both the BCSFB – increasing the peptide concentration in the CSF – and the BBB, allowing it to reach a wider number of targets, deeper in the brain parenchyma. For instance, a subcutaneous ghrelin injection of 60pmol/g in wild type mice has been shown to activate the neurones in the ARC and promote food intake without significantly affecting other brain areas, while a much higher dose of 300pmol/g activates neurones of the paraventricular area (which also express GHS-R1a) to modulate food-related stress response, independently from the ARC (Cabral et al. 2016). The hypothesis that ghrelin can access numerous brain structures, depending on its plasma concentration, is consistent with the fact that GHS-R1a is expressed almost ubiquitously in the brain (this will be discussed in section 1.4.4), including areas that are usually less accessible to circulating peptides, such as the hippocampus. Moreover, this theory debunks the hypothesis from Wellman and colleagues (Wellman and Abizaid 2015), previously described, that GHS-R1a-mediated effects in the hippocampus are solely caused by its constitutive activity.

Another possibility is that ghrelin distribution in the brain may prioritise areas that are most active, therefore its transport would be based on *need*, rather than location. This hypothesis originates from the fact that ghrelin crosses the BBB via a saturable mechanism (Banks et al. 2002) and it would most likely require specific signals to access certain areas of the brain. However, it is also possible that regulating the expression of GHS-R1a in specific areas of the brain, – rather than regulating the amount of ghrelin that is transported there – would be enough to determine ghrelin's activity in that area. Indeed, a recent study showed that ghrelin transport across the BBB occurs independently from the amount of GHS-R1a expressed from the target cells (Rhea et al. 2018). This suggests that the regulation of ghrelin transport is not influenced by signals coming from the brain parenchyma, rather occurs independently from it: after ghrelin is distributed in the brain, according to its plasma concentration and brain permeability, the regulation of GHS-R1a expression could be an additional mechanism of regulation in certain areas or cell types. Interestingly, it has been shown that GHS-R1a is internalised approximately 20 minutes after ghrelin's binding in order to protect the receptor from overstimulation, a mechanism that is shared by several other GPCRs (Tolle et al. 2001; Camiña et al. 2004). Approximately 360 minutes after agonist removal,

GHS-R1a levels on the cell membrane are restored and the cycle can start again (Camiña et al. 2004). The quick internalisation reduces the ability of the cell to further respond to ghrelin in the short term – indicating that the *need* for ghrelin of that cell has been fulfilled.

Overall, these evidences show that the mechanisms that regulate ghrelin entry into the brain have a major role in determining its activity, and that paradigms like CR or diseases that may modify its plasma concentration, are able to influence the extent of ghrelin's presence, and therefore its function in specific brain areas.

1.4.2 Human hippocampus anatomy and structure

The human hippocampus is a 4-4.5cm long and 1-2cm thick arched bilateral structure (figure 1.8), localised under each hemisphere's cortex in the medial temporal lobe. It is part of the limbic lobe, a C-shaped region on the medial surface of the hemispheres that surrounds the midbrain, identified in 1878 by Paul Broca and named from the Latin *limbus*, that means edge, since this region represent the curved rim of the cortex (Broca 1878).

The term *hippocampus*, first used in the XIV century (Arantius 1587), is ancient Greek for seahorse, due to its resemblance with the sea creature with whom it shares an arched physiognomy. Indeed, the hippocampus folds on itself during development, although this process remains incomplete in most mammals and only reaches its maximum extent in humans: the rat hippocampus remains dorsal to the thalamus, while in humans it curves beneath (Tilney 1939). Interestingly, the size of the hippocampus, compared to body weight, generally increases in higher species, with the largest being found in primates (Stephan 1983). Due to its connection with memory and learning, it has been hypothesised that the size of the hippocampus in the different species may correlate with increased cognitive abilities. However, some highly evolved species such as dolphins and whales, have a small hippocampus if considered in proportion to the size of their brain (Patzke et al. 2015). Moreover, certain hippocampal areas such as the CA1 and the dentate gyrus, are much larger in humans and monkeys, in relation to the size of the whole hippocampus, compared to rodents and other species (Stephan and Manolescu 1980), further reinforcing the idea of the unicity of the structure of the human hippocampus.

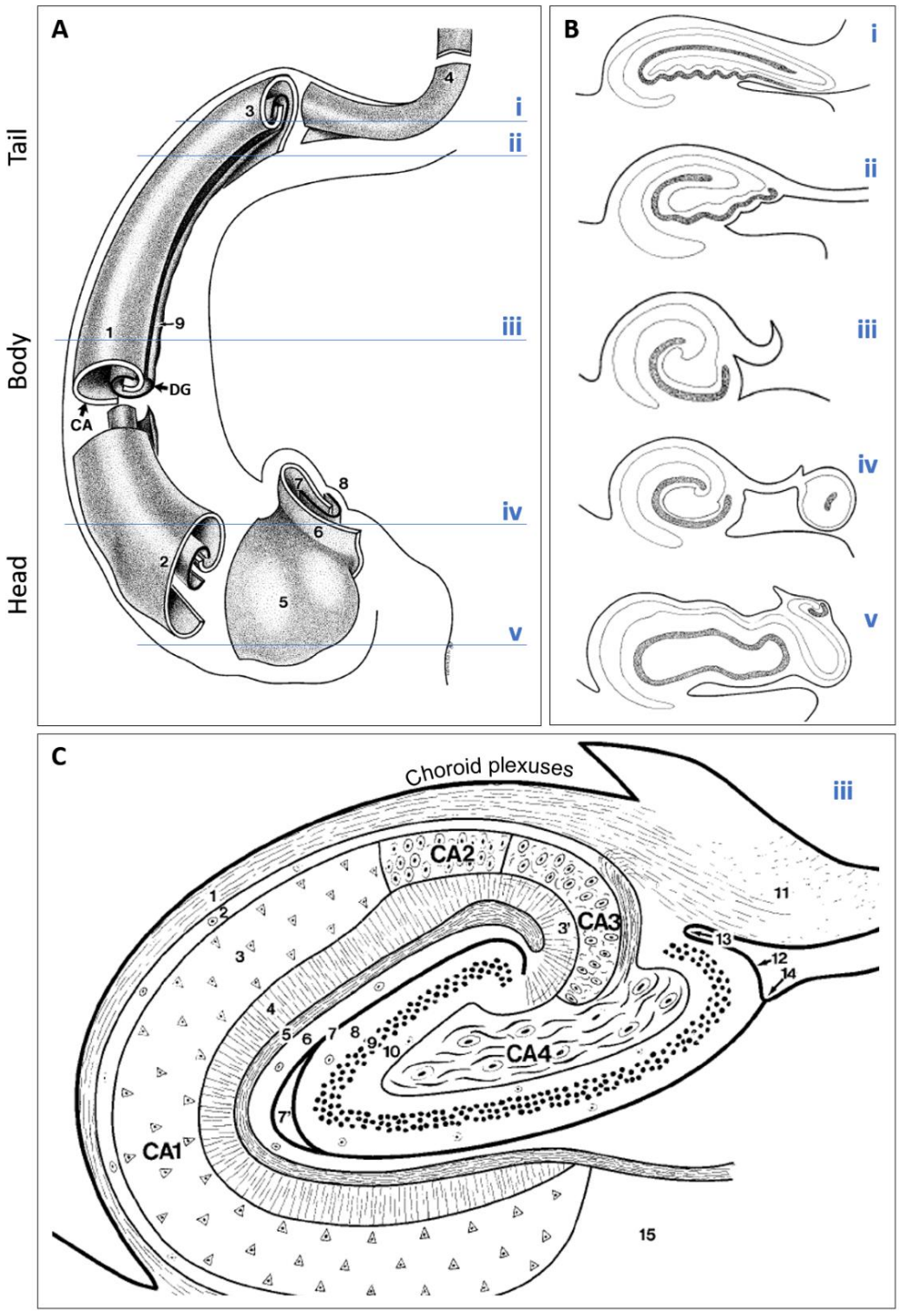


Figure 1.8 Structure of the human hippocampus

(**A**) Representation of a single human hippocampus, with the CA and the DG folding on each other. (**B**) The typical "C-like" aspect of the GCL (dark grey) on coronal sections at different positions (from i to v). (**C**) Coronal section from the hippocampus body (iii) (adapted with permission from Duvernoy et al. 2013, Springer licence number 4967160485075) *(continues in the next page)*.

Figure 1.8 Structure of the human hippocampus (continuation)

(**A**) 1 body; 2 head; 3 tail; 4 terminal segment; 5 *digitationes*; 6 vertical digitation; 7 *uncus*; 8 band of Giacomini; 9 *margo denticulatus*. (**C**) 1 *alveus*; 2 *stratum oriens*; 3 *stratum pyramidale*, or pyramidal layer; 3 *stratum lucidum*; 4 *stratum radiatum*; 5 *stratum lacunosum*; 6 *stratum moleculare*; 7 vestigial hippocampal sulcus; 8 *stratum moleculare*, or molecular layer; 9 *stratum granulosum*, or granular layer; 10 polymorphic layer; 11 fimbria; 12 *margo denticulatus*; 13 fimbrio-dentate sulcus; 14 superficial hippocampal sulcus; 15 *subiculum*.

On the medial axis (antero-posterior), the human hippocampus can be divided in three segments: head, or anterior segment; body, middle segment; and tail, posterior segment (figure 1.8 A) (Duvernoy et al. 2013). On the lateral axis (left-right) it consists of two layers, or laminae: the *Cornu Ammonis* (CA) or hippocampus proper, and the dentate gyrus (DG) or *fascia dentata*. The two laminae are folded inside each other, so that, in coronal sections, they appear as two intersecting "C" letters separated by the hippocampal sulcus (figure 1.8 B and C). From the intersection of the two laminae, several layers of distinct cells can be identified in coronal sections (figure 1.8 C, layers 1 to 10). The largest and most evident one is the pyramidal cell layer (figure 1.8 C, layer 3), whose cells are easily recognisable by their large triangular soma and dense ramifications. The heterogeneity of this region led anatomists to divide it in 4 distinct parts: the CA1 is a large area that continues from the *subiculum* (Latin for support), a wide structure that seem to physically sustain the hippocampus in place and whose fibers connect it directly to the entorhinal cortex; the CA2 is a small transition region with densely packed cells; the CA3 corresponds to the curve of the lamina and is rich in non-myelinated mossy fibers originating in the DG; lastly the CA4 – sometimes called *hilus* or the end-blade of the hippocampus – is the region underlying the DG curvature and is scarcely dense of cellular soma but rich of myelinated mossy fibers.

The DG lamina is also divided in 3 layers: the thick molecular layer (figure 1.8 C, layer 8) that mostly receive fibers from the entorhinal cortex via the perforant pathway; the granule cell layer (figure 1.8 C, layer 9), or GCL, that contains plenty of small, round and densely packed neuronal cell bodies, which form the smaller 'C' in coronal sections; lastly, the polymorphic layer (figure 1.8 C, layer 10), a very thin layer that contains the initial part of the GCL cells' axons – those mossy fibers that will then cross the CA4 and CA3.

Lastly, between the DG and the CA, there is often another visible layer, the hippocampal sulcus, that sometimes forms vestigial cavities (figure 1.8 C, layer 7) (Duvernoy et al. 2013). Ultimately, the CA and the DG form two continuous laminae on the longitudinal axis, resulting in the hippocampus being essentially the same throughout its length.

1.4.3 Hippocampus and mnemonic functions

The first evidence that connected the human hippocampus to mnemonic function dates back to 1953, when a surgeon performed a bilateral temporal lobe resection on patient H.M., relieving him of an uncurable epilepsy, but causing a severe memory loss (Scovielle and Milner 1957). Since then, many studies investigated the role of hippocampus on memory formation, consolidation and recall in rodents (Aggleton et al. 1999; Clark et al. 2000; Prusky et al. 2004), primates (Zola et al. 2000; Nemanic et al. 2004), and humans (McKee and Squire 1993; Pascalis et al. 2004; Kirwan et al. 2010; Lad et al. 2019).

The totality of the information coming from the environment, are collected by specialised sensorial organs associated to specific cortical structures: for instance, the parietal cortex is mostly activated upon reception of visual signals (Sakata and Kusunoki 1992), the temporal cortex after auditory signals (Scheich et al. 1998), and so on. Then all the signals converge to the entorhinal cortex, and later to the hippocampus, where information most recently acquired are "stored" for short-term recall – opposite to older "long-term" memories that are stored in the cortex (Duvernoy et al. 2013).

The human hippocampus has been associated to the processing of space (Hassabis et al. 2009) and time (Umbach et al. 2020); decision-making processes (Bach et al. 2014); episodic memory, such as recall of autobiographic memory (personally experienced events, set in a precise space-temporal context) (Chadwick et al. 2010)

and the emotions associated to them (Zhu et al. 2019); visual and odour recognition (Levy et al. 2003); pattern separation for overlapping memories, for example recognition of details across different memories (Chadwick et al. 2011); and many more. Significantly, the hippocampus has been shown to be the main regulator of coordinating separate elements from divergent type of memories, into one whole representation (Backus et al. 2016). Moreover, the hippocampus has also an important function in regulating endocrine processes such as stress management, fear and anxiety, for instance in post-traumatic stress disorder (Astur et al. 2006; Milad et al. 2007).

The hippocampus ability to create and retain new memories, mostly involves the neuronal progenitor cells in the GCL layer, that are able to generate new neurones through lifetime (Boldrini et al. 2018; Moreno-Jiménez et al. 2019). These cells are involved in memory and plasticity, but also mood and anxiety. Interestingly, antidepressant drugs like serotonin reuptake inhibitors and tricyclic antidepressant, specifically stimulates neuronal progenitors of the GCL in the brain of patients with major depressive disorder (Boldrini et al. 2009; Anacker et al. 2011), suggesting that targeting these cells may have beneficial effects on the hippocampal function.

Despite the consistency of its anatomical structures, the three main hippocampal regions – head, body, and tail – have been suggested to be noticeably different when it comes to their function. Fifty years ago, a study showed that the dorsal and ventral regions of the mouse hippocampus have distinct input and output connections (Swanson and Cowan 1977). Since then, numerous studies performed in mice by selectively lesioning individual hippocampal regions, reported that the ventral segment modulates emotional behaviour and affective processes, including stress response (Henke 1990) and fear (Maren and Holt 2004), while the dorsal area is involved in cognitive and mnemonic function, including navigation and spatial processes (Moser et al. 1995). Interestingly, Bast and colleagues identified a third functionally distinct region in the intermediate segment, involved in translating cognitive and spatial knowledge into motivation and action (Bast et al. 2009), although the existence of such region is still debated.

Several studies confirmed this regionalisation in the human hippocampus, where the most posterior (dorsal) segment – the tail – has been associated to spatial

navigational strategy and visuo-spatial cognition (Brunec et al. 2018) and showed greater connectivity with medial and lateral parietal cortex (Adnan et al. 2016; Vogel et al. 2020); whereas the most anterior (ventral) region – the head – is associated to imagination and episodic memory (Barry et al. 2019), and is highly connected to temporal and orbitofrontal cortex (Adnan et al. 2016), that is also involved in social and motivational behaviours (Vogel et al. 2020).

However, few studies using electrophysiological recordings in rodents (Kjelstrup et al. 2008) and fMRI imaging in humans (Collin et al. 2015), suggest that the hippocampus longitudinal axis is functionally organized along a gradient, rather than being arranged in discrete regions. Moreover, left and right hippocampi in human seem to have differential activations.

Studies performed in patients with unilateral temporal lobotomy, identified a correlation between the right hippocampus and patients' visuo-spatial memory abilities – the ability to recall objects and their location (Smith and Milner 1981), while the lobectomy of the left hippocampus has been associated to impaired working memory capacity and verbal or narrative memory (Frisk and Milner 1990). Subsequent fMRI studies confirmed this hypothesis: for instance, recalling a complex driving route through a city, preferentially activates the right posterior hippocampus (Maguire et al. 1997), while recall of verbal material (Greicius et al. 2003) and the acquisition of new conceptual information (the acquisition of rules that can guide behaviour in novel situations) (Kumaran et al. 2009), mostly activate the left posterior hippocampus.

Overall, the hippocampus multifaceted role in memory and cognition, makes it significantly relevant in the study of ageing and neurodegeneration.

Interestingly, structural abnormalities have been observed in a variety of neuropsychiatric diseases: unilateral decrease of the hippocampal volumes has been reported in major depression (Bremner et al. 2000) and bipolar disorder (Swayze et al. 1992); bilateral volume loss was reported in schizophrenia (Heckers 2001) and alcoholism (Sullivan et al. 1995); while increased volume was reported in some genetic disorders like X-fragile syndrome (Reiss et al. 1994). Ageing has also been associated to a slight decrease of the hippocampal size (Convit et al. 1995b), although there are contradictory evidences on this matter (Sullivan et al. 2005). Indeed, the human hippocampus shows selective vulnerability to age-

related diseases that are unique to humans. For instance, spatial and episodic memory impairments are the first symptoms of AD, whose main feature is cellular damage to the medial temporal lobes, including the hippocampus (Moss et al. 1986; Laakso et al. 1996). Moreover, hippocampal cell loss is also present in other neurodegenerative disorders, like PD (Laakso et al. 1996; Camicioli et al. 2003), and Huntington (Rosas et al. 2003).
Ultimately, the hippocampus plays a pivotal role in human cognition and memory and its impairment in certain disease makes it a valuable pharmacological target.

1.4.4 The ghrelinergic system in the human brain

Acyl-ghrelin has been shown to affect memory and attention, two cognitive functions that are associated to the hippocampus. However, there is general consensus that acyl-ghrelin is not produced in the brain (Kojima et al. 1999; Banks et al. 2002; Wortley et al. 2004; Grouselle et al. 2008; Kageyama et al. 2008; Yang et al. 2008a; Sakata et al. 2009; Furness et al. 2011; Cabral et al. 2017) – with the possible exception of two regions: the hypothalamus, whose neurones show dense ghrelin^{+} vesicles in presynaptic terminals (Russo et al. 2017), and its tiny neighbour, the septum pellucidum (Cowley et al. 2003). Since septal neurones project to the hippocampus (Huh et al. 2010), it may be hypothesised that ghrelin reaches hippocampal GHS-R1a following release from presynaptic septal neurones, in which it may be endogenously produced. Nevertheless, ghrelin production in the septum was not confirmed by other studies.
Notably, numerous researches report that acyl-ghrelin crosses both BBB and BCSFB, as shown in section 1.4.1. Eventually, the latest proposed model of acyl-ghrelin accessibility to the brain (Perello et al. 2019), states that central ghrelin is exclusively produced peripherally, and later transported in the brain so that its availability in the deepest areas of the parenchyma is almost exclusively dependent on its plasma concentration (see section 1.4.1).

Notably, another regulatory mechanism has recently emerged that could represent a turning point in understanding the ghrelinergic activity in the brain. As mentioned in section 1.2.5, GOAT is the only enzyme known to catalyse ghrelin acylation, and ghrelin is the only known substrate of GOAT. Since ghrelin is not produced in the brain, and numerous evidences show that peripheral GOAT

expression perfectly matches that of ghrelin (Sakata et al. 2009; Lim et al. 2011), it seems unlikely that GOAT would be expressed in the brain. However, recent studies detected GOAT mRNA in the mouse hippocampus; even more interestingly, using mouse hippocampal slices and a fluorescent acylation reporter, GOAT – most likely located in the plasma membrane – was able to re-acylate ghrelin *in-situ* (Murtuza and Isokawa 2018). These data significantly change the previous knowledge about the dynamics of the ghrelinergic axis; indeed, the fact that unacylated ghrelin is subject to cell target-mediated activation, provides a novel potential mechanism for manipulating ghrelinergic activity in the brain.

This process has also been shown to occur in peripheral tissues. For instance, our collaborators recently reported that the pro-adipogenic effect observed after bone marrow infusions with unacylated ghrelin, was completely abolished in GOAT KO mice, whilst the same effect reported after acyl-ghrelin infusion was not dependent on the presence of GOAT (Hopkins et al. 2017). These data suggest that the effect itself, apparently attributed to unacylated ghrelin, is actually dependent on ghrelin being re-acylated by GOAT in the tissue.

Another study showed that the administration of unacylated ghrelin to cultured cardiomyocytes increases their uptake of MCFA, that are essential for several metabolic functions but are also important for ghrelin acylation itself. Indeed, the authors detected GOAT expression in these cells, hinting at the possibility that unacylated ghrelin is converted to acyl-ghrelin in the cytoplasm of these cells (Lear et al. 2010).

Noteworthily, full length GOAT proteins were detected in the crude protein fraction from plasma of fasted mice and rats (Stengel et al. 2010) and in human plasma (Stengel et al. 2013). The authors suggest that GOAT released depends on the individual's energetic status: its plasma concentration significantly increases in rodents after 24 hours of fasting (Stengel et al. 2010), and correlates with acyl-ghrelin plasma levels in obese and anorexic patients (Stengel et al. 2013). However, to the best of my knowledge, no study was performed to determine whether circulating GOAT is fully functional. Besides, since GOAT is a transmembrane protein, its release is most likely mediated by small lipidic vesicles (Hopkins et al. 2017), that could represent an obstacle for GOAT binding with circulating unacylated ghrelin in the blood. Indeed, unacylated ghrelin represents 70 to 90% of the total circulating ghrelin, as shown in section 1.2.7, suggesting that GOAT re-

acylation activity in the circulation may be poorly efficient, or may undergo strict regulation.

Whilst ghrelin acylation has been confirmed to occur in the brain, and possibly in the circulation, hardly any information is known about its deacylation. APT1 mRNA transcript has been almost ubiquitously detected in rodents (Allen Cell Types Database - RNA-Seq; Wang et al. 1999; Won et al. 2017), whilst APT1 protein has been found in various cell types, including neurones (Duncan and Gilman 1998; Toyoda et al. 1999). Notably, APT1 has also been shown to be expressed on circulating macrophages, hinting to the possibility that acyl-ghrelin may be deacylated in the circulation.

Lastly, several authors reported that GHS-R is highly expressed in the brain, by using a combination of *in situ* detection of mRNA and immunoreactivity assays or using fluorescent GHSR-GFP mice. Specifically, GHS-R1a was abundantly detected in the ARC of the hypothalamus, the SN, the DG of the hippocampus, the VTA and the dorsal raphe nucleus, while almost no expression was detected in the striatum (Howard et al. 1996; Bennett et al. 1997; Guan et al. 1997; Gnanapavan et al. 2002; Diano et al. 2006; Zigman et al. 2006; Albarrán-Zeckler and Smith 2013; Mani et al. 2014a; Hornsby et al. 2016).

1.5 Aims of the project

PD and AD are age-related neurodegenerative diseases, and represent the most common cause of dementia, affecting several million people worldwide.
Acyl-ghrelin is a versatile hormone that has been shown to illicit numerous protective functions in the body. More specifically, it has been shown to exert a neuroprotective function in mouse models of both PD and AD, making it a potential therapeutic compound against neurodegeneration and dementia.
Furthermore, ghrelin-mediated activation of its receptor, GHS-R1a, induces the pAMPK pathway and mediates mitochondrial function, contributing to the maintenance of the cellular homeostasis.

Despite numerous evidences reporting ghrelin neuroprotective effects in rodents and *in vitro*, very little is known about this pathway in humans. This thesis will try to compensate the lack of information, by characterising the ghrelinergic axis in *post-mortem* brains of healthy and diseased patients, whilst testing the hypothesis that ghrelin reduces oxidative stress and modulates mitochondrial homeostasis in human neuronal cells.

The main aims of this thesis are:
- To characterise the expression of the key enzymes that participate in the ghrelin axis (GHS-R1a, GOAT and APT1) in the aged healthy human hippocampus and in human stem-cell-derived mature neurones.
- To determine whether the ghrelinergic axis may be impaired in the hippocampus of patients affected by PD and PD with dementia, and to characterise how ghrelin modulates mitochondrial health *in vitro*, using human stem-cell derived neurones treated with rotenone.
- To investigate the ghrelinergic axis in the hippocampus of patients affected by AD at early or late stages, and to characterise mitochondrial health in human stem-cell derived neurones treated with amyloid-β oligomers.

Chapter 2

Materials and methods

2.1 Human brain tissue

2.1.1 Aged, PD and PDD subject

The PD and PDD brain tissues used in this thesis, were donated to the UK Parkinson's Disease Society Tissue Bank at Imperial College, London, with ethical approval (07/MRE09/72).

Fixed frozen *post-mortem* hippocampal tissue slices (6-8μM thick) were received onto Superfrost + glass slides. Upon arrival, tissue was stored at -70°C and sections from across the anterior-posterior extent of the hippocampus were visually determined, before being used in IHC and BaseScope, as described in sections 2.7 and 2.9 respectively.
A total of 17 subjects were included in the analysis: healthy controls (n=5), with no evidence of degenerative disease or cognitive decline, participants diagnosed with PD (n=7) and participants diagnosed with PDD (n=5) as shown in table 1.

Un-fixed *post-mortem* hippocampal tissue slices (5μM thick) were received in Eppendorf tubes (approximately 25mg per tube). Upon arrival, tissue was stored at -70°C. AllPrep extraction kit was used to extract RNA and proteins from each tube, as described in section 2.3.
A total of 18 subjects were included: healthy controls (n=6), participants diagnosed with PD (n=6) and PDD (n=6) as shown in table 1.

	fixed-frozen				fresh			
	Case	**Gender**	**Age**	**pmi (h)**	**Case**	**Gender**	**Age**	**pmi (h)**
ctr	C08	F	93	9	PDC92	M	79	25
	C025	M	35	22	PDC91	M	85	29
	PDC016	F	93	13	PDC107	F	87	15
	PDC022	M	65	12	PDC88	F	96	24
	PDC026	F	80	23	PDC86	M	89	18
					PDC87	F	92	24
PD	PD081	M	73	19	PD239	M	81	8
	PD086	F	87	22	PD261	M	84	21
	PD022	F	76	14	PD275	M	79	22
	PD051	M	80	7	PD247	F	88	12
	PD074	M	85	17	PD184	M	71	24
	PD107	F	76	n/n	PD86	F	87	22
	PD109	M	72	9				
	PD101	F	79	41				
PDD	PD28	M	82	14	PD28	M	82	14
	PD63	F	80	10	PD93	F	81	22
	PD079	F	78	22	PD99	M	82	10
	PD093	F	81	22	PD79	F	78	22
	PD099	M	82	10	PD122	M	92	21
	PD076	M	61	14	PD106	M	75	3
	PD078	M	79	24				

Table 1 Human brain samples from PD and PDD patients

(Legend: ctr = controls; pmi = *post-mortem* interval; h= hours; n/n data not known)

2.1.2 Young, aged, early AD and late AD subjects

Human brain tissue donated to Brains for Dementia Research (BDR) (London – City and East NRES committee 08/H0704/128+5) was used in this thesis, and ethical approval obtained for all our analysis (07/MRE09/72).

Fixed-frozen paraffin-embedded (FFPE) *post-mortem* hippocampal tissue slices, mounted onto Superfrost + glass slides, were received from Edinburgh BDR brain bank. Upon arrival, tissue was stored at +4°C before being used in IHC, as described in section 2.6.
A total of 20 subjects were included: healthy young controls (ctr) (n=5), healthy aged ctr (n=5), participants diagnosed with early AD (n=5) and late AD (n=5), as shown in table 2.

Un-fixed *post-mortem* hippocampal tissue were received frozen in OCT medium blocks from Oxford and Newcastle BDR banks. Upon arrival, tissue was stored at -70°C, then cryo-sectioned and 25mg of tissue per each block was stored in Eppendorf tubes, also stored at -70°C before use. AllPrep extraction kit was used to extract RNA and proteins from each tube, as described in section 2.3.
A total of 20 subjects were included: healthy young ctr (n=5), healthy aged ctr (n=5), participants diagnosed with early AD (n=5) and late AD (n=5), as shown in table 2.

	FFPE				fresh			
	Case	**Gender**	**Age**	**pmi (h)**	**Case**	**Gender**	**Age**	**pmi**
young ctr	SD038/17	M	34	99	039/13	M	41	1d
	SD029/17	M	40	103	103/13	F	48	2d
	SD026/16	F	37	126	156/13	M	45	2d
	SD022/16	M	39	86	099/11	F	41	2d
	SD031/15	F	40	89	167/11	F	43	2d
	SD061/13	F	40	77				
aged ctr	SD030/19	M	82	56	2006/17	M	100	3d
	SD042/18	F	73	74	2006/53	M	71	2d
	SD030/18	M	63	115	2006/57	M	85	2d
	SD011/18	F	61	80	2006/116	F	89	1d
	SD046/17	F	65	76	2005/115	M	93	2d
	SD036/17	M	71	71				
early AD	SD001/17	F	76	29	2006/051	M	79	4d
	SD025/19	M	86	123	2003/84	F	89	5d
	SD023/19	M	77	7	2005/63	F	88	3d
	SD016/19	F	79	19	2002/54	M	87	2d
	SD008/18	F	69	94	2002/65	F	90	1d
	SD001/18	M	72	103				
late AD	SD031/19	F	90	52	91/1250	F	73	1d
	SD005/19	M	74	75	RI 92/1087	F	90	54h
	SD004/19	M	66	49	RI 91/1112	F	64	65h
	SD037/18	M	72	103	RI 92/1125	F	70	144h
	SD021/18	F	90	76	RI 91/1313	F	83	42h
	SD012/18	F	85	80				

Table 2 Human brain samples from young, aged, early AD and late AD patients

(Legend: ctr = controls; pmi = *post-mortem* interval; h = hours; d = days)

2.2 ReNcells ™ VM

2.2.1 Plate coating and cell reviving

ReNcells™ VM are neural stem cells obtained from the ventral mesencephalon of the developing human brain. This cell line was generated by transduction with the transcription factor c-myc that confers extended lifespan and stable genotype and phenotype (Donato et al. 2007). Moreover, ReN cells can replicate for a large number of cycles, without undergoing genomic instability (appendix 1).
Cells were purchased from Merck Millipore (SCC008), stored in liquid nitrogen at low passages (between 4 and 7). In order to maintain the cells in proliferation, fresh growth factors, EGF and bFGF, should be added every 48 hours at least. However, cells are very sensitive to changes in their environment, so low plating densities or increased cell death, would spontaneously trigger cell differentiation.

Cells were grown as a monolayer in a laminin-coated plastic dish.
Laminin (AMSbio 3400-010-02) was stored at -70°C after purchase. Before use, one tube (1mg/1ml) of laminin was thawed at +4°C, then resuspended in 9ml of DMEM/F12 (Gibco), for a final concentration of 20µg/ml (coating media). This solution was always kept in ice during use, then stored at +4°C for up to 1 week. Once resuspended, laminin solution could be used up to 2 times.
The day before cell plating, the appropriate volume of coating media was added in every plate (table 3), then plates were incubated at +37°C and 5% CO_2 for 24 hours. After incubation, laminin was removed from the plate and stored at +4°C for a second use. Plate was washed twice with 1xPBS (Gibco), then fresh proliferating media (APM) was added. Cells were removed from liquid nitrogen, and quickly thawed in the water bath at +37°C, until almost all ice was thawed. Then the content of each vial was gently transferred into a coated T75 plate with APM, without vortexing or centrifuging to prevent cell death. Then plates were immediately moved at +37°C and 5% CO_2, overnight.
Complete APM was prepared as shown in table 4 and kept at +4°C for up to 4 weeks. Growth factors EGF and bFGF were added fresh on the day.

Plate/flask	Growth area (cm^2)	Minimum coating volume (ml)	Trypsin/EDTA (ml)	Media volume (ml)	Approx. cell n. at confluency
T75	75	6,5	3	15-20	$20x10^{e6}$
T25	25	3	1	7-10	$9x10^{e6}$
6wp	9,5	1	0,5	1,5-2	n/a
96wp	0,32	0,05	n/a	0,1	n/a

Table 3 Plate volumes

2.2.2 Cell growth and passaging

The next day, half of the media was removed, and the same volume of fresh APM was added. ReN cells, as most cultured cells, release in the culture media variable amounts of soluble factors. Interestingly, in the case of ReN-derived neurones, some of those factors prevent cells to undergo cell death. Indeed, ReN cells die when the whole media is entirely replaced with fresh one, even in the presence of growth factors. To overcome this issue, I developed a protocol where only half media is replaced during cell growth, all the way until maturation.

When ReN stem cells reached 70-80% confluence, they were passaged as follow. Cell monolayer was quickly washed with 1xPBS (Gibco), and immediately treated with 1-3ml of trypsin/EDTA solution (Gibco), using the volumes shown in table 3. Cells were incubated at +37°C and 5% CO_2 for 2 minutes, then quickly removed from incubator and gently agitated to favour cell detachment. 10ml of APM (per 1ml of trypsin) was quickly added to neutralise its enzymatic activity. Then all cells were collected from the surface and transferred in a 15ml tube to be centrifuged at 300g for 5 minutes at RT. Supernatant was removed and pellet was resuspended in fresh APM, then plated in a newly coated flask. For differentiation, cells were counted using a haemocytometer and plated as described in section 2.2.4.
In order to maintain the cells in a proliferative state, they were passaged as follow: 1in3 dilution, in order to have them 70% confluent the next day; or 1in20 dilution,

to reach confluency in 3 days (i.e., Friday to Monday). These dilutions were optimised to ensure the maximum cell cycle efficiency. Indeed, if cells were seeded at lower densities, the increased room between the cells would trigger differentiation and cell death.

Lastly, due to the short half-life of the growth factors in the media and in order to prevent spontaneous differentiation, half media was changed every other day with freshly added growing factors. Indeed, leaving the cells in the same media for longer than 60-70 hours would trigger the differentiation process.

2.2.3 Cells freezing and storage

ReN cells can grow for a short number of passages before they start to spontaneously differentiate in neurones (data not shown). Proliferating ReN cells can be stored in liquid nitrogen for several years, whilst neither differentiating nor mature ReN neurons can be revived after frozen (data not shown), hence the survival of the clone is entirely dependent on the stem cells.

ReN cells freezing media was purchased by Merk Millipore (SCM007) then quickly thawed at RT (never at +37^{o}C). Each confluent T75 plate of ReN stem cells was washed once with 1xPBS, then incubated with the appropriate volume of trypsin/EDTA, collected and centrifuged, as described in the previous section. For the freezing process to be more efficient, as much supernatant as possible must be removed from the pellet. Then, the pellet can be resuspended in 1ml of freezing media, per each 70% confluent T75 plate that was processed. Since freezing media contains high concentrations of DMSO, that is toxic for the cells at RT, it is important to move them as quickly as possible at lower temperatures. More specifically, the cryovials were moved to -80^{o}C using a freezing container (i.e., Mr Frosty, Thermo Fisher 5100-0001) filled with isopropanol, to ensure a gradual reduction of the temperature. Then, the next day, cells were moved in liquid nitrogen for long term storage.

2.2.4 ReN neurones differentiation

Before inducing neuronal differentiation, cells need to be passaged at a density between 60,000 and 600,000 cells/ml. Lower seeding density (60,000) were used for long-term cultures (i.e., 28 days) and for imaging analysis to allow better cell-cell resolution. However, due to large cell death observed in the first days of the protocol, very few cells are expected towards the end of the differentiation protocol; hence higher seeding density (600,000) were required for experiments including quantitative analysis (qPCR, WB).

24 hours after plating, APM was removed and cells were quickly washed once with differentiation media (ADM), then the media was entirely replaced with ADM (table 4). The day in which the APM was entirely replaced by ADM was marked as the day 0 of the differentiation process (day *in vitro* 0, DIV0). Half media was changed every other day until DIV14, to counteract the increase of media pH caused by the cell death. After DIV14, cell number was stabilised, hence half media was changed twice a week (i.e., Monday and Friday), until the end of the differentiation protocol.

❖ **Differentiation Media (ADM)**	
Advanced DMEM/F12 (Gibco 12634)	47.5ml
1% Penicillin-Streptomycin (Gibco)	0,5ml
4mM glutamine (Gibco)	1ml
1xB27 supplement (Gibco 17504-044)	1ml
50µg/ml gentamicin (Gibco 15750037)	50µl
10U/ml (50µg/ml) Heparin (Sigma H3393)	50µl
♦ Sterile-filter the media (22µm filter) and store at +4°C for up to 1 week	
❖ **Proliferation Media (APM)**	
ADM	50ml
20ng/ml bFGF (preprotech AF-100-18B)	10µl
20ng/ml EGF (preprotech AF-100-15)	10µl

Table 4 ReNcell media composition

2.2.5 ReN neurones treatment and AβOs preparation

After confirming that ReN neurones express neuronal maturation markers, as well as GHS-R1a (that starts being detectable around DIV22-24) (chapter 3), the next experiments were designed as follow. First, cells were pre-treated with acylated or unacylated ghrelin every day for 5 days, from DIV24 to DIV28; this pre-treatment intended to recreate the effects of a short-term CR, without affecting the nutrient composition of the media. Then, neurones at DIV28 were treated for 24 hours with either rotenone or amyloid-β oligomers (AβOs), at two different concentrations – 1 or 10μM – in order to induce mild oxidative stress and simulate the earlier stages of PD and AD respectively (figure 2.1).

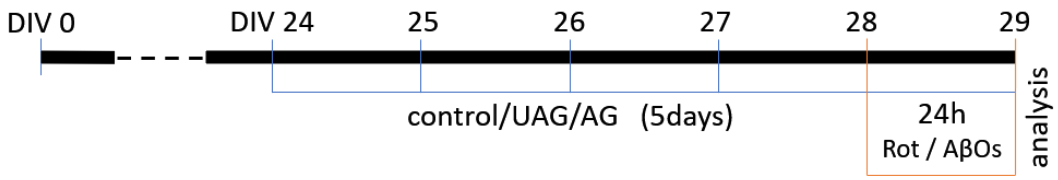


Figure 2.1 ReN Neurones treatment timeline

Since only half of the media can be removed from each well, as described in section 2.2.2, stock solutions of each compound are prepared containing double the concentration (2x) needed.

Following supplier's guidelines, acylated ghrelin (Tocris) and unacylated ghrelin (Tocris) were resuspended in water, while rotenone (Sigma) was resuspended in DMSO; all three compounds were stored at -20°C in small stock aliquots of 100μM. Before use, compounds were resuspended in ADM at 2μM (and 20μM rotenone), before being diluted 1in2 with the cell media that was already contained in the well.

AβOs were prepared as described in Dr. Klain's lab protocol (Lambert et al. 2001). Briefly, 1μg of full-length Aβ peptide (1-42) (Aβ42) was resuspended under a chemical fume hood in 1ml ice-cold 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), a highly volatile fluorinated solvent that allow slow monomerization of the structure.

After 1 hour incubation at RT with the lid of the reaction tube firmly closed, the solution was placed on ice for 1 hour before being aliquoted on ice to prevent evaporation. Each aliquoted tube was then left over-night at RT with the lid open under a chemical fume hood to allow complete evaporation of the solvent – that is highly toxic. To ensure complete evaporation, the next morning each tube was fast-spinned through a speed vacuum for 10 minutes with the lid open. At the end of this process, Aβ monomers appeared as a small transparent film, barely visible at the bottom of each tube. Aβ pellets were frozen at -80°C and thawed as needed.

The day before treatment, one or more tubes were thawed and quickly resuspended with freshly opened DMSO to a 100μM concentration. After a gentle vortex to ensure the pellet was lifted from the tube, the DMSO was further diluted to a final concentration of 2μM (for 1μM treatments) or 20μM (for 10μM treatment) in ADM. To allow slow and controlled oligomerisation, the pellet was incubated at +4°C for 16-18 hours.

For every new batch of Aβ42 powder purchased, we tested the oligomerisation process to exclude any batch-to-batch variation. The validation was performed by our collaborators and consisted of high-resolution scanning of the oligomers using atomic force microscopy (AFM), to determine particle size and shape (Gazze et al. 2021). Only batches that produced oligomeric particles of circular shape and regular size (approximately 10nm of diameter) were used for cell treatment, whilst preparation containing fibrils were discarded (see chapter 5).

2.3 Protein and nucleic acids extraction

2.3.1 AllPrep (QIAGEN) extraction kit from cells and tissues

The Qiagen AllPrep DNA/RNA/Protein mini kit (80204) enables the simultaneous extraction and purification of genomic DNA, total RNA, and total protein from the same sample, ensuring high level of efficiency and great purity.
The protocol was performed according to manufacturer's instructions.

- Tissue rupture

The cell pellet or up to 30mg of tissue was transferred in a tissue disruptor tubes from the QIAmp Fast DNA kit (Qiagen, 51404). Then 600µl buffer RLT was added per sample and the tubes were vortexed for approximately 30 seconds until complete homogenisation. The lysate was then centrifuged at 20,000 x g for 3 minutes at RT and transferred to an AllPrep DNA spin column. This was centrifuged for 30 seconds at 8,000 x g at RT and stored at 4°C for later DNA purification. The flowthrough was used for RNA and protein purification.

- RNA isolation

430µl of 100% ethanol was added to the flow-through and mixed well by pipetting, then added to a RNeasy spin column and centrifuged for 15 seconds at 8,000 x g at RT. The flow-through was transferred to a separate tube and stored at 4°C for later protein purification. 700µl buffer RW1 was added to the RNeasy spin columns, centrifuged and the flow-through discarded. Subsequently, 500µl of buffer RPE was added and centrifuged. This was repeated a second time, with a longer 2 minutes centrifuge at 8,000 x g at RT to thoroughly wash the spin column. The spin column was placed in a new 2ml collection tube and centrifuged at full speed for 1 minute to spin dry. The RNeasy spin column was placed in a new 1.5ml collection tube and 30µl of RNase-free water was added directly to the column membrane, then centrifuged for 1 minute at 8,000 x g at RT to collect the eluted RNA. RNA was treated with DNase (Ambion, AM1906) as described in section 2.4.1.

- Protein isolation

1000µl of buffer APP was added to the flow-through from the RNA purification step, mixed vigorously and incubated at RT for 10 minutes to precipitate the protein. This was centrifuged 20,000 x g for 10 minutes, and the supernatant carefully decanted. 500µl of 70% ethanol was added to the protein pellet and centrifuged at 20,000 x g for 1 minute. As much liquid as possible was removed and the pellet left to dry at RT for 5-10 minutes. 5% Sodium dodecyl-sulfate (SDS) (Sigma, 436143) was prepared by dissolving 5g of SDS powder in 100ml of water. The protein pellet was re-suspended in approximately 250µl 5% SDS and stored at -20°C.

- DNA isolation

500µl of buffer AW1 was added to the AllPrep DNA spin column (set aside in the first step), then centrifuged for 15 seconds at 8,000 x g and the flow-through discarded. Next, 500µl buffer AW2 was added and centrifuged for 2 minutes at 20,000 x g. The tube was centrifuged for an additional 1 minute at 20,000 x g to spin-dry. Finally, the DNA was eluted by the addition of 100µl of buffer EB (pre-heated to 70°C), incubated for 2 minutes and centrifuged at 8,000 x g for 1 minute.

2.3.2 Protein quantification

Protein quantification was performed using the Pierce BCA assay kit (Thermo Fisher, 23227).

Briefly, a standard curve was generated using the albumin standards provided in the kit, diluted with 5% SDS (2.00, 1.50, 1.00, 0.750, 0.500, 0.250, 0.125, 0.025, 0.0µg/µl). 25µl of sample/standard was added per well in duplicate. A blank well was added using the buffer diluent. Working reagent was made by combining reagent A + B (50:1), then 200µl was added per well. The plate was then incubated for 30 minutes at 37°C and absorbance read at 562nm using a plate reader (PolarStar Omega plate reader). Sample concentrations were calculated by extrapolating against the standard curve line of best fit using the equation (y=mx+c).

2.4 Quantitative real-time PCR

2.4.1 DNase treatment

DNase treatment was conducted to minimise gDNA contamination, according to manufacturer's instructions (DNA-free TM kit; Ambion cat: AM1906).

5µl 10X DNase I buffer and 1µl rDNAse I was added to each RNA sample and mixed gently. Samples were incubated in a water bath at 37°C for 30 minutes. 0.1 volume DNase inactivation reagent was added to each sample and mixed well, incubated at RT for 2 minutes with occasional mixing. Samples were centrifuged at 10,000 x g for 1.5 minutes and transferred to a fresh tube.

2.4.2 Reverse transcription

Extracted and purified RNA were reverse-transcribed to cDNA using the Primer Design nanoScript2 RT kit.

900ng of RNA were used per sample. 1µl of the oligo-dT primers and 1µl of the random nonamer primers were added per sample and made up to 10µl total volume with RNAse/DNAse-free water. Samples were heated to 65°C using a thermal cycler (BioRad T100), then quickly stored on ice. The rest of the reagents were added according to table 5. A "no-RT" sample was prepared as a control for gDNA contamination. All samples were then placed in a thermal cycler (BioRad T100) with the following conditions: +20°C for 5 minutes, +42°C for 20 minutes, +75°C for 10 minutes, +4°C until collection. Samples were then stored at -40°C.

Reagent	RT sample	No-RT sample
nanoscript2 4x buffer	5µl	5µl
dNTP mix	1µl	1µl
Nanoscript2 enzyme	1µl	---
water	3µl	4µl
TOTAL	10µl	10µl

Table 5 Reverse transcription

2.4.3 SYBR green qPCR

Each cDNA sample was made into a 1:5 dilution with water. A 10µl reaction volume was used with universal SYBR-green (Primer Design, PPLUS), as shown in table 6. Primer sets were used at 1µM concentration. Samples were processed in a BioRad CFX96 Real-Time PCR Detection System, with the following conditions: +95°C for 2 minutes followed by 40 cycles of +95°C for 10 seconds, +60°C for 30 seconds, +72°C for 15 seconds. Melt curve analysis was also performed (appendix 2). Results were quantified using the ΔΔCt method, normalised against the reference genes (β-actin and β2-microglobulin, β2M), and presented as fold change. Statistical analysis was performed using the ΔCt values.

$$\Delta Ct = Ct\ (\text{gene of interest}) - Ct\ (\text{reference genes})$$

$$\Delta\Delta Ct = \Delta Ct\ (\text{treatment}) - \Delta Ct\ (\text{vehicle})$$

$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

Reagent	Volume (µl)
Sample (1:5)	2.5
Primers (1µM)	2.5
SYBR-green master mix	5
TOTAL VOLUME	10

Table 6 Reagents used in qPCR reaction

2.4.4 Primer's optimisation

Primer's optimisation was performed as described elsewhere (Bustin et al. 2009). Briefly, each primer was tested to determine the optimum melting temperature, by applying a temperature gradient to the plate (+50°C to +70°C). Then cDNA from the control samples, was used to determine the optimal cDNA dilution (range between 1:2 and 1:20). In my investigations, all primers were found to be optimal at the temperature of +60°C. The melting curves show no primer dimer formation (appendix 2). All primer sequences are shown in appendix 2.

2.5 Western blot

2.5.1 SDS-page

Moulding glass plates were cleaned with 70% ethanol and rinsed with water before use, then constructed into the gel frame. The separating gel with the appropriate percentage of acrylamide, was then prepared according to table 7 with 10% ammonium persulfate (APS) (Sigma, 215589) and TEMED (Sigma, T9281) added immediately before casting. Water-saturated butanol (Sigma, 28154) was added on top of the gel to ensure an even gel edge. The gel was then left until complete polymerisation (approx. 15-30 minutes), then the butanol removed and rinsed with water. The stacking gel was prepared according to table 7, and added on top of the separating gel, with a comb inserted to form the lanes. Once fully polymerised, the comb was removed, and the wells rinsed with water. The gel was then assembled in the Mini-PROTEAN tetra system (Bio-Rad) electrode assembly clamping frame and filled with running buffer (1.5M Tris-HCl pH 8.8, 0.4% SDS).
20 µg of each protein sample was combined with 4X sample loading buffer (BioRad, 1610747) and made up to a final volume of 25µl with water, boiled at 100°C for 5 minutes before being carefully loaded into the 50 corresponding wells. 5µl of 11-245kDa protein molecular-weight marker (NEB, P7712S) was also added. The whole assembly was connected to a powerpack Basic (BioRad) and run at 150V for approximately 1 hour or until bands had sufficiently resolved.

reagent	**Separating gel**				**Stacking gel**
	7.5%	**10%**	**12%**	**15%**	
Water	5ml	4.2ml	3.5ml	2.5ml	1.75ml
4x Tris and SDS	2.5ml	2.5ml	2.5ml	2.5ml	0.75ml
30% acrylamide	2.5ml	3.3ml	4ml	5ml	0.5ml
10% APS	100µl	100µl	100µl	100µl	100µl
TEMED	10µl	10µl	10µl	10µl	10µl

Table 7 WB gel preparation reagents

2.5.2 Transfer and visualisation

Polyvinylidene difluoride (PVDF) membrane (BioRad, 1620177) was first washed in methanol for 1 minute, then water for 2 minutes and then incubated for 30 minutes in transfer buffer (25mM Tris, 190mM Glycine, 20% Methanol w/v). Also, two pieces of Criterion size Extra thick blot paper (BioRad, 1703967) and the gel was incubated in transfer buffer at this time. One piece of blot paper was laid down in the Trans-Blot Turbo transfer system (BioRad) and a roller used to reduce air bubbles. The membrane was laid on top of this, with the gel carefully on top of the membrane and another piece of blot paper applied on top of this, with a final gentle rolling to reduce air bubbles. The transfer machine's lid was locked into place and run at 15V for 75 minutes.

Once the transfer was complete, the membrane was incubated in 5% bovine serum albumin (BSA) (Sigma, A9418) dissolved in TBS-tween20 (20mM Tris, 150mM NaCl 0.1% Tween 20) (TBS-T) for 1 hour. The appropriate concentration of primary antibodies was then added (table 8), diluted in 5% BSA-TBS-T together with the membrane and sealed in a small plastic bag, incubated overnight on a rocker at +4°C. The next day, the membrane was washed (5x5 minutes washes) with TBS-T. This was repeated for the appropriate secondary antibody.

Enhanced chemiluminescence detection using the Amersham ECL Select (GE Healthcare, RPN2235) was added to the membrane to visualise the protein bands. The ECL select solution was prepared using a 1:1 combination of reagent A and reagent B. Typically, 500µl was added per whole membrane, incubated for 2 minutes, and visualised using the BioRad ChemiDoc XRS.

2.5.3 Densitometric quantification: Image J

The quantification of the WB data was performed using the open-source software Fiji (by ImageJ). Briefly, each band was flagged as a region of interest (ROI) of fixed size. Then the software measured the optical density of the target protein bands relative to the reference protein (housekeeper) of the same samples.

2.6 Immunohistochemistry

2.6.1 ImmPACT DAB (Vector lab) immunohistochemistry

3,3'-diaminobenzidine (DAB) is oxidized in the presence of peroxidase and hydrogen peroxide resulting in the deposition of an alcohol-insoluble precipitate at the site of enzymatic activity. The reaction product is insoluble, stable, and not light sensitive so stained samples may be preserved for extended periods of time without losing sensitivity and quality of prepared tissue samples.

The ImmPACT DAB Peroxidase (HRP) (Vector Lab) yield a typical brown coloured product and is 2-4 folds more sensitivity than the original DAB substrate kit.

To investigate the distribution of the two major enzymes of the ghrelinergic system in the aged brain, DAB-immunohistochemistry (IHC-DAB, or simply IHC) was performed using anti-GOAT or anti-APT1 antibodies (table 8) on wither fixed frozen tissue from PD and PDD patients, or formalin-fixed paraffin-embedded sections of human hippocampus from early and late AD.

2.6.2 Fixed-frozen tissue (PD)

Sections were removed from -80°C freezer, air dried for 1-3 minutes then draw around the section with a hydrophobic pen (ImmEdge™ PAP Pen, Vector lab). Once the moisture was disappeared from the tissue, each slice was covered with 250µl of 10% NBF, then incubated for 1 hour at RT. This extra-fixation step was necessary due to the frailty of the tissue, in order to prevent detaching in the following steps. After incubation, tissue was washed twice in 1xPBS, then incubated with 1% H_2O_2 in PBS-triton (1xPBS + 0.1% Triton100) (PBS-T) for 8 minutes to inactivate endogenous peroxidases. Sections were quickly washed twice in 1xPBS, then a specific antibody-binding was prevented by incubating the tissue with 10% normal Donkey serum (NDS) (Sigma Aldrich, St Louise, USA) in PBS-T for 20 minutes at RT. None of the antibody used in this thesis required antigen retrieval. Sections were incubated with the appropriate primary antibody in PBS-T overnight at +4°C, at the concentrations shown in table 8.

The following day, sections were washed twice in 1xPBS, then incubated with biotinylated secondary antibody (against the host species of the primary) at 1:500 dilution in PBS-T for 1 hour at RT. In the meantime, the avidin-biotin complex

(ABC) solution was prepared, following manufacturer's guidelines (Vectastain® ABC Kit PK-6100, Vector lab). Sections were washed twice in 1xPBS and promptly incubated with pre-mixed ABC solution for 1 hour at RT. After two more washes in 1xPBS, ImmPACT DAB (Vector lab) was prepared and applied quickly. During the first steps of antibody optimisation, colour development was observed under optic microscope to determine the optimal incubation time (10 seconds to 2 minutes). All the antibodies used in this thesis were found to be optimally detectable after 40 seconds incubation with DAB. Then sections were quickly placed in warm tap water to stop further colour development. Counterstain was performed using Gill's Haematoxylin, diluted 1in2 with distilled water, for 30 seconds at RT. After few more washes sections were increasingly dehydrated: 70% for 3 minutes; 100% twice for 5 minutes; then Histoclear twice for 1 minute. Lastly, the glass was covered with the Entellan mounting media and left to dry overnight before inspecting.

2.6.3 FFPE tissue (AD)

FFPE tissue requires an additional de-paraffination step, at the beginning of the protocol, since the wax seal physically impede the antibodies to reach their targets on the tissue.

To remove the paraffine, sections were placed into preheated oven at 60°C for 30 minutes. Then slides were incubated in Xylene, a paraffine solvent, under fume hood for 30 minutes. After the incubation, sections were re-hydrated with decreasing concentrations of ethanol (100% → 95% → 70%) for two minutes each, then placed in tap water. Antigen retrieval was performed with warmed citraconic anhydride 0.05% (0.5g in 1ml ddH2O) in the steamer (100%) for 1 hour. Sections were removed from the steamer and quickly placed into warm tap water, then the area surrounding the tissue was marked with the hydrophobic pen and sections were moved in 1xPBS.

Once the paraffine was removed from the tissue, sections were incubated in H_2O_2, followed by blocking buffer and primary antibody, as shown in the previous section.

2.6.4 Cell count: QuPath

Using a Zen Axio microscope system (ZEISS) (Bioimaging facility, Cardiff University), stained sections were scanned with a 40x objective (400x magnification), and the resulting images (>100 per section) were automatically stitched from the microscope software in order to produce a single file (>1GB of size). The files were then acquired using the open-source QuPath software. The DG area was manually drawn in each section, and the positive cells manually counted in the ROI, then normalised for the area size.

2.7 Immunofluorescence

2.7.1 ICC protocol

ReN neurones maturation was assessed by investigating the expression of specific maturation markers.

Briefly, ReN neurones were grown in 96 well plates, then fixed at different stages of differentiation with 10% NBF for 15 minutes at RT. Fixed cells were washed several times with 1xPBS, then blocked with 5% NDS at RT for 1 hour. Primary antibodies were applied, and cells were incubated overnight at 4°C (table 8). The following day, cells were washed in 1xPBS, then incubated with secondary antibody for 1 hour at RT in the dark. Excess antibody was removed, and cells were stained with the nuclear dye Hoechst, diluted 1:5000 in 1xPBS for 5 minutes. Immunofluorescence examination and imaging were carried out on a Zen LSM710 confocal microscope (ZEISS) with AxioObserver.

target	source	IHC	WB	ICC	supplier	catalogue number
human APT1	rabbit monoclonal	1:500	1:1000	---	Abcam	ab91606
human GOAT	rabbit polyclonal	1:500	---	---	Phoenix pharmaceutical	H-032-12
human* GOAT	rabbit polyclonal	---	1:1000	---	bioss	BS-13355R
human GHS-R1a	goat polyclonal	---	1:200	---	Santa Cruz	sc-10359 (discontinued)
human total ghrelin	mouse monoclonal	---	1:500	---	Millipore	MAB10404
human GAPDH	rabbit monoclonal	---	1:1000	---	Abcam	ab181602
human Nestin	mouse monoclonal	---	---	1:200	Millipore	MAB5326
human Ki67	rabbit monoclonal	---	---	1:250	Abcam	ab16667
human VGluT2	rabbit polyclonal	---	---	1:400	Alomone	AGC-036
human TH	rabbit polyclonal	---	---	1:500	Abcam	ab6211
human β3-tubulin	rabbit monoclonal	---	---	1:400	Cell signalling	5568
human GFAP	rabbit polyclonal	---	---	1:1000	Abcam	ab7260
human 6E10	mouse monoclonal	---	---	1:100	Biolegend	SIG-39320

* predicted

Table 8 Antibodies for WB, IHC and ICC

2.8 Mitochondria homeostasis assays

2.8.1 MitoTracker™ orange (Thermo Fisher)

MitoTracker™ Orange CMTMRos (Thermo Fisher) is a useful tool for *in vivo* characterisation of mitochondrial mass and oxidative activity. It consists of a fluorescent dye that is internalised in live cells, due to its lipophilic nature, and is incorporated in the mitochondria matrix where it accumulates proportionally to their membrane potential.

ReN neurones were treated in a 96-well plate, as shown in figure 2.1. Then at DIV28 were treated with MitoTracker following the manufacturer instruction and incubated 30 minutes in the dark. After incubation cells were fixed with 10% NBF containing 1:5000 Hoechst, for 15 minutes in the dark. After few 1xPBS washes, cells were imaged immediately on a Zen LSM710 confocal microscope (ZEISS). Analysis were performed with the open-source software Cell Profiler, using a specific pipeline designed to detect mitochondria in fixed neurones and recently published from our group (Rees et al. 2020).

2.8.2 CellTiterGLO™ (Promega)

ATP cellular content was detected with the CellTiterGLO™ luminescent assay (Promega), consisting of a single reagent that is applied directly to live cells. The reagent contains a lysis buffer that can break the cellular membrane and induce the release of intracellular ATP in in the luciferase-containing.

Fluorescence was measured using a plate reader and reported as fold change normalised to protein content (measured with BCA assay, section 2.3.2).

2.9 BaseScope™ Assay

2.9.1 RNA-ISH in fixed-frozen human tissue

The BaseScope™ Assay in situ hybridization (ISH) technology (ACDbio/biotechne) is a powerful tool to detect gene expression *in situ*, within the spatial and morphological context of the tissue section (Wang et al. 2012a). The proprietary probe is designed to allow significant signal amplification and increased sensitivity and reproducibility, for the detection of the target RNA with single-cells resolution. In this thesis I used a variant of the original protocol, designed to improve tissue stability, and prevent detaching, whilst allowing high quality stain in old and fragile human brain tissue (Carisì et al., in preparation). Briefly, tissue sections were removed from the freezer and one extra-fixation steps were performed, similarly to what shown in section 2.6.2. Then, BaseScope protocol was carried out by incubating the tissue with H_2O_2, to inhibit endogenous peroxidases, and with a proprietary protease that contributes to the antigen unmasking. Finally, oligonucleotide probes were hybridised onto the tissue, targeting either GHS-R1a mRNA or the housekeeper gene peptidyl-propyl isomerase B (PPIB) as positive control, and revealed by secondary detection to yield small, fuchsia-coloured dots, counterstained with Gill's Haematoxylin.

2.9.2 Analysis: QuPath

As previously described, a Zen Axio microscope system (ZEISS) (Bioimaging facility, Cardiff University) was used to scan the sections. The resulting files were then acquired using the QuPath software, the DG area was manually drawn, and the dots manually counted, as previously described in section 2.6.4. GHS-R1a dots in the DG were normalised for the housekeeping gene PPIB (Carisì et al., in preparation).

2.10 Statistical analysis

The experiments in this thesis were performed with a variable number of biological replicates, indicated in each figure legend with the letter *n*. *In situ* experiments on human brains were performed using 1 tissue section per sample (donor), except for the BaseScope assay that was performed on three tissue sections per sample. *In vitro* experiments included two or three biological replicates, with each replicate including three distinct wells on the same plate (three technical replicates).

Statistical analyses were performed using the GraphPad Prism 8.0 software. Outliers were detected using the ROUT method, setting the maximum desired false discovery rate at Q=1%.

Data distribution was assessed using D'Agostino-Pearson's normality test. For normally distributed data, statistical differences between two groups were identified by using a two-tailed unpaired Student's *t*-test. When comparing more than two groups, a one-way ANOVA test, followed by Tukey's post-hoc test, was used to determine statistical differences. Data displaying non-Gaussian distribution (non-parametric) were analysed with the Kruskal-Wallis test, followed by Dunn's multiple comparison test to determine statistical differences.

All data are presented as mean ± standard error of the mean (SEM). P values of <0.05 (*), <0.01 (**), <0.001 (***) and <0.0001 (****) were considered significant.

More details regarding the number of biological repeats and the specifics of each statistical test, can be found in the relative results chapters.

Chapter 3

Ghrelinergic system in human hippocampus and mature neurones

3.1 Introduction

3.1.1 Ghrelinergic system and cognition during ageing

The ageing process consists in a progressive accumulation of intracellular molecular changes and oxidative stress, to a point where the cells are no longer able to defend themselves from stress and damage. In humans, ageing is typically accompanied by reduced immune response and increased tissue inflammation (Fagiolo et al. 1993; Bruunsgaard et al. 2001; de Gonzalo-Calvo et al. 2010), hormonal dysfunctions (Simon et al. 1992; Andrawis et al. 2000; Díez et al. 2003; Collins et al. 2019), and reduced appetite (Chapman 2004; Pilgrim et al. 2015; Giezenaar et al. 2016). Brain function is also affected, since aged individuals manifest impaired sleep (Ehlers and Kupfer 1989), reduced CSF production (May et al. 1990), and a decline in overall brain activity (Cummins and Finnigan 2007). Mostly healthy aged adults experience a subtle decline in episodic and working memory – functions that are supported by cortical and medio temporal circuits (Bailey et al. 2013; Votruba et al. 2016). Indeed, some studies report progressive brain atrophy during ageing, especially involving the frontal lobe and the hippocampus (Convit et al. 1995a; Enzinger et al. 2005), whose size reduction correlates with mild impaired cognitive functions (Kalpouzos et al. 2012). Moreover, the aged brain show increased inflammation and T cells infiltration in rodents (Dulken et al. 2019), monkeys (Batterman et al. 2021), and humans (Gemechu and Bentivoglio 2012; Dulken et al. 2019), due to increased vascular permeability, although the exact mechanism of this process is still debated: Yang and colleagues suggested a shift from ligand-specific receptor-mediated transport,

that physiologically occurs in the healthy brain, to a non-specific transcytosis (Yang et al. 2020), while Dulken and colleagues suggested the existence of defined molecular signals that are able to guide the T-cells in precise brain locations (Dulken et al. 2019). Interestingly, a study recently showed that aged mice undergoing CR for 8 weeks, have significantly reduced immune cell infiltration in the aorta and other peripheral arteries, in addition to reduced production of pro-inflammatory cytokines (Trott et al. 2018). Although this study did not take in consideration T cell infiltration in the BBB or BCSFB, this report suggests a potential additional protective mechanism by CR in the aged brain.

Along with a general deterioration of physiological functions, the ghrelinergic axis has also been shown to be impaired in ageing. Aged mice exhibit reduced plasma acyl-ghrelin compared to younger animals (Takeda et al. 2010). Similarly, plasma acyl-ghrelin levels in normal weight healthy elderly subjects (range 60 to 90 years of age) are significantly lower than in younger subjects (range 20 to 40 years) (Rigamonti et al. 2002; Akamizu et al. 2004; Schutte et al. 2007; Spitznagel et al. 2010). This is consistent with the aforementioned studies showing that the elderly show a physiological decrease in appetite, sleep and memory – functions that are modulated by acyl-ghrelin activity (Chapman 2004).

As previously mentioned, several studies performed in rodents reported that central administration of acyl-ghrelin improves memory retention, increases dendritic spine density, and stimulates synapse formation, highlighting the importance of ghrelin in regulating memory and hippocampal function (recently reviewed in Buntwal et al. 2019). Interestingly, acyl-ghrelin levels in plasma seem to directly correlate with cognitive function in both young and aged humans. Xu and colleagues measured cognitive function in 150 individuals aged 18-65, in the three months following a mild brain injury, reporting a significant correlation between cognitive decline and reduced plasma acyl-ghrelin level (Xu et al. 2014). A similar study, performed in a cohort of 260 subjects between 60 and 85 years of age, identified a positive correlation between total ghrelin plasma level (AG+UAG) and memory performance in both diabetic and non-diabetic subjects (Sang et al. 2018), while Ngo and colleagues showed that acyl-ghrelin levels in plasma of amyotrophic lateral sclerosis patients, inversely correlates with the severity of the disease (Ngo et al. 2015). These evidences suggest the use of acyl-ghrelin detection

in plasma as a predictive biomarker for brain and cognitive deterioration. Likewise, it has been shown that total ghrelin is significantly decreased in the plasma of epileptic patients, and its level can be partially restored after treatment (Greco et al. 2005; Dag et al. 2010); interestingly, these studies also showed that total ghrelin can be isolated and measured from salivary samples, since ghrelin has been shown to be produced in salivary glands (Aydin et al. 2005), hence suggesting ghrelin extracted from saliva could represent a more easily obtainable, although equally effective, biomarker.

Yet, some studies reported contradicting results. Spitznagel and colleagues measured acyl-ghrelin plasma level in a small group of 35 elderly subjects (age range 60-85), adjusting the values for waist-to-hip ratio, cholesterol, and fasting insulin levels of the subjects (on the ground that previous reports correlated these three variables to ghrelin levels). Their research showed that increased plasma acyl-ghrelin levels – normalised for the variables above – is associated with poorer cognitive and memory functions (Spitznagel et al. 2010). For this particular study, the authors refer to their results as "partial inverse correlation", because of the complex normalisation that may have biased the results. Few other conflicting results have been published: one reported worse overall cognitive performance in twenty-one young subjects (range 20-40 years) injected with two doses of 100µg/km of acyl-ghrelin, over a 3 minutes time period (Kunath et al. 2016); another study reported enhanced memory performance, although only for food-related pictures, in twenty young subjects (range 20-30 years) after two 500µg/kg acyl-ghrelin injection, over a 15 minutes time period (Malik et al. 2008). The variability in the methods used, as well as the small number of participants, may explain the many discrepancies among different studies.

Most importantly, hardly any studies in literature have focused on unacylated ghrelin levels, and this could lead to the exclusion of significant information from the greater picture. Indeed, our group recently showed that peripheral administration of unacylated ghrelin in mice, reduces AHN – the main process underlying memory formation – (Hornsby et al. 2020), confirming previous reports in which unacylated ghrelin has been shown to counteract the beneficial effects of acyl-ghrelin in several animal models (Matsuda et al. 2006; Kumar et al. 2010; Stevanovic et al. 2014; Fernandez et al. 2016). Moreover, we also showed that acylated/unacylated ghrelin (AG:UAG) ratio is impaired in the plasma of PD with

dementia patients, but not in cognitively normal PD subjects (Hornsby et al. 2020), as will be better discussed in chapter 4. This suggests that, measuring AG:UAG ratio – rather than acyl-ghrelin levels by itself – may better represent the changes occurring to the ghrelinergic system during ageing.

Another important element often missing from ghrelin research, is the investigation of the enzymes involved in the ghrelinergic axis – GHS-R1a, GOAT and APT1. Indeed, these enzymes are essential in determining circulating AG:UAG ratio, hence affecting ghrelinergic signalling in the brain. Notably, acyl-ghrelin relocates from the circulation to the brain, where it binds GHS-R1a in several regions, including the hippocampus, the occipital cortex, and the hypothalamus, as shown in section 1.4.4. Sun and colleagues studied the expression levels of GHS-R1a mRNA in the mouse brain during ageing (range 1-30 months of age). While they were able to detect significant decrease of plasma total ghrelin and close-to-significance decrease of acyl-ghrelin, they could not identify any significant change in GHS-R1a mRNA in total brain extracts, at any age (Sun et al. 2007b). Singularly, while GHS-R1a mRNA expression is physiologically very low in fat tissue of young mice, its expression increases during ageing and is associated with impaired thermogenesis and pro-inflammatory response (Lin et al. 2011) – in contrast with the well-known anti-inflammatory properties of acyl-ghrelin (as shown in section 1.3.6) – suggesting a GHS-R-dependent, ghrelin-independent mechanism of thermoregulation and inflammation in fat tissue during ageing (Ma et al. 2011).
GOAT has also been shown to be expressed in the human brain, as well as in rodents where it can re-acylate unacylated ghrelin, as shown in section 1.4.4. However, to my knowledge, no one has investigated expression of GOAT in the brain during ageing – except one single report, showing that GOAT increases in total brain lysate of aged chicken (Kitazawa et al. 2015).
Furthermore, hardly anything is known about APT1 expression, localisation, and activity in the human brain. As a result, the goal of this chapter is to investigate and compare the expression of GHS-R1a, GOAT and APT1 between the young and the aged human brain.

3.1.2 *In vivo* and *in vitro* models of brain diseases

The human hippocampus (sections 1.4.2 and 1.4.3) differs from most of the other species not only because of its size and anatomical features, such as neurotransmitters distribution and differential connections between different areas, but also for its involvement in highly complex cognitive tasks, some of which are unique to humans. Indeed, while several other species are capable of complex processes such as communication and memorisation, humans can also engage in distinctive forms of social cognition, such as elaborating the intentions behinds a person behaviour and learning from them, in order to develop a sense of collective culture (Tomasello and Rakoczy 2003). This process, known as the "theory of mind", is based on two processes; the "mentalisation", defined as the ability of recognising the others as distinct individual, and the "episodic autobiographical memory", which specifically involves the human hippocampus and defines a human's ability to recall knowledge of the past and to use it to understands other's people intentions (Perry et al. 2011a). So, while on one side the hippocampus contributes to our species identity, on the other side its complexity makes it selectively vulnerable to diseases that are unmatched in the animal kingdom, like neurodegenerative diseases. Indeed, hardly any cases of neurodegenerative-like disorders have been ascertained in nature. The closest example is a genetic disorder of wild Japanese baby macaques, that causes progressive neurological impairment and death at early age (McBride et al. 2018). Other neurodegenerative-like diseases have been identified in domestic animals, although these are very rare and mostly caused by parasite infections or genetic mutations, aggravated by centuries of genetic selections (Sisó et al. 2006). Hence, neurodegenerative disorders – intended as the consequence of progressive, mostly age-related, accumulation of neuronal and cognitive defects – are almost exclusive to humans, therefore human, or humanised, models are needed to thoroughly investigate and characterise these diseases.

Nevertheless, the use of genetic mice models to study human neurodegenerative disorders has always been favoured due to the difficulty of accessing – or successfully reproducing – the human brain, as well as the simplicity in handling small animals, such as rodents, that exhibit quicker breeding capability and shorter life cycle (18-22 months).

As mentioned before, the use of human stem cell-derived neurones steadily increased in the last few decades and numerous companies invested in the production and commercialisation of human stem cells lines readily available for differentiation. One of such models is the ReNcell™ VM stem cell line (from now on referred to as ReN cells), generated from the ventral mesencephalon (VM) region of a 10-week-old gestational human brain, and immortalised by insertion of the v-myc transcription factor gene (Donato et al. 2007). Although myc genes are oncogenes, hence traditionally associated with uncontrolled tumorigenesis (Dang et al. 1999), stem cell transfected with either c-myc or v-myc, have displayed long-term cell expansion capacity (or "stemness"), while maintaining a stable phenotype and without developing karyotypic abnormalities (Villa et al. 2004; Takahashi and Yamanaka 2006) (appendix 1). Notably, ReN cells differentiation occurs upon removal of the mitogens EGF and FGFb from the culture media, and has been extensively characterised (Hoffrogge et al. 2006; Donato et al. 2007; Pai et al. 2012; Stevanato et al. 2015; Song et al. 2019). The resulting ReN cells-derived neuronal cultures (from here on referred to as ReN neurones) are actually a mixed population of post-mitotic cells such as astrocytes, neurones, and oligodendrocytes in variable amounts, although specific protocols have been designed to favour single cell subtypes, such as dopaminergic (Seiz et al. 2012) or oligodendrocytes (Li et al. 2017). Ultimately, the variability of the culture composition and the cell frailty – being post-mitotic cells – make their usage challenging, albeit they more closely resemble primary cell cultures in term of cell type composition, differentiation fate and genomic stability.

In alternative, one of the most common cell line in neuroscience research is the human SH-SY5Y, obtained in 1970 from a bone marrow biopsy of a metastatic neuroblastoma, and characterised by high expression levels of tyrosine hydroxylase (TH) and dopamine-β-hydroxylase, two key enzymes of the catecholaminergic system – hence can synthesize both dopamine (DA) and noradrenalin (NA) (Biedler and Schachner 1978). These cells are highly stable, easy to handle and can divide numerous times without losing their genetic and phenotypic characteristics. Interestingly, SH-SY5Y cells can also be differentiated to a more mature neuron-like phenotype, and are especially employed as models of PD (Encinas et al. 2000).

Alas, SH-SY5Y cell cultures are not entirely neuronal, and some cells exhibit epithelial-like characteristics (Ross et al. 1983). Furthermore, their cancerous origin may influence differentiation, growth and metabolism; indeed, reports on their exact phenotype are contradictory (reviewed in Xicoy et al. 2017).

Lastly, human *post-mortem* brain tissues are another important research tool to investigate brain diseases. However, whilst the mouse brain can be collected, sliced, and cultivated *alive* in culture media for a short amount of time (Hatton et al. 1980), human brain can only be collected after death – hence cannot be used as an actively responding model. Although it is probably the most reliable source of information, being the closest model possible, *post-mortem* brain tissue has numerous disadvantages; firstly, very few people decide to donate their brains after death, and very few organs are in good enough conditions to be used for research. Indeed, a sudden or violent death may significantly affect cellular structure, for instance in case the brain cells suffered a significant lack of oxygen before death; moreover, the time window between the subject death and the tissue collection, known as *post-mortem* interval (pmi), may also significantly affect the structure of the tissue, producing unwanted molecular changes and increasing samples variability (Barton et al. 1993; Harrison et al. 1995; Li et al. 2003). Secondly, although different fixation techniques are available, each one of them is only suitable for certain type of experiments; for instance, formalin-fixed paraffine-embedded (FFPE) tissue maintains high structural integrity therefore is recommended for *in situ* detection of macromolecules, however it is challenging to use for protein and/or nucleic acids extraction, since the paraffin removal step requires harsh solvents that cause the loss of some antigens and impairs the integrity of nucleic acids (Wang et al. 2013). On the contrary, fresh-frozen tissues – stored at very low temperatures immediately upon collection – present lower structural integrity, since freeze-thaw cycles may impact 3D structure of the cells, but preserve protein antigens and allow extraction of very high purity RNA/DNA (Kelly et al. 2019).

Ultimately, there are numerous *in vivo* and *in vitro* models that can – and are being – successfully used in neuroscience research. However, due to the many limitations of each of these models, using a combination of *in vitro* and *ex vivo* may be more effective into recapitulating the complexity of the system.

3.2 Aims

Acyl-ghrelin has been shown to exert neuroprotective effects in cellular and animal models of neurodegenerative diseases (section 1.2.2), being able to cross both the BBB and the BCSFB (section 1.4.1) and to bind and activate GHS-R1a in the brain (section 1.4.4).

Since our group recently reported that acyl-ghrelin is significantly reduced in the brain of PD patients diagnosed with dementia, it is imperative to investigate the extent to which the enzymes of the ghrelinergic axis – GOAT and APT1 – and ghrelin receptor – GHS-R1a – are expressed in the human hippocampus during ageing, in order to establish the potential for a pharmacological intervention for neurodegenerative diseases.

The aims of this chapter are:

- To characterise the expression and the spatial localization of ghrelinergic axis proteins (GOAT, APT1 and GHS-R1a) in the human hippocampus from healthy young and aged subjects.
- To quantify the expression of these proteins (GOAT, APT1 and GHS-R1a) in order to determine their variation between the young and aged human hippocampus.
- To assess maturation of human stem-cell derived neurones and expression of ghrelinergic axis proteins (GOAT, APT1 and GHS-R1a).

3.3 Results

3.3.1 Ghrelinergic system components are expressed in distinct compartments in the aged human hippocampus

GOAT immunopositive cells are present throughout the human hippocampus and the surrounding regions in young (not shown) and aged healthy brain (figure 3.1 A). The immunopositivity is higher in regions with greater nuclei density, like the GCL (figure 3.1 B), and lower in areas with less cells, like the CA4 (figure 3.1 C), suggesting that GOAT is proportionally distributed between brain regions.
In comparison to GOAT, APT1 protein expression does not appear to be uniform; instead, the pattern of immunoreactivity is variable across the brain (figure 3.2 A). In particular, the highest anti-APT1 immunoreactivity was found in the hippocampal sulcus (figure 3.2 B), the CA4 area (figure 3.2 C), and the vestigial cavity (figure 3.2 D). APT1 immunopositive cells are scarce in other locations of the tissue (not shown), except for the area surrounding vasculature and for the rim of ependymal and sub-ependymal tissue contiguous to the ventricles and the cells adjacent the choroid plexus (figure 3.2 E, black arrows). These results suggest that APT1 is expressed in discrete regions in the aged healthy human brain.

In contrast, studying the distribution of ghrelin receptor is quite challenging since the commercially available antibodies are not 100% effective (as described in section 6). Therefore, I performed the BaseScope assay in the aged brains: oligonucleotide probes targeting GHS-R1a mRNA can be seen on the tissue as small, fuchsia-coloured dots, with each dot representing the amplification of a single molecule of GHS-R1a mRNA. The puncta were not clearly visible under a 4x objective (40x magnification) (figure 3.3 A); however, using a 40x objective (400x magnification) the GHS-R1a probe dots were clearly visible around the nuclei. The stain was particularly evident in the GCL (figure 3.3 B, black arrowheads), while it was less consistently observed in other areas of the hippocampus, such as the CA4 (figure 3.3 C), suggesting that GHS-R1a is also expressed in discrete patterns in the aged healthy human brain.

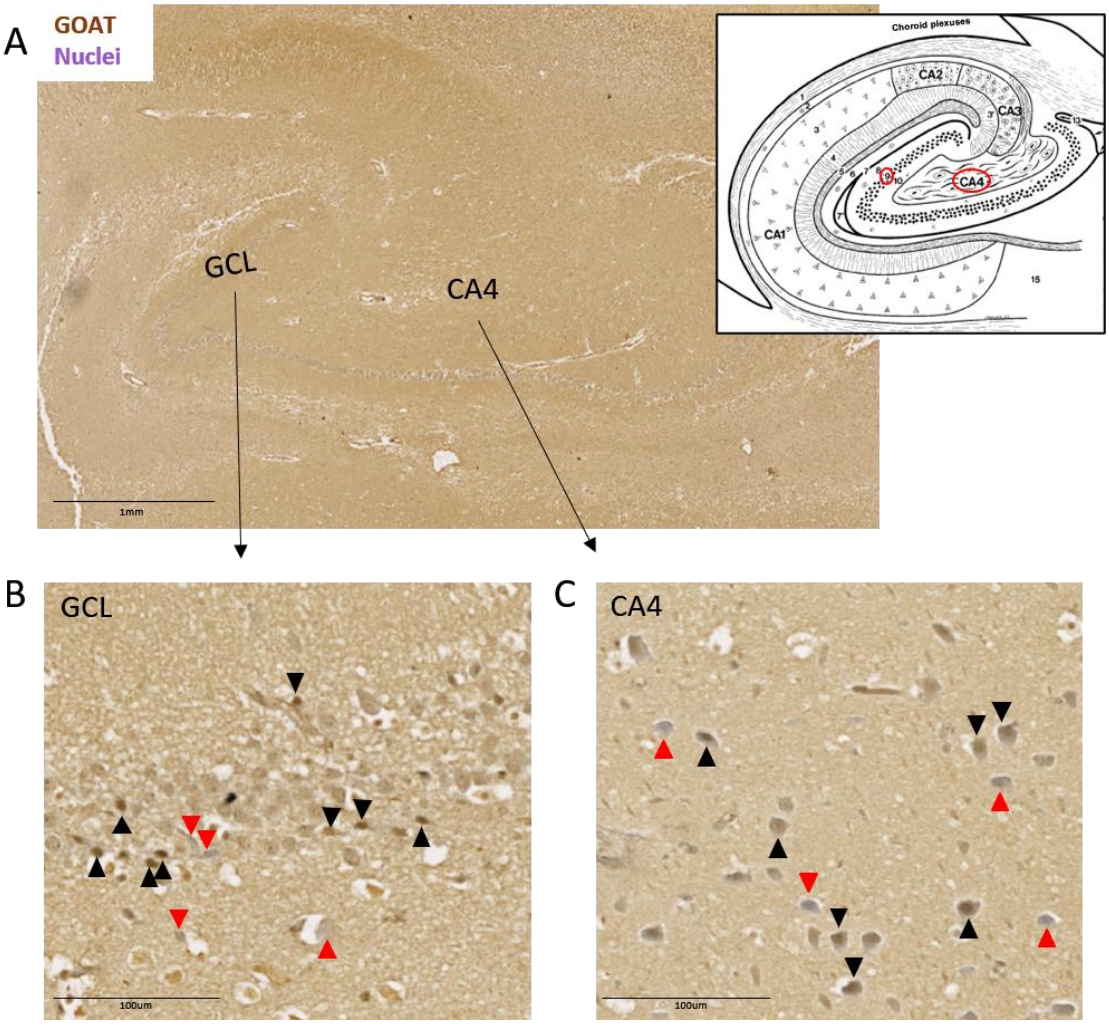


Figure 3.1 GOAT localization in the aged human hippocampus

GOAT immunoreactivity detected with IHC-DAB (dark brown, black arrowheads) in the aged healthy human hippocampus, seen at (**A**) 40x magnification and (**B, C**) 400x magnification. Nuclei are stained with haematoxylin (purple, red arrowheads). (Atlas adapted with permission from Duvernoy et al. 2013, Springer licence number 4967160485075)

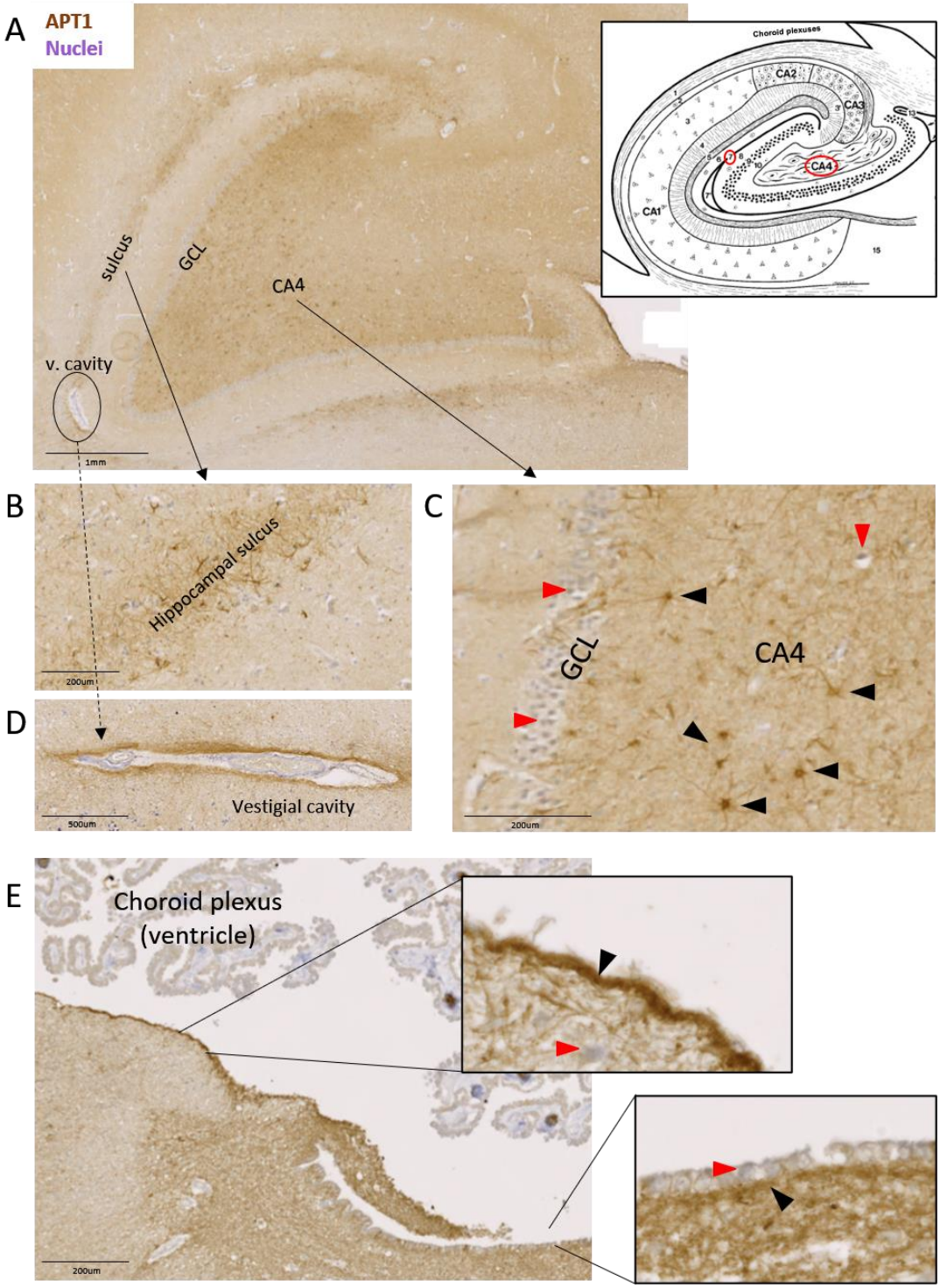


Figure 3.2 APT1 localization in the aged human hippocampus

APT1 stained with IHC-DAB (brown, black arrows) in the aged healthy human hippocampus, seen at (**A**) 40x magnification and (**B, C, D**) 400x magnification. (**E**) APT1 immunopositive stain is also clearly visible in the rim of the sub-ependymal tissue, underneath the choroid plexus and adjacent to the ventricle. Nuclei are stained with haematoxylin (purple, red arrows). (Atlas adapted with permission from Duvernoy et al. 2013, Springer licence number 4967160485075).

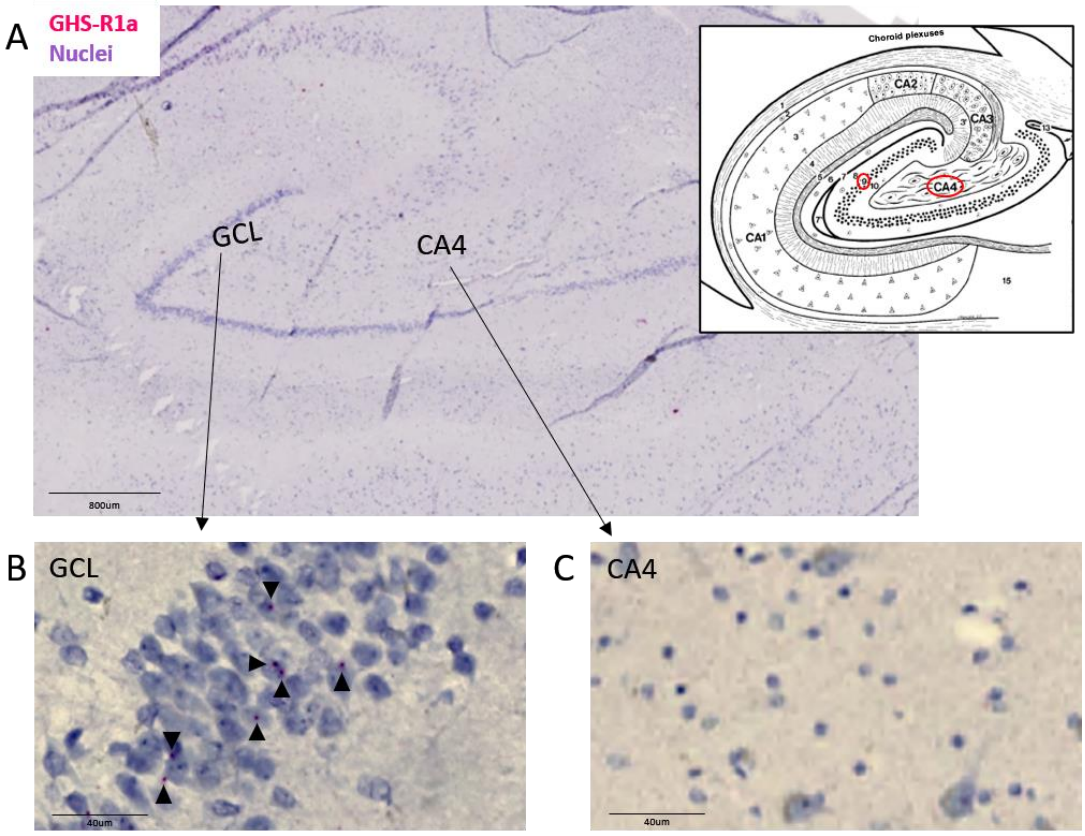


Figure 3.3 GHS-R1a mRNA localization in the aged human hippocampus

GHS-R1a mRNA expression (fuchsia dots, black arrows) in the aged human hippocampus, detected using BaseScope, is shown at (**A**) 40x magnification, and (**B**, **C**) 400x magnification. Nuclei are stained with haematoxylin (purple). (Atlas adapted with permission from Duvernoy et al. 2013, Springer licence number 4967160485075).

3.3.2 Ghrelinergic system is intact with ageing

Anti-GOAT immune-positivity was identified in the GCL of the hippocampus by the round aspects, with the brown precipitate of chromogen tightly packed around the nuclei (figure 3.4 A, black arrowheads). GOAT immunopositive cells were counted inside the GCL (delineated by red lines), then normalised for the area of the region in mm^2; this was done to account for natural variations in size of the hippocampus that may occur between subjects (donors) as well as the natural shrinkage of the DG associated with aging (see section 1.4.2). After normalisation, the analysis revealed 804.4± 159.5 GOAT positive cells per mm^2 of DG in young brains (n=6), and 433.3 ± 160.8 cells per mm^2 of DG in aged brains (n=4), with no significant difference between groups (p=0.1551) (figure 3.4 B). Western Blot protein quantification, reported as fold change to GAPDH, confirmed no significant changes between young (0.9942 ± 0.1994) (n=5) and aged (0.9616 ± 0.09621) (n=5) subjects (p=0.8864) (figure 3.4 D and E). Lastly, quantification of MBOAT4 mRNA transcript from tissue lysates, reported as fold change to β-actin, also showed no significant difference between young (ΔCt=7.163 ± 0.1743) (n=5) and aged (ΔCt=7.025 ± 0.2016) (n=5) subjects (p=0.6187) (figure 3.4 C). These results suggest that GOAT expression in the hippocampus does not vary significantly with ageing.

APT1 immunopositive cells were identified in the CA4 area of the hippocampus, by their star-like morphology (figure 3.5 A, black arrowheads), then normalised for the area in mm^2 of the CA4. 76.22 ± 17.22 cells per mm^2 were counted in young brains (n=5), and 134.3 ± 56.62 cells per mm^2 in aged brains (n=4), with no significant difference between groups (p=0.3132) (figure 3.5 B). Western Blot protein quantification, reported as fold change to GAPDH, confirmed no significant changes in APT1 between young (1.417 ± 0.1345) (n=5) and aged (1.205 ± 0.2499) (n=5) brains (p=0.4784) (figure 3.5 D and E). Lastly, LYPLA1 gene expression from tissue lysates, reported as fold change to β-actin, showed no significant difference between young (ΔCt=5.941 ± 0.2075) (n=5) and aged (ΔCt=5.780 ± 0.1409) (n=5) subjects (p=0.5387) (figure 3.5 C), suggesting that APT1 expression does not vary between young and aged hippocampus.

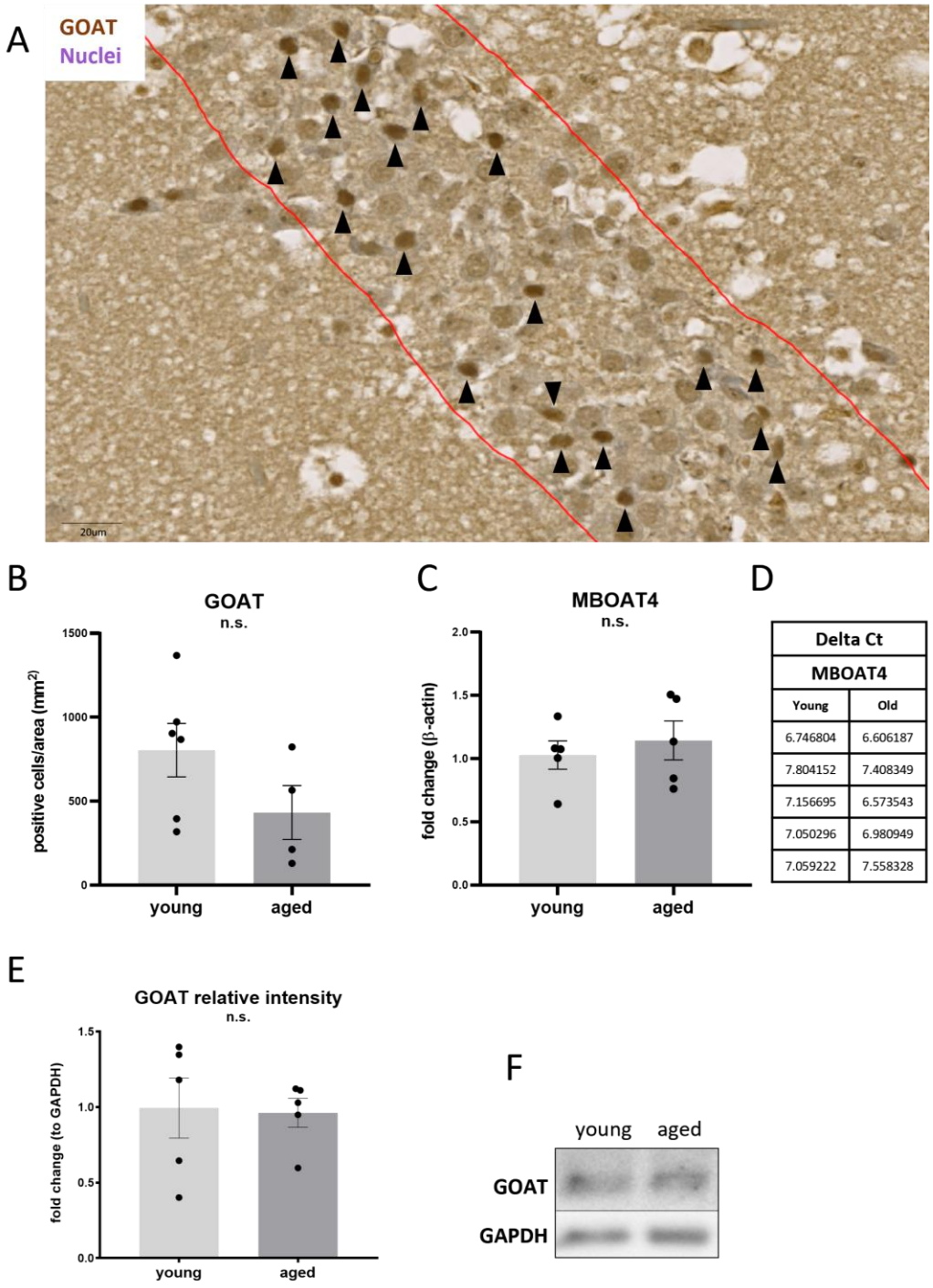


Figure 3.4 GOAT expression in young and aged human brain

(**A**, **B**) Anti-GOAT immunoreactivity in the GCL (circled in red) was determined by IHC, quantified, and normalised for the area of the GCL (in mm^2); (**C**) MBOAT4 gene expression was measured by RT-qPCR; (**D**, **E**) GOAT protein relative quantification in the tissue was performed by western blot. ROUT analysis was performed to remove any outliers from the groups. Significance was measured with unpaired two-tailed *t*-test (n.s. not significant). Data are reported mean ± SEM.

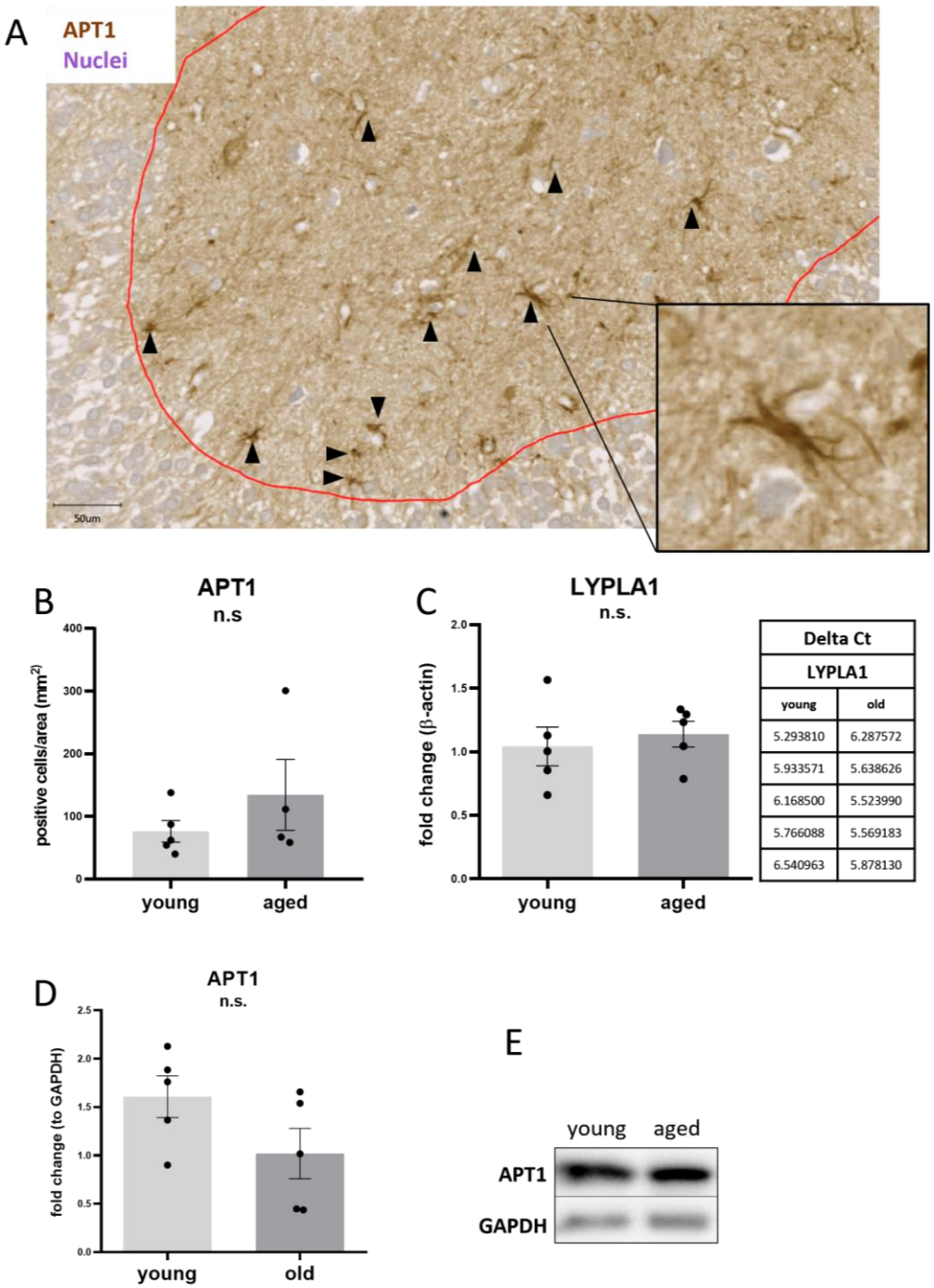
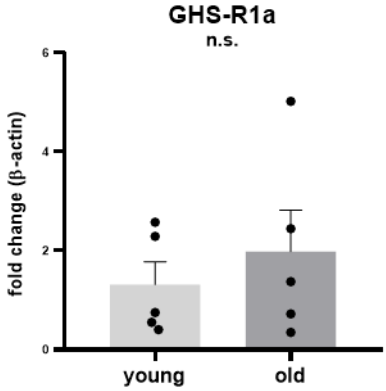


Figure 3.5 Expression of APT1 in the young and aged human brain

(**A, B**) Quantification of anti-APT1 immunoreactive cells, detected in the CA4 (circled in red) by IHC and normalised for the area of the CA4 (in mm^2); (**C, D**) relative quantification of LYPLA1 gene expression by RT-qPCR; (**E, F**) relative quantification of APT1 protein by western blotting. ROUT analysis was performed to remove any outliers from the groups. Significance was measured using an unpaired two-tailed *t*-test (n.s. not significant). Data are reported as mean ± SEM.

Lastly, due to the incompatibility of the BaseScope assay with our FFPE sections, GHS-R1a mRNA expression was analysed via RT-qPCR. Gene expression in tissue lysates, reported as fold change to β-actin, showed no significant difference between young (ΔCt=6.867 ± 0.5421) (n=5) and aged (ΔCt=6.447 ± 0.6679) (n=5) subjects (p=0.6385) (figure 3.6), suggesting that GHS-R1a mRNA expression is maintained and is constant during ageing.



Delta Ct	
GHS-R1a	
Young	old
7.276295	5.576097
8.167620	6.407590
5.501309	8.379328
5.671294	7.332841
7.717407	4.538188

Figure 3.6 GHS-R1a mRNA expression in young and aged human brain

GHS-R1a gene expression was measured by RT-qPCR. ROUT analysis was performed to remove any outliers from the groups. Significance was measured using an unpaired two-tailed *t*-test (n.s. not significant). Data are reported as mean ± SEM.

3.3.3 ReN neurones reach maturation and express key enzymes of the ghrelinergic axis, after 28 days of differentiation *in vitro*

ReN stem cells (DIV0) grow uniformly and consistently, exhibiting typical fibroblast-like shape and size (figure 3.7 A). At this stage, cells express "stemness" markers such as the neuroepithelial stem cell protein, Nestin (figure 3.7 B) and the proliferation antigen Ki67 (figure 3.7 C).

Upon growth factors withdrawal, cells started differentiating; after 4 days into the differentiation process (DIV4), they begin to show a more elongated morphology (figure 3.7 D) and express neuronal precursor markers such as the microtubule-associated protein 2 (MAP2) (figure 3.7 E) and the "neuronal nuclei" antigen, NeuN (figure 3.7 F).

After 28 days (DIV28), ReN neurones exhibit long prolongments and a mature neuronal morphology (figure 3.7 G); moreover, some cells express markers associated to specific neuronal phenotypes, such as the vesicular glutamate transporter 2 (VGluT2), marker for glutamatergic neurones (figure 3.7 H), and the tyrosine hydroxylase (TH) enzyme (figure 3.7 I) of DA/NA neurones. They also express more generic markers such as the axonal microtubule protein β3-tubulin, that is expressed by all neurones regardless of the cellular sub-type, and the glial fibrillary acidic protein (GFAP) expressed by astrocytes (figure 3.7 K).
More specifically, 32.9% ± 0.417 of the total cells were found to be β3-tubulin$^+$ (neurones), while 63.3% ± 3.33 were GFAP$^+$ (astrocytes) (n=3) (figure 3.7 J). No overlap between the two stain was detected. The remaining cells ("others") were not further characterised.

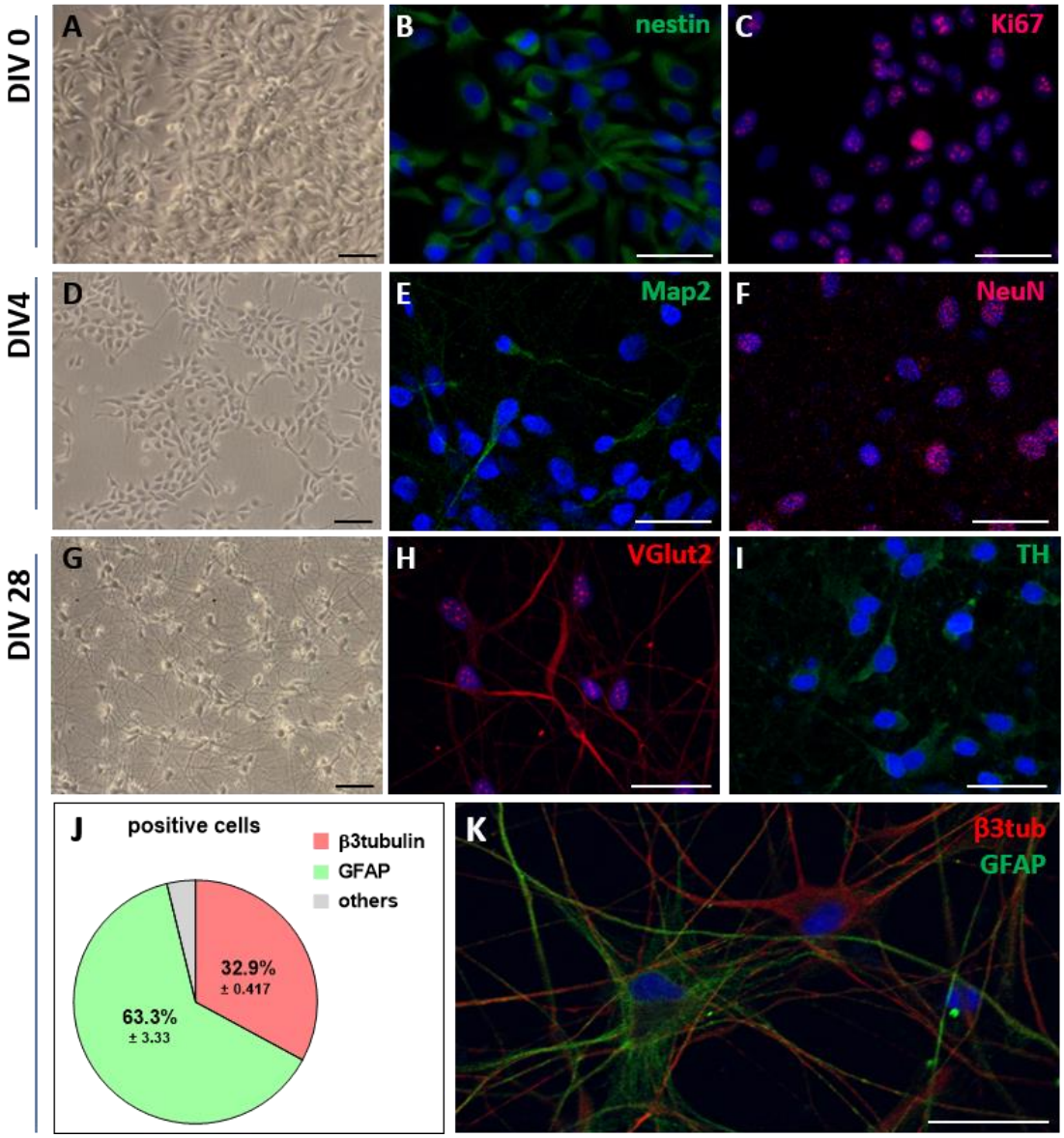
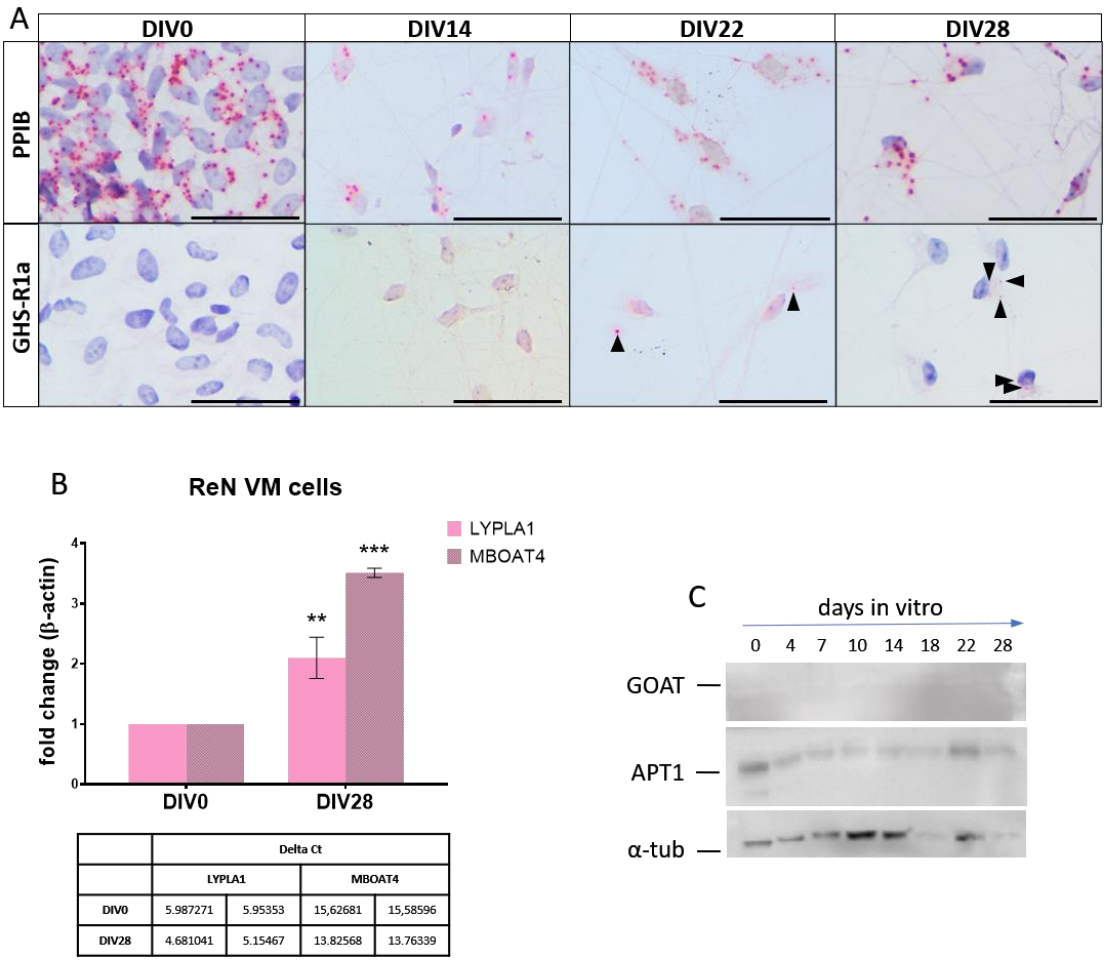


Figure 3.7 ReN cells express neuronal markers during differentiation

(**A**) ReN cells (DIV0) express proliferation markers such as (**B**) Nestin (green) and (**C**) Ki67 (purple). (**D**) At day 4 of the differentiation process (DIV4), ReN cells start showing elongated morphology and expressing neuronal precursor markers such as (**E**) MAP2 (green) and (**F**) NeuN (purple). (**G**) After 28 days (DIV28), ReN neurones exhibit mature morphology and express markers of different neuronal sub-populations, such as (**H**) VGlut2 for glutamatergic neurones (red) and (**I**) TH for dopaminergic neurones (green). (**J**, **K**) Quantification of neuronal cells (immunopositive for β3-tubulin, red), and astrocytes (positive for GFAP, green) (n=3). Scale bar 50μM.

ReN cells and ReN neurones were investigated for the expression of key enzymes of the ghrelinergic system.
Using the BaseScope assay, no GHS-R1a stain was detected at DIV0 and DIV14, while some dots appeared at DIV22 and became more evident at DIV28 (figure 3.8, black arrowheads), suggesting that GHS-R1a is expressed in the later stages of neuronal maturation, but not in stem cells nor during early differentiation.

Both GOAT and APT1 mRNA expression were detected by RT-qPCR in stem cells (DIV0), while mature neurones (DIV28) exhibit 2-times higher (**p=0.0069) and 4-times higher (***p=0.0009) expression level, respectively, compared to DIV0. Similarly, APT1 protein was detected in ReN cells during differentiation, between DIV0 and DIV28. On the contrary, GOAT protein was not detectable using WB in cell lysates at any of the analysed timepoint, in contrast with the whole brain tissue lysate that was used as a positive control (not shown), suggesting a differential regulation of the protein expression in this specific cell type.



	Delta Ct			
	LYPLA1		MBOAT4	
DIV0	5.987271	5.95353	15,62681	15,58596
DIV28	4.681041	5.15467	13.82568	13.76339

Figure 3.8 ReN neurones express key proteins of the ghrelinergic system

(**A**) BaseScope assay of PPIB (housekeeper) and GHS-R1a (black arrowheads) on ReN cells at different stage of differentiation. (**B**) RT-qPCR analysis of LYPLA1 (APT1) and MBOAT4 (GOAT) gene expression at DIV0 and DIV28 (n=2). (**C**) Western blot of GOAT and APT1 in differentiating ReN cells at different timepoints (n=1). Scale bar 50µM. **p=0,0069; ***p=0,0009.

3.4 Discussion

One of the major candidates identified as a protective agent against age-related diseases in the brain, is the hunger hormone ghrelin, whose complex signalling, described in sections 1.2.9 and 1.3.6, is mediated by its receptor GHS-R1a. In several mice models, acyl-ghrelin has been shown to exert an important protective effect against neurodegeneration as shown in sections 1.2.2. Furthermore, its levels in humans are reduced during ageing, as described in section 3.1.1. To better understand the extent of this impairment and the potential consequences to brain health, my first aim was to characterise the expression of the three key players of the ghrelinergic system in the human hippocampus – GOAT, APT1 and GHS-R1a – and to determine whether the ghrelinergic system is affected during ageing. Moreover, since *post-mortem* brain tissue can only partially recapitulate the complexity of the mature brain, I also differentiated and characterised the human ReN stem cell line. I was able to confirm their maturation into neurones by showing the expression of relevant maturation markers after 28 days of differentiation *in vitro* (figure 3.7), as previously extensively reported (Donato et al. 2007). These data confirm that ReN neurones could represent a comprehensive model of neuronal maturation, hence I further investigated the expression of key enzymes of the ghrelinergic axis.

3.4.1 GOAT

My data show that GOAT is homogeneously expressed in the human hippocampus (figure 3.1), as previously reported in rodents (Murtuza and Isokawa 2018). The pattern of immunostaining is well defined and tightly concentrated around the cell nuclei, consistent with GOAT localisation in the membranes of the ER (Taylor et al. 2013). Interestingly, GOAT protein and mRNA levels do not vary significantly with ageing, in any of my analyses (figure 3.4).

As previously mentioned, GOAT can acylate unacylated ghrelin in the mouse hippocampus (Murtuza and Isokawa 2018). Moreover, several studies reported that ghrelin is the only known substrate of GOAT, as shown in section 1.2.5. These studies, in combinations with my data about GOAT expression in the aged human brain, could indicate that ghrelin is re-acylated *in situ*, similarly to what occurs in rodents. Alas, these data are circumstantial and not sufficient to confirm this

hypothesis; moreover, even though the data show that GOAT expression levels are maintained throughout ageing, its enzymatic activity may still vary with time (i.e., reduced efficiency, inhibition, etc.), therefore it is essential to determine the conditions required for its activation. This is impossible to investigate directly in the human brain, however it could be possible to measure GOAT activity *in vitro* using GOAT-expressing human stem cell-derived neurones and a fluorescent acylation probe, similarly to what shown by Murtuza and colleagues in mouse brain slides (Murtuza and Isokawa 2018). ReN neurones could potentially be used for this purpose, since my data show GOAT mRNA expression after 28 days of differentiation (figure 3.8 B). Strangely, GOAT protein could not be detected in the same cells (figure 3.8 C), suggesting that the protein may undergo specific post-translational regulation. Interestingly GOAT expression in rodents has been shown to be enhanced by CR (Reimer et al. 2010; Stengel et al. 2010; Zhao et al. 2010), hence future work should investigate whether cultivating ReN neurones in a nutrient-restricted media may favour GOAT protein expression.

Additionally, it would be interesting to investigate GOAT cell-specificity. This information could be obtained by performing double immunostaining *in situ* in the human brain, for several neuronal-subtype markers – such as GFAP for astrocytes, VGlut for glutamatergic neurones or TH for dopaminergic neurones. This information could be particularly relevant in the context of specific neurodegenerative disorders, in which single cell types are more effected than others, such as dopaminergic (DA) neurones in PD. For instance, in the context of PD, if GOAT were to be found in a single cell type, such as DA cells, it could predict an impairment of the ghrelinergic system specifically in this disease, due to the specific degradation of this cell type and subsequent loss of GOAT. On the contrary, if GOAT expression were to be found in diverse cell types, a compensatory mechanism could occur upon death of affected neurones, allowing regular ghrelinergic activity despite the cell loss. Lastly, identifying cell specificity of GOAT expression, may also give information about the brain areas where ghrelin could be locally re-acylated, hence further extending our knowledge about acyl-ghrelin availability in the brain.

3.4.2 APT1

A study reported that following intracranial-delivery, unacylated ghrelin persists for a longer period of time in the CSF of mice, compared to acyl-ghrelin – which is most likely used or degraded (Banks et al. 2002). This evidence suggests that acyl-ghrelin deacylation may occur locally and, since unacylated ghrelin is unable to activate GHS-R1a (Shiimura et al. 2020), it is vital to characterize APT1 protein expression and activity in the brain, to understand the mechanisms that determine acyl-ghrelin bioavailability. Indeed, very few studies have shown APT1 mRNA and/or protein expression in the CNS.

In the mouse brain, APT1 mRNA expression was detected by single-cell RNA sequencing (Linnarsson lab Mouse Brain Atlas) and RNA in situ hybridisation (RNA-ISH) (Siegel et al. 2009; Tatro et al. 2013), while protein expression was reported in a single study, using IHC (Tatro et al. 2013). Similarly, I was able to detect *in vitro* APT1 mRNA and protein expression in mature ReN neurones at DIV28 (figure 3.8 B and C), suggesting these cells retain the ability of de-acylate ghrelin. Interestingly, APT1 protein expression has only been shown once before in the human brain, performing WB on the whole brain lysates (Wang et al. 1999), while the mRNA was detected in a large study using RNA-ISH to analyse thousands of genes simultaneously (Allen Human Brain Atlas). Hence, to my knowledge, the data presented in this thesis are the first evidence specifically focusing on showing the localisation of APT1 protein in the human hippocampus (figure 3.2). Interestingly, my data show that APT1 is distributed in discrete layers of the hippocampus (figure 3.2) suggesting a clear and defined pattern of action. The highest expression was found in three areas: the CA4 (figure 3.2 C), the hippocampal sulcus and vestigial cavity (figure 3.2 B and D), and in the ependymal and sub-ependymal tissues of the inferior blade of the lateral ventricles (figure 3.2 E). Notably, in most of my tissue samples, it was not possible to identify the polymorphic layer (figure 1.8 C, layer 10) - a very thin transition layer that runs between the GCL and the CA4 - partly because of the reduced size of the layer, and partly because it is scarcely populated, being mostly a crossroad for the axons originating from the GCL above. Either way, since the anti-APT1 stain looked very consistent in that region (figure 3.2 C), I used the term “CA4” to refer to the whole area immediately inferior to the GCL, with the assumption that this would include the polymorphic layer.

A novel element that stands out, is the atypical, branched aspect of the APT1^{+} cells in the CA4 (figure 3.5 A), whose appearances closely resemble those of astrocytes. To my knowledge, there is no study specifically focusing on APT1 expression in astrocytes in any species, although murine stem-cell derived astrocytes have been shown to express both APT1 and APT2 (Kong et al. 2013). Interestingly, based on the data from a large mouse RNA-seq database, it seems that APT1 is expressed in astrocytic cells in the region of the hippocampus (*dropviz.org*). However, in order to confirm this hypothesis, future work should investigate co-localisation between APT1 and specific cellular sub-types, using molecular markers such as GFAP for astrocytes, NeuN for neurones, and more.

The hippocampal sulcus is a foetal structure, generated during development. When the two hippocampal laminae have completed their folding, the sulcus extremities start fusing and the fissure disappears shortly after, except for a small vestigial region in the most medial part of the hippocampus (Duvernoy et al. 2013). After development, this structure does not retain any function. However, being a continuation of the superficial hippocampal sulcus (figure 1.8 C, layer 14) that is in contact with the ventricle, it could be possible that the small vestigial cavity, could also be filled with CSF. Notably, an enlargement of the cavity has been associated with Alzheimer's disease, consistent with the massive cell loss occurring in the brains of the affected patients (De Bastos-Leite et al. 2006). Further studies will be needed to clarify its function and the role of APT1 positive cells in this region.

Interestingly, APT1 is also selectively expressed in the rim of the cells immediately next to the choroid plexus, suggesting that the enzyme may indeed have a key role in determining acyl-ghrelin availability in the CSF. The analysis of APT1 gene expression patterns from the mouse brain atlas and other databases, confirmed that APT1 mRNA was detected in choroid plexus epithelial cells (*mousebrain.org*, *dropviz.org*), although to my knowledge no previous studies have acknowledged this expression pattern in humans. As shown in section 1.4.1, acyl-ghrelin crosses the BBB via a saturable receptor-mediated mechanism, while accessibility of the unacylated form is most likely dependent on a different non-saturable mechanism.

Moreover, peripherally injected fluorescent acyl-ghrelin has been shown to be internalised by the ependymal cells of the CP and the hypothalamic pericytes, although the exact uptake mechanism has not been investigated (Uriarte et al. 2019). Since APT1 is known to catalyse ghrelin de-acylation, its presence in proximity of the BCSFB may indicate a role as a crossing guard: by de-acylating ghrelin before it could access the brain parenchyma, APT1 would represent an important key regulator of acyl-ghrelin accessibility in the brain. Interestingly, chemoreceptors like Tas1r1 and Tas1r3, that are responsible for ghrelin sensing in the gastrointestinal tract, are also expressed in the brain, including in the hypothalamic tanycytes and in the CP, suggesting a role in the chemo surveillance system of the brain (Santos et al. 2019). Future studies on co-localization between APT1, GOAT and ghrelin chemoreceptors in the ependymal and sub-ependymal tissues of the lateral ventricles, may suggest an additional role for local acylation/deacylation.

Indeed, as previously mentioned, acyl-ghrelin is quickly de-acylated after intra cranial injection (Banks et al. 2002); in order to investigate whether APT1 enzymatic activity may be responsible for this, a possible approach would be to inject fluorescent acyl-ghrelin (McGirr et al. 2011) in the CSF of APT1 KO mice, to determine whether APT1 is actively implicated in acyl-ghrelin import in the brain.

Lastly, according to the mouse atlas, APT1 is also expressed in the cells of the diagonal band of Broca, a bundle of cholinergic nerve fibers that innervates the hippocampus (Senut et al. 1989). These fibers, are considered to be the generators for low frequency neuronal waves, called *theta* rhythms, that occur in the mouse hippocampus during active exploration (Green and Arduini 1954).

Theta waves play an essential role in learning and memory (Winson 1978; Wang et al. 2015a), particularly the formation of new memories associated to experience (Feng et al. 2015). Since acyl-ghrelin role in improving hippocampal-dependent memory formation in rodents has been extensively reported (Kent et al. 2015), future work could investigate the presence of APT1 in these neurones, and its role in acyl-ghrelin mediated memory formation.

My data show that APT1 protein and mRNA levels, do not vary with ageing in the DG of the human hippocampus (figure 3.5). Interestingly, a previous report showed that APT1 mRNA is increased in the CA1 and DG of aged mice compared to young controls (Tatro et al. 2013); moreover the microRNA miR-138, that has been shown to downregulate APT1 expression (Siegel et al. 2009), is highly expressed in the mouse hippocampus at the dendritic spines – structures located at the synaptic terminals that are involved in the formation of new memories (Tatro et al. 2013). Interestingly, this study also reported a positive correlation between miR-138 expression in the brain, and short-term memory function in the aged mice (Tatro et al. 2013). Similarly, a study recently showed that APT1 inhibitor peptides are neuroprotective in a mouse model of Huntington disease (Virlogeux et al. 2021). Although these studies did not investigate ghrelin levels, the results are in line with the well-known beneficial function of acyl-ghrelin on memory formation. Unfortunately, I was not able to quantify APT1 immunoreactivity in other areas of the brain, such as the ependymal cells next to the choroid plexus, due to technical difficulties; future work could utilise diverse assays, such as BaseScope, to quantify APT1 in such areas, to determine whether its level may be altered in ageing or in the context of neurodegenerative disorders.

Overall, these data suggest the involvement of local APT1 in determining availability of acyl-ghrelin in the brain. More studies are needed to determine its role in the cognitive process, and to investigate whether a therapeutic intervention aiming at reducing APT1 expression or activity (hence increasing circulating acyl-ghrelin) could be beneficial for cognitive function.

3.4.3 GHS-R1a

GHS-R1a was known to be expressed in the brain (Howard et al. 1996) long before the endogenous ligand, ghrelin, was ever discovered (Kojima et al. 1999). Numerous studies detected GHS-R1a mRNA in the hippocampus of mice (Zigman et al. 2006) and humans (Guan et al. 1997; Gnanapavan et al. 2002). Using the BaseScope assay, I was also able to detect GHS-R1a mRNA in the healthy aged human brain. More specifically its expression appeared to be restricted to defined areas, such as the GCL of the DG (figure 3.3 B), while little to no immunoreactivity was detected in the CA4 (figure 3.3 C) and surrounding regions (not shown). This

is consistent with previous reports showing GHS-R1a is mostly expressed in the DG in rodents (Diano et al. 2006; Hornsby et al. 2016).

Since all commercially available anti-GHS-R1a antibodies are poorly specific (Ratcliff et al. 2019), I was only able to detect the mRNA of the receptor, hence these data do not give any information on protein localisation. Notably, using a GHS-R1a-GFP mice, our group recently showed that GHS-R1a is expressed in mature neurones, since GFP immunoreactivity co-localises with $NeuN^+$ cells in the mice hippocampus; however, no colocalization was identified with stem cells ($Nestin^+$) or astrocytes ($GFAP^+$) (Hornsby et al. 2016), suggesting acyl-ghrelin activity in the mouse brain is restricted to determined cell types. This could be addressed in future experiments, for instance by multiplexing the BaseScope assay for GHS-R1a mRNA with a classic antibody-based immunostaining *in situ* in the human brain.

Lastly, my data report no significant changes in GHS-R1a mRNA expression level with ageing (figure 3.6), although the impossibility to analyse its protein level, prevented a more comprehensive analysis. Either way, if confirmed, my data showing the intact expression of GHS-R1a in the aged human brain, are highly encouraging; indeed, the development of a therapeutic strategy aimed at increasing acyl-ghrelin level and activity in the brain could be only possible in the presence of a functional ghrelin receptor and depletion of GHS-R1a in the aged or diseases brain would discourage the use of such strategy.

3.4.4 Conclusions

Ultimately, this chapter shows that the key enzymes of the ghrelinergic system are expressed in the human hippocampus, and their expression levels are maintained during ageing, suggesting the feasibility of a therapeutical intervention aimed at increasing ghrelinergic tenor in the brain.

Moreover, ReN neurones at DIV28 reach full maturation and express GHS-R1a and APT1, confirming their suitability as a human neuronal model of the ghrelinergic system.

3.5 Key findings

- GOAT is homogeneously expressed in the DG and the CA1 of the human hippocampus (figure 3.1).
- GOAT protein and mRNA levels in the DG do not vary with ageing (figure 3.4).
- GOAT mRNA is detectable in ReN neurones (DIV28), but GOAT protein is not (figure 3.8).

- APT1 is expressed in discrete regions in the human hippocampus – most notably CA4, the vestigial sulcus and the rim of tissue next to the CP. Moreover, APT1$^+$ cells show an atypical, branched aspect (figure 3.2).
- APT1 protein and mRNA levels in the DG do not vary with ageing (figure 3.5).
- APT1 mRNA and protein are both detectable in ReN neurones (DIV28) (figure 3.8).

- GHS-R1a mRNA is detectable in the DG of human aged hippocampus (figure 3.3).
- GHS-R1a mRNA level does not vary with ageing (figure 3.6).
- GHS-R1a mRNA is not expressed in ReN stem cells, nor at the early stages of the differentiation, but it is detectable at DIV22 and DIV28.

- Approximately 40% of cultured ReN cells at DIV28, are neurones. Moreover, other markers of neuronal maturation, such as VGlut2 and TH are detectable.
- ReN neurones start expressing GHS-R1a around DIV22 of the differentiation process. APT1 and GOAT mRNA are both detectable in stem cells and mature neurones. Surprisingly, while APT1 protein is detectable throughout the differentiation, GOAT protein cannot be detected at any of the timepoint analysed.

Chapter 4

Ghrelinergic system in Parkinson's Disease

4.1 Introduction

4.1.1 Pathogenesis, anatomy, and dementia

Parkinson's disease (PD) is the second most common neurodegenerative disorder in the world. It is a chronic and progressive disease, characterized by bradykinesia, akinesia, resting tremor, postural instability, and muscular rigidity (Parkinson 1817). Its aetiopathogenesis is unknown and there is currently no cure; most cases are sporadic – a combination of genetic susceptibility and environmental risk factors – and 1.4 times more frequent in men than women. The onset generally occurs after the age of 50, with global prevalence of ~0.5% at age 65, and ~2% at age 85. In Europe, where life expectancy is higher than the global average, the prevalence reaches ~1.8% and ~2.6%, respectively. Indeed, with a worldwide increase of life expectancy in the past 30 years, the number of PD cases has now reached 6 million and it is expected to double by 2050 (Dorsey et al. 2018), making it one of the highest health care cost burden in modern society.

The two main pathological changes that occur in the brain of patients are the development of abnormal intracellular aggregates known as Lewy Bodies (Gibb and Lees 1988), and the loss of dopamine-producing (DA) neurones in the SN pars compacta (Yahr and Bergmann 1987). The direct consequence of this selective degeneration is the impairment of the motor system (Schultz 1986). However, the failure of the dopaminergic system usually precedes the visible symptoms by several decades, due to the redundance within the system: in earlier stages, the neuronal loss is compensated by the surviving neurones, and the system is functionally maintained. When the cell loss is too conspicuous – usually around

70/80% – the remaining dopaminergic cells cannot maintain nigro-striatal signaling and the first motor symptoms appear (Bernheimer et al. 1973). As a consequence, diagnosis of PD usually occurs when the disease has already reached an advanced stage of neurodegeneration, contributing to the difficulties in identifying an effective therapeutic strategy. The current therapies focus on increasing the dopaminergic tenor in the brain, for management and relief of the symptoms. The gold standard is the dopamine precursor, 3,4-dihydroxy-L-phenylalanine (L-DOPA), whose function is to replace the loss of dopamine in the brain of affected patients, but its activity is quite limited, and the dosage need to be increased constantly to counteract the neurodegenerative process. This molecule was identified over 60 years ago (Carlsson et al. 1957), and has not yet been replaced, despite that its long-term use has been associated with severe side effects and toxicity (Ahlskog and Muenter 2001).

Although motor dysfunctions are more notably associated with the disease, PD patients also experience a variety of non-motor symptoms (NMS) (Politis et al. 2010). Some of them, such as sensorial impairment (i.e. the loss of the sense of smell), muscular pain, mood disorders and reduced sleep quality, may anticipate the motor symptoms of a few years, but are often missed or misinterpreted (Stamey et al. 2008; Postuma et al. 2012). Since L-DOPA is not effective against NMS (Fabbri et al. 2017), and in some cases it may even worsen some of them (Eskow Jaunarajs et al. 2010), it is believed that the dopaminergic failure is not responsible for the NMS, that are most likely associated with a failure of the cholinergic autonomic nervous system (reviewed in Wolters 2009). Indeed, synthetic cannabinol analogues are being tested for NMS management alongside L-DOPA, and preliminary data look promising (Peball et al. 2020).

Approximately 52% of PD patients also experience significant weight loss (Abbott et al. 1992), although the exact causes are uncertain. Some studies report loss of appetite (Politis et al. 2010), reduction in sense of taste (Wszolek and Markopoulou 1998), and variations in energy expenditure at rest (Markus et al. 1992). Moreover, PD patients exhibit severe deficits in gastrointestinal function due to a malfunctioning in the enteric nervous system and damage of the dorsal motor nucleus of the vagus nerve (Halliday et al. 1990). Ultimately, PD patients report

attenuated ghrelin-mediated response to food (Song et al. 2017), suggesting an impairment in the ghrelinergic axis that will be described in section 4.1.3.

During the later stages of PD, more severe neurological dysfunctions occur that interfere with the daily functioning and impact the life quality of the patients and their families (Schrag et al. 2000). These include anxiety (Henderson et al. 1992), depression (Barone 2011), pain (Defazio et al. 2008), confusion and hallucinations (Mosimann et al. 2006) and severe cognitive deficits, such as language deficit (Matison et al. 1982), spatial impairment (Hovestadt et al. 1987), and memory loss (Weintraub et al. 2004). It has been estimated that at least 20-40% of the diagnosed PD patients, exhibit signs of dementia a few years after diagnosis (Cummings 1988; Hobson and Meara 2004; Athey et al. 2005; De Lau et al. 2005), and up to 70% of them would develop dementia within 10 years (Portin and Rinne 1987; Aarsland et al. 2003; Hely et al. 2005). This condition is defined as Parkinson's disease with dementia (PDD). Due to the high variability of the diagnostic criteria, in 2007 the Movement Disorder Society recruited a task force of scientist and clinicians from all over the world, to define the first official guideline to clinically identify PDD (Dubois et al. 2007; Emre et al. 2007). The PDD diagnosis is based on a numeric score attributed to patients' performances on various cognitive tests, including memory, language, and attention, in combination with fMRI scans. Interestingly, volumetric MRI studies have shown that brain volume loss is a measure of cognitive decline during PD progression (Hu et al. 2001). More specifically, mildly impaired PD patients and PDD patients, have shown significant hippocampal atrophy compared to cognitively functional PD patients (Weintraub et al. 2011). In addition, PDD patients exhibit significant atrophy of the medial temporal lobe, particularly in the entorhinal cortex (Kenny et al. 2008; Weintraub et al. 2011), as well as parietal-temporal and prefrontal cortex (Jokinen et al. 2009). Since PD with mild cognitive impairment often deteriorates in PDD (Janvin et al. 2006), hippocampal atrophy has been suggested as a predictive biomarker of cognitive decline in PD, possibly anticipating a more severe PDD condition (Weintraub et al. 2011).

Due to the increasing average lifespan, and consequently the number of PD cases worldwide, the identification of the molecular pathways underlying this disease has become a priority for neuroscientists all over the world.

4.1.2 Molecular pathways and mitochondrial impairment

Although the exact aetiopathogenesis of PD is unknown, 10–20% of PD patients have a family history. Notably, specific PD-causing mutations have been identified in the genes of α-synuclein (SNCA), leucine-rich repeat kinase 2 (LRRK2), phosphatase and tensin homolog (PTEN)-induced novel kinase 1 (PINK1) and parkin (PARK2) (Tan et al. 2019). Other genes, such as deglycase 1 (DJ-1) (Bonifati et al. 2003) and β-glucocerebrosidase (GBA1) (Swan et al. 2016), have also been associated with increased risk of developing the disease. Studies performed on these mutations and the molecular pathways regulated by these genes, allowed the delineation of a wide, although not yet complete, picture of the molecular mechanisms that may be involved in PD and PDD (figure 4.1).

Four factors are considered the principal contributors to PD at a molecular level: activation of the ER stress response (Hoozemans et al. 2007); impairment of the intracellular protein degradation systems (McNaught and Jenner 2001; Cuervo et al. 2004); oxidative stress and mitochondrial dysfunction (Schapira et al. 1992); neuroinflammation and glial cell activation (Ouchi et al. 2005). In order to fully understand the disease, it is imperative to consider these elements not as distinct pathways, rather as a combination of overlapping events. Because of that, a deficit in any one of the pathways is enough to trigger a cascade reaction, affecting downstream signalling of the other pathways, ultimately causing DA neurones to die (Klemann et al. 2017).

The key molecular player in PD pathogenesis is the formation of Lewy Bodies (figure 4.1, point 1) (Lewy 1912), intracellular inclusions made of abnormally aggregated α-synuclein protein, associated with others such as ubiquitin (Tofaris et al. 2003) and tau (Ishizawa et al. 2003) in variable amounts. α-synuclein is a multi-functional protein, mostly located at the synaptic densities, where it is involved in vesicles motility and dopamine uptake/release (Wang et al. 2014b). Genetic mouse models carrying PD-relevant SNCA mutations, showed an alteration in the distribution of presynaptic vesicles that correlates with decrease in dopamine release and impairment in neuronal firing properties (Janezic et al. 2013). Interestingly, in the presence of damaged DNA, normal α-synuclein is rapidly recruited to the nuclei where it coordinates DNA repair, restoring homeostasis (Schaser et al. 2019). Mutations in the SCNA gene, elicit abnormal

aggregation of α-synuclein (Conway et al. 1998), possibly causing a reduction in its presence in the nucleus, contributing to cell death (figure 4.1, point 2).
There are two main intracellular mechanisms of degradation for aberrant or excessive proteins; the macro-autophagy or autophagy-lysosome pathway (ALP), (figure 4.1, point 3) (De Duve 1963; Takeshige et al. 1992) and the ubiquitin-proteasome system (UPS) (figure 4.1, point 4) (Hershko et al. 1978; Hershko et al. 1979). Both clearance processes are activated by misfolded proteins carrying unique "tags", made of covalently bonded polyubiquitin chains. The two systems usually target different proteins, however they can also work concurrently; for instance, intracellular α-synuclein is preferentially degraded by the UPS system, but an increase in protein misfolding will also recruit the ALP pathway in parallel (Ebrahimi-Fakhari et al. 2011). Moreover, in case of dysfunction in one of the two systems, the other one is able to compensate (Ding et al. 2007; Shen et al. 2013).

When the protein load is too high, both systems fail, and misfolded proteins accumulate inside the cell. The increasing aggregation of aberrant proteins, triggers a cascade mechanism known as ER stress, or unfolded protein stress response (UPR), whose function is to reduce the load of misfolded proteins by inhibiting unnecessary gene expression and protein translation (figure 4.1, point 5) (Zhang et al. 2000). Since the ER is involved in the production and maturation of several proteins including lysosomal enzymes, impairment in the ER secretory pathway results in further lysosomal dysfunction, increased stabilisation and consequential accumulation of the mutated proteins, that contribute to the cycle (Mazzulli et al. 2016). Ultimately, prolonged activation of the ER stress response pathway, has been shown to induce cell death via apoptosis (figure 4.1, point 6) (Wang et al. 1996).
Another important PD-related mutation occurs in the gene LRRK2, that encodes a cytosolic protein kinase whose targets are involved in autophagosome maturation and upregulation of autophagy-associated genes (Reinhardt et al. 2013). Mutated LRRK2 abnormally hyper-phosphorylates its targets, often leading to lysosomal abnormalities (Sánchez-Danés et al. 2012), further contributing to ER stress and cell death.

The loss of DA neurones, and the consequential reduction in dopaminergic signalling, induces the surviving neurones to increase dopamine metabolism (oxidation) (figure 4.1, point 7), a process that increases oxidative stress, ROS production and causes mitochondrial impairment (Burbulla et al. 2017). Moreover, increased dopamine oxidation, has been shown to significantly increase the misfolding of α-synuclein (Pham et al. 2009), leading to its further accumulation inside the Lewy bodies.

DA neurones death can also occur as a consequence of an exaggerated immune response from microglia (figure 4.1, point 8), a population of macrophage-like cells involved in brain homeostasis and immune surveillance. Physiological functions of brain microglia include clearance of unnecessary synapses (pruning) (Schafer et al. 2012), myelin remodelling during development (Hughes and Appel 2020), activation of defence mechanisms against pathogens (Olson and Miller 2004), and scavenging debris from dying neurones to prevent damage to neighbouring cells (Damisah et al. 2020). In the brain of PD patients, immune cells are abnormally activated from an early stage of the disease (Gerhard et al. 2006). In particular, microglia cells express SNCA and LRRK2 genes, that have been shown to directly affect microglial function in models of PD (Austin et al. 2006; Kim et al. 2020). Extracellular aggregates of α-synuclein promote the activation of neighbouring microglia; however if this fails to remove the insoluble proteins from the extracellular space, it results in increased tissue inflammation and cell damage (Choi et al. 2019). Additionally, damaged and dying neurones release specific antigens and molecular signals, like ATP, that induce activated microglia to attempt to remove the defective cells (Davalos et al. 2005), creating a vicious cycle of cell death and immune cell activation.

Lastly, Parkin/PINK1-mediated mitophagy, previously described in section 1.3.3, has been extensively reported to be impaired in PD; indeed, PARK2 mutations are the first cause of early onset PD, accounting for 77% of the familial cases and 10-20% of the sporadic ones, while PINK1 mutations are the second, accounting for 2-4% of the sporadic cases. Furthermore, malfunctional mitochondria trigger the release of cytC from the IMM to the cytoplasm, where it interacts with various heat shock proteins and recruits caspase-9 and 3, ultimately leading to cell death (Ott et al. 2002).

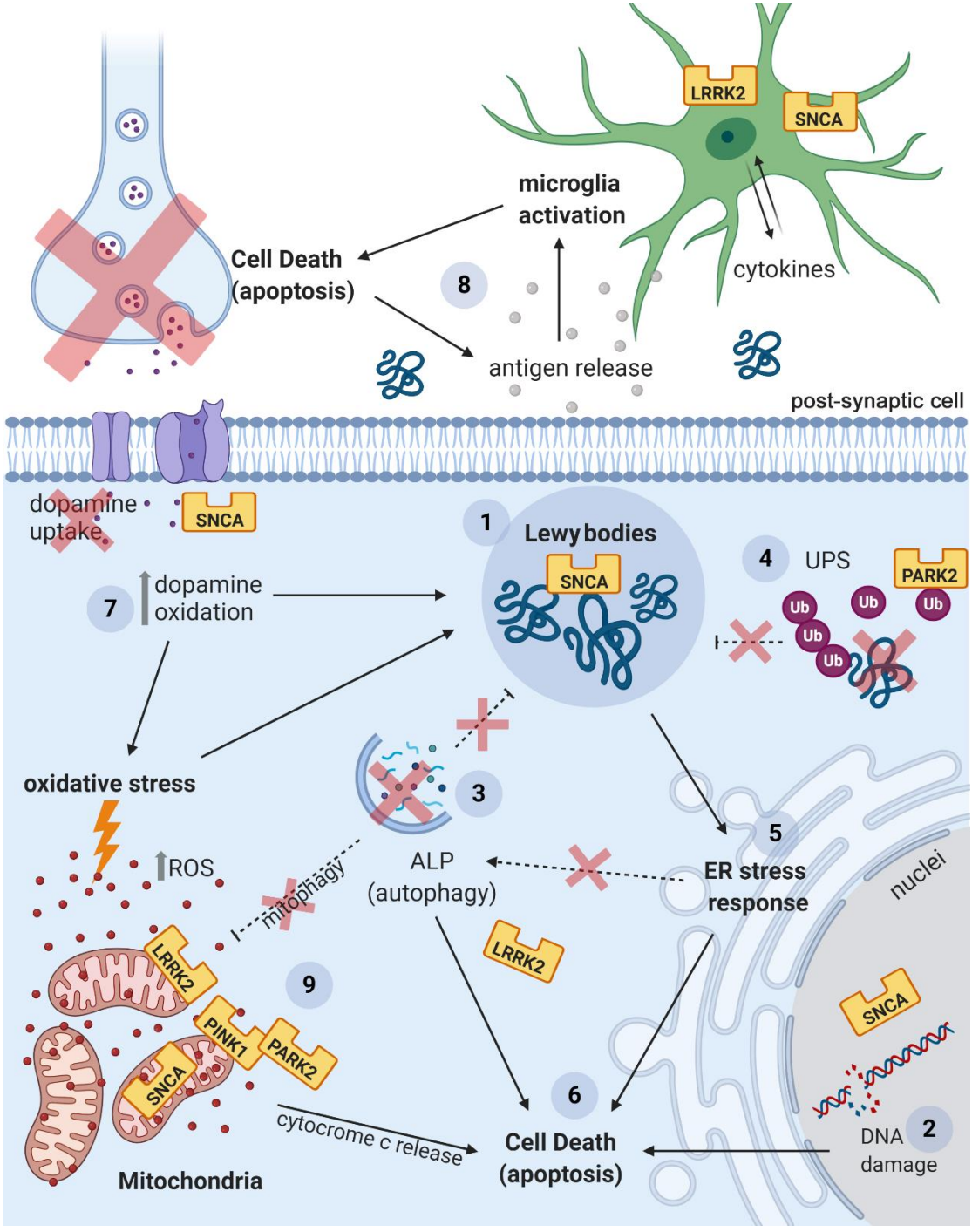


Figure 4.1 PD molecular pathways

Schematic representation of dying DA neurones and the main molecular pathways impaired in PD: (**1**) protein accumulation, (**2**) DNA damage, (**3**) autophagy impairment, (**4**) UPS impairment, (**5**) ER stress response, (**6**) apoptosis, (**7**) increase in dopamine oxidation, (**8**) immune response, (**9**) mitochondrial impairment. Yellow boxes show the localization of the four major PD-associated gene products (*created with BioRender.com*).

In addition to PINK1 and PARK2, LRRK2 and α-synuclein are also involved in mitochondrial functions. LRRK2 has been recently shown to play a major role in regulating mitochondrial biogenesis, even though its localisation is mostly cytosolic (Toyofuku et al. 2020), while α-synuclein aggregates are associated with the IMM in the brain of PD patients, where they bind to TOM20 and impair the import of mitochondrial protein from the cytosol (Devi et al. 2008; Di Maio et al. 2016).

Mitochondrial complex I deficits have also been extensively reported in *post-mortem* PD brains (Schapira et al. 1990; Hattori et al. 1991; Janetzky et al. 1994; Keeney et al. 2006) and are associated with dementia in PDD patients (Gatt et al. 2016). *Post-mortem* brains of PD patients exhibit disrupted mitochondria turnover (Berenguer-Escuder et al. 2020), conspicuously reduced levels of PGC1α and impaired downstream signalling (Zheng et al. 2010), increased markers of oxidative damage (Isobe et al. 2010) and extensive mtDNA mutations (Bender et al. 2006; Kraytsberg et al. 2006), indicating that deficient mitochondrial quality control is a main component in PD pathogenesis.

The first evidence of impaired mitochondrial function in PD was obtained by accident. Three medical reports described parkinsonism in six young individuals (20 to 40 years of age) with no PD familiarity, as a result of the abuse of a new illicit drug, the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Ziering et al. 1947; Davis et al. 1979; William Langston et al. 1983). The first symptoms – rigidity, tremor and bradykinesia – appeared just a few weeks after the drug injection, and disease progression was slowed by treatment with L-DOPA (William Langston et al. 1983). This sparked the interest of the scientific community as further studies revealed that the toxin is selectively taken by DA neurones via the dopamine transporter, DAT (Javitch et al. 1985). Moreover, MPTP principally target mitochondria: the molecule accumulates inside their membrane where it is metabolised by mitochondrial enzymes into a more toxic form, MPP^+ (Heikkila et al. 1984), that specifically impairs complex I of the electron transport chain (Ramsay et al. 1986). These studies suggested that the MPTP toxin may recapitulate some of the pathogenic mechanisms that occur in the brain of PD patients, hence making it a highly valuable tool for research and drug testing.

Indeed, mitochondrial toxins have long been considered the gold standard to recapitulate complex I dysfunction and PD pathogenesis in laboratory models. In most animal studies, MPTP is largely used as it induces selective degradation of DA neurones in rodents (Heikkila et al. 1985; Hamre et al. 1999; D. S. Prediger et al. 2011) and primates (Burns et al. 1983; Russ et al. 1991; Chassain et al. 2001). Similarly, 6-hydroxyl-dopamine (6-OHDA), a synthetic dopamine analogue, is also internalised by DAT and causes selective neuronal degeneration and PD-like symptoms (Perese et al. 1989; Eslamboli et al. 2003; Iancu et al. 2005). *In vitro*, one of the most common, non-genetic, cellular models of PD is treatment with rotenone, a compound extracted from the roots of several plants and extensively used as an insecticide and pesticide (Sherer et al. 2002; Ahmadi et al. 2003; Radad et al. 2006). Interestingly, the use of rotenone as a pesticide has been associated with higher incidence of sporadic PD among rural populations (Priyadarshi et al. 2001; Dhillon et al. 2008). Like MPTP, rotenone is a selective inhibitor of mitochondrial complex I and has been shown to induce parkinsonism in rats (Betarbet et al. 2000; Alam and Schmidt 2002; Fleming et al. 2004).

In summary, the four aforementioned mechanisms – defective degradation systems, ER stress response, microglia activation and mitochondrial impairment – work together to contribute to PD pathogenesis. However, mitochondrial toxicity better and more comprehensively recapitulates PD pathology in most models. Hence, mitochondria-specific molecular pathways have been pharmacologically targeted in PD models in order to improve the symptoms. For instance, small compounds targeting the transcription factor nuclear factor E2 (Nrf2), which is involved in mitochondria biogenesis and the antioxidant system, have been shown to exert an anti-apoptotic effect and to reduce oxidative stress in a neuronal cell line treated with MPTP (Feng et al. 2019a). Generic antioxidant (Roghani and Behzadi 2001), and mitochondrial-targeted peptides, such as the Mito-Q10 (Yang et al. 2009; Ghosh et al. 2010), have also proven to be effective in reducing mitochondrial stress *in vitro* and improving PD symptoms *in vivo,* suggesting that the mitochondria may indeed be considered a promising therapeutic target in the fight against PD.

4.1.3 Ghrelin and PD

Acyl-ghrelin is an important regulator of mitochondrial health via pAMPK signalling (section 1.3.6). Interestingly, acyl-ghrelin has been shown to exert neuroprotective function *in vivo* and *in vitro* in numerous animal models of PD.

Using MPTP-treated PD mice, several groups reported that intra-cranial (Jiang et al. 2008; Andrews et al. 2009) and peripheral (Moon et al. 2009; Bayliss et al. 2016a) injections of acyl-ghrelin significantly reduced TH^+ cell death in the SN. More specifically, acyl-ghrelin improved DA neurones firing rate and enhanced dopamine tenor – impoverished by the degenerative process (Andrews et al. 2009); prevented microglia and astrocyte activation, reducing the release of pro-inflammatory cytokines (Moon et al. 2009; Bayliss et al. 2016a); increased LC3-II-mediated protective autophagy (Bayliss et al. 2016a); and attenuated cytC release, hence preventing caspase3-mediated apoptosis (Jiang et al. 2008). Similar neuroprotective and anti-inflammatory functions have also been shown in MPTP-treated mouse cell lines (Moon et al. 2009; Bayliss et al. 2016a; Wang et al. 2020). Interestingly, acyl-ghrelin neuroprotective effects in the SN of MPTP-treated mice, were totally abrogated in UCP2 double KO mice (Andrews et al. 2009), or in AMPK KO mice – in which AMPK was selectively deactivated in dopaminergic neurones (Bayliss et al. 2016a). Indeed, acyl-ghrelin has been previously shown to upregulate UCP2 mRNA levels in the hypothalamus, enhancing mitochondrial respiration and protecting neurones from oxidative stress (Andrews et al. 2008), suggesting that the AMPK/UCP2 pathway is an essential mediator of acyl-ghrelin neuroprotective effects in the brain.

Pharmacological activators of the pAMPK pathway, such as Resveratrol, a diet supplement that has been shown to induce SIRT1 and PGC1α pathways (Sun et al. 2007b; Wu et al. 2011; Wang et al. 2015b; Chiang et al. 2018; Pineda-Ramírez et al. 2020) and Metformin, currently used in clinics against type II diabetes (Dulovic et al. 2014; Patil et al. 2014; Bayliss et al. 2016c; Chiang et al. 2016; Ozbey et al. 2020), were found to be protective against DA neurones degeneration in PD models. Since both CR and ghrelin are strong activators of pAMPK signalling, these studies suggest that every intervention aimed at restoring ghrelinergic levels in the aged human brain, could potentially represent a novel strategy against PD.

DA neurones from GHRL KO mice or from GHS-R KO mice, exhibit increased susceptibility to death after MPTP treatment (Andrews et al. 2009). Notably, plasma ghrelin levels are significantly reduced in aged individuals (section 3.1.1), and AMPK activity is also significantly reduced in aged rats – with lower AMPK level correlating with reduced mitochondria biogenesis (Reznick et al. 2007). Lastly, PD patients show impaired response to food (Unger et al. 2011) and exhibit reduced plasma ghrelin levels, compared to age-matched healthy subjects (Song et al. 2017; Hornsby et al. 2020; Pietraszko et al. 2020). These data suggest that an impairment of the ghrelinergic system may contribute to increased vulnerability of DA neurones. Consistently, obesity – a disease associated with lowered plasma ghrelin levels (Tschöp et al. 2001) and ghrelin resistance (Andrews 2019) – together with diabetes and elevated body mass index, increase the risk of developing PD at later age (Palacios et al. 2011; Cereda et al. 2013; Llorens-Arenas et al. 2015). Moreover, some of the most common NMS of PD patients such as weight loss, cognitive deficit, sleep disorders and depression (Bachmann and Trenkwalde 2006; Storch et al. 2008; Fiszer et al. 2010; Unger et al. 2011; Jiao et al. 2017; Song et al. 2017; Masala et al. 2020), are all associated with physiological processes that are mediated by acyl-ghrelin, as seen in section 1.2.2.

Indeed, acyl-ghrelin has been shown to be highly interconnected with the dopaminergic system of the SN; firstly GHS-R1a is known to dimerise with the DA receptors D1R and D2R (as seen in section 1.2.9), that are involved in the regulation of feeding and food reward (Szczypka et al. 2001; Hnasko et al. 2006; De Melo Martimiano et al. 2015); then, acyl-ghrelin is also known to exert an anti-depressive function (Lutter et al. 2008) by specifically interacting with the DA neurones in the midbrain (Abizaid et al. 2006), and PD is also associated with depression (Storch et al. 2008).

In sum, ghrelin seems to be a valuable therapeutic treatment for PD, not only because of its protective activity towards DA cells degeneration, but also in its capability of promoting NMS such as food intake, mood, and depressive symptoms.

4.2 Aims

In order to develop an effective strategy to modulate the ghrelinergic system in the brain of PD patients, we first need to characterise the expression and function of ghrelinergic axis in the diseased brain.

However, PD is a complex disorder with a wide variety of symptoms – from motor dysfunctions to cognitive impairment. Since acyl-ghrelin has been associated with improved memory function in rodents (see section 1.2.2), and since only PDD patients exhibit evident cognitive impairments (see 4.1.1), it is important to differentiate between PD and PDD patients when investigating the ghrelinergic axis in the diseased brain. More specifically, since cognition and memory functions are strongly associated to the hippocampus, this chapter will look at *post-mortem* hippocampus tissue samples from aged controls, PD and PDD individuals. Secondly, since *post-mortem* tissue samples can only give partial information about the mechanisms of the disease (section 1.3.5), human stem cell-derived mature neurones will also be used to model oxidative stress in PD.

The aims of this chapter are:

- To quantify the expression of GOAT, APT1 and GHS-R1a in the human hippocampus from PD and PDD patients, compared to age-matched healthy individuals.
- To investigate the mitochondrial health of ReN neurones pre-treated with acylated or unacylated ghrelin for 5 days (to simulate medium-length CR regimen), and then treated with rotenone for 24 hours.

4.3 Results

4.3.1 The ghrelinergic system is impaired in the hippocampus of PDD patients

GOAT immunoreactivity in the GCL of the human hippocampus, was identified as shown in chapter 3. To account for size variations of the hippocampus due to aging or disease, $GOAT^+$ cells (figure 4.2 A, black arrowheads) were counted in the GCL then normalised for the area of the region. 817.9 ± 70.33 cells per mm^2 of GCL were counted in controls (n=5) and 748.9 ± 161.7 cells in PD (n=6), with no significant difference compared to controls (p=0.5131). While PDD brains had 461.1 ± 66.11 cells per mm^2 of GCL (n=6), a significant reduction compared to controls (*p=0.0335), but no difference compared to PD (p=0.1227) (figure 4.2 B). Western blot analysis of GOAT, reported as fold change to GAPDH, demonstrated a significant reduction between controls (1.226 ± 0.1278) (n=5) and PD (0.7081 ± 0.1782) (n=6) (*p=0.0415), and PDD (0.6870 ± 0.1221) (n=6) (*p=0.0415), but no changes between PD and PDD (p>0.9999) (figure 4.2 D and E), suggesting an impairment in GOAT protein expression in both PD and PDD. On the contrary, MBOAT4 gene expression from tissue lysates, reported as fold change to β-actin, showed no significant differences between control (ΔCt=7.360 ± 0.3432) (n=6) and PD (ΔCt=7.048 ± 0.4386) (n=6) (p=0.5164) nor PDD (ΔCt=7.458 ± 0.2327) (n=6) (p=0.8711), and no difference between PD and PDD (p=0.4173) (figure 4.2 C). Overall, these results suggest that GOAT protein levels, but not mRNA expression, are significantly impaired in the hippocampus of diseased patients.

APT1 immunopositive cells in the CA4 (figure 4.3 A, black arrowheads) were quantified to be 279.6 ± 59.32 cells per mm^2 of CA4 in control brains (n=4), 302.1 ± 111.5 cells per mm^2 of CA4 in PD brains (n=6), and 283.2 ± 42.26 cells per mm^2 of CA4 in PDD brains (n=5), with no significant difference reported against the control (p>0.9999 for PD; p=0.6171 for PDD) and between the two groups (p=0.5796) (figure 4.3 B). Western blot protein quantification, reported as fold change to GAPDH, showed that APT1 expression in PDD (0.5802 ± 0.1259) (n=6), was about 39% lower than controls (0.9452 ± 0.1179) (n=6), although not significantly different (p=0.0836). PD group (0.6969 ± 0.1330) (n=6) was not

different from control (p=0.2342), nor from PDD (p=0.5887) (figure 4.3 D and E). Lastly, LYPLA1 gene expression from tissue lysates, reported as fold change to β-actin, showed no difference between controls (ΔCt=6.126 ± 0.2945) (n=6) and PD (ΔCt=5.975 ± 0.1993) (n=6) (p=0.6653), or PDD (ΔCt=6.158 ± 0.1344) (n=6) (p=0.9139), nor between PD and PDD (p=0.5887) (figure 4.3 C). Overall, these data report no significant difference in APT1 protein and mRNA levels between diseased and control groups.

GHS-R1a mRNA in the GCL was detected using the BaseScope assay (figure 4.4 A, bottom row, black arrowheads), normalised to both the area of the region and the positive housekeeper PPIB (figure 4.4 A, top lane), as described in section 2. Data are reported as positive dots per mm^2 of the GCL. No significant differences were found between controls (287,271.00 ± 96,923.00) (n=5) and PD (171,357.00 ± 48,304.00) (n=7) (p=0.3536), or PDD (552,279.00 ± 243,983.00) (n=5) (p=0.5730), nor between PD and PDD (p=0.1244) (figure 4.4 B). Similarly, RT-qPCR analysis of GHS-R1a, reported as fold change to β-actin, showed no differences between controls (ΔCt=7.455 ± 0.6320) (n=6) and PD (ΔCt=6.716 ± 0.6509) (n=6) (p=0.4173), nor PDD (ΔCt=7.484 ± 0.3467) (n=6) (p=0.7456), and no difference between the two diseased groups (p=0.2561) (figure 4.4 C), suggesting that GHS-R1a expression is not affected in PD or PDD.

Interestingly, total ghrelin (tot G) protein level from tissue extract, assessed by western blot, was significantly decreased in PDD (0.4389 ± 0.05152) (n=6) compared to PD (0.9962 ± 0.2483) (n=5) (*p=0.0383), confirming other reports showing that ghrelinergic system is impaired in dementia. However, no difference was detected between controls (1.105 ± 0.2901) (n=6) and PDD (p=0.1530), nor PD (p=0.4786), probably due to the large spread of the data within the control group (figure 4.4 E).

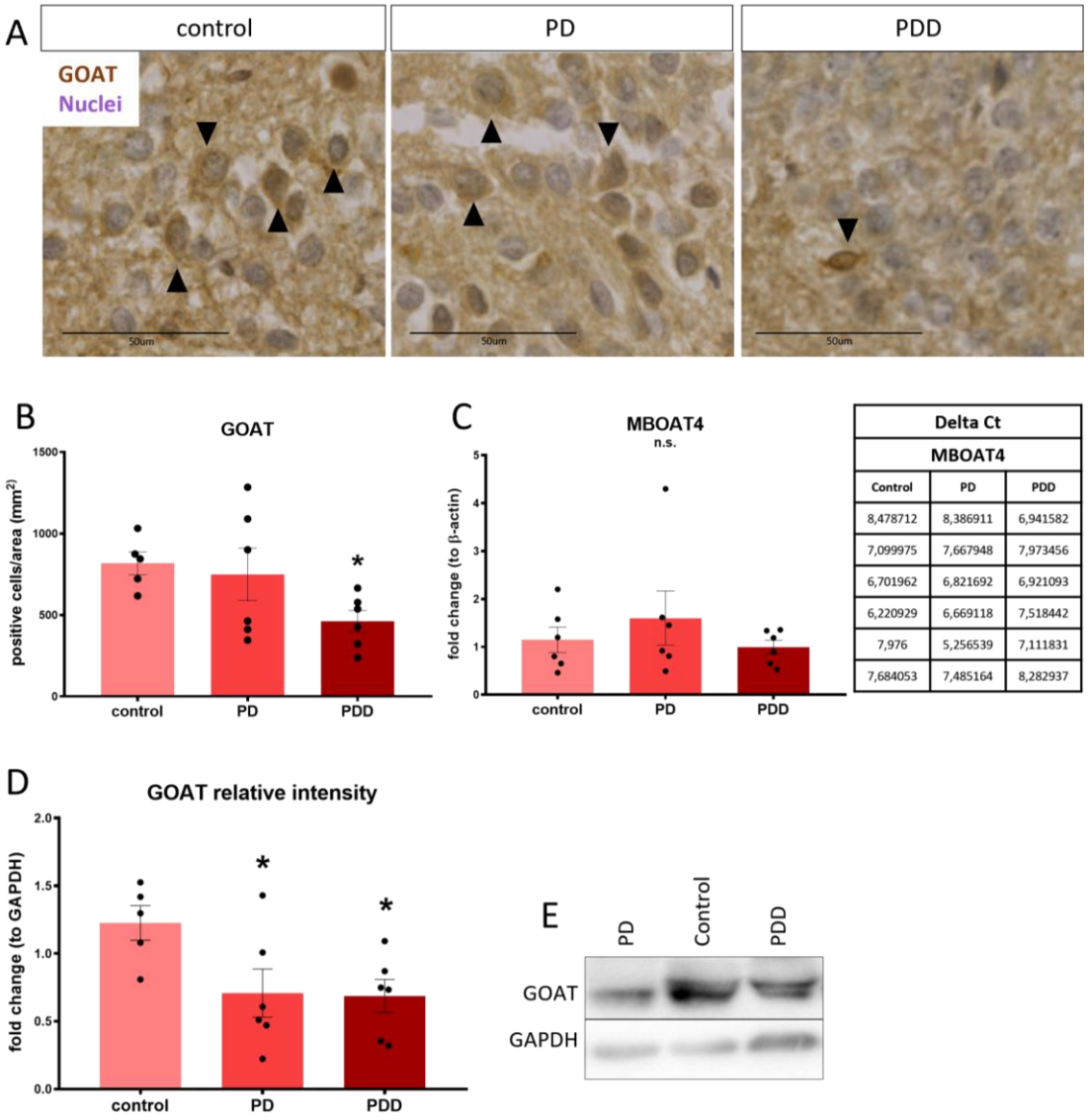
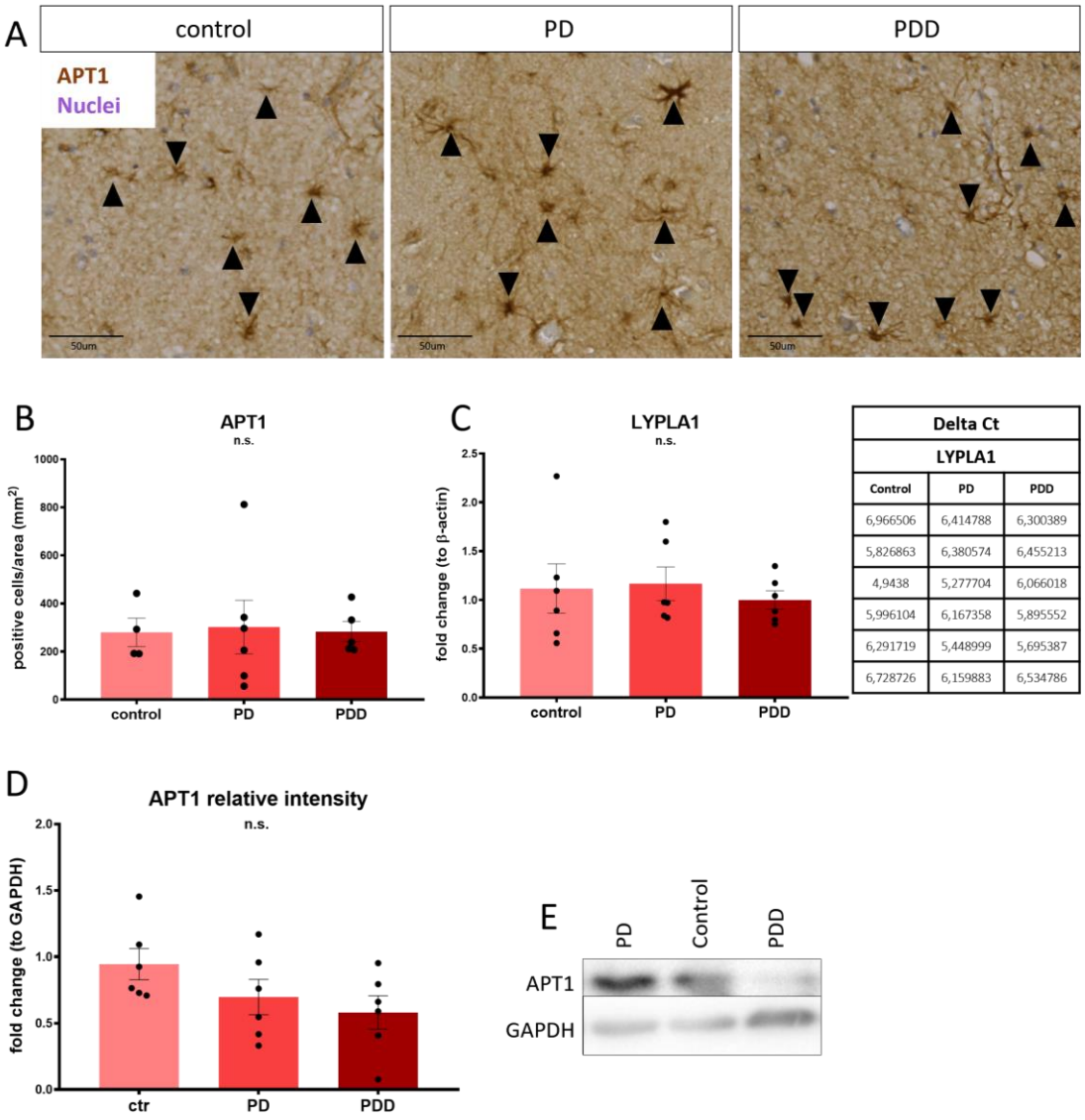


Figure 4.2 GOAT expression in PD

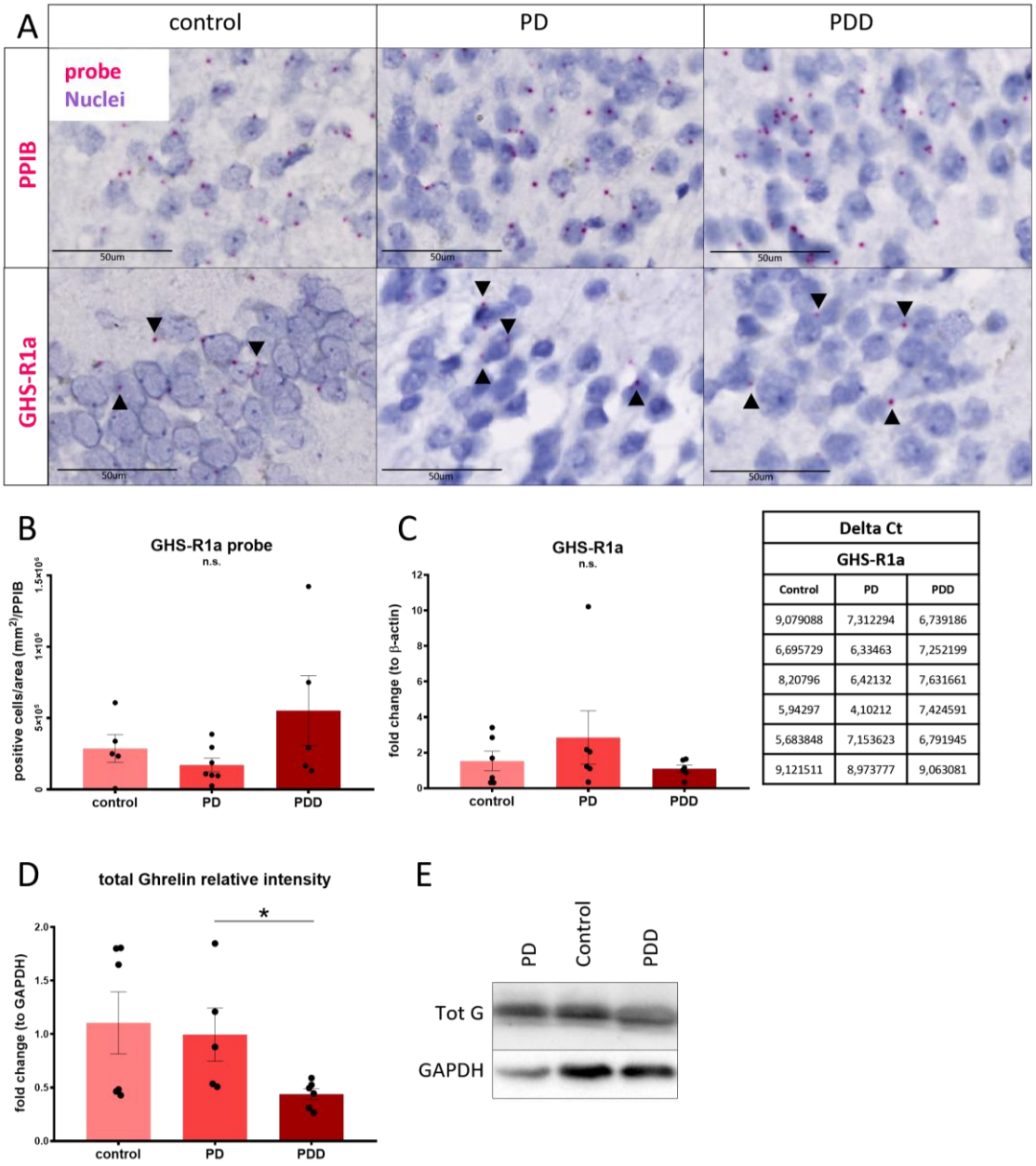
(**A**) GOAT immunoreactivity by IHC (in brown, black arrowheads) was identified in the GCL of controls (left), PD (centre) and PDD (right). (**B**) Positive cells were counted and normalised for the area of the GCL (in mm^2). (**C**) MBOAT4 mRNA expression was quantified via RT-qPCR; (**D, E**) GOAT relative protein expression was quantified by WB. ROUT analysis was performed to remove any outliers from the groups. Significance was measured by Kruskal-Wallis test with Dunn's multiple comparison. Data are reported as mean ± SEM. (n.s. not significant; *p≤0.05)



Delta Ct		
LYPLA1		
Control	PD	PDD
6,966506	6,414788	6,300389
5,826863	6,380574	6,455213
4,9438	5,277704	6,066018
5,996104	6,167358	5,895552
6,291719	5,448999	5,695387
6,728726	6,159883	6,534786

Figure 4.3 APT1 expression in PD

(**A**) APT1 immunoreactivity (in brown, black arrowheads) was identified in the CA4 of controls (left), PD (centre) and PDD (right). (**B**) Positive cells were counted and normalised for the CA4 area (in mm^2). (**C**) LYPLA1 mRNA expression was quantified via RT-qPCR while (**D, E**) APT1 relative protein expression was quantified by WB. ROUT analysis was performed to remove any outliers from the groups. Significance was measured by Kruskal-Wallis test with Dunn's multiple comparison. Data are reported as mean ± SEM. (n.s. not significant)



Delta Ct		
GHS-R1a		
Control	PD	PDD
9,079088	7,312294	6,739186
6,695729	6,33463	7,252199
8,20796	6,42132	7,631661
5,94297	4,10212	7,424591
5,683848	7,153623	6,791945
9,121511	8,973777	9,063081

Figure 4.4 GHS-R1a expression in PD

(**A**) GHS-R1a mRNA dots (fuchsia dots, bottom lane, black arrowheads) were identified in the GCL of controls (bottom left), PD (bottom centre) and PDD (bottom right), (**B**) quantified as number of dots per mm^2 of GCL and normalised for the positive control, PPIB (A, top lane). (**C**) GHS-R1a mRNA expression was also quantified via RT-qPCR (**D**, **E**) while total ghrelin relative protein expression was quantified by WB. ROUT analysis was performed to remove any outliers from the groups. Significance was measured by Kruskal-Wallis test with Dunn's multiple comparison. Data are reported as mean ± SEM. (n.s. not significant; *p≤0.05)

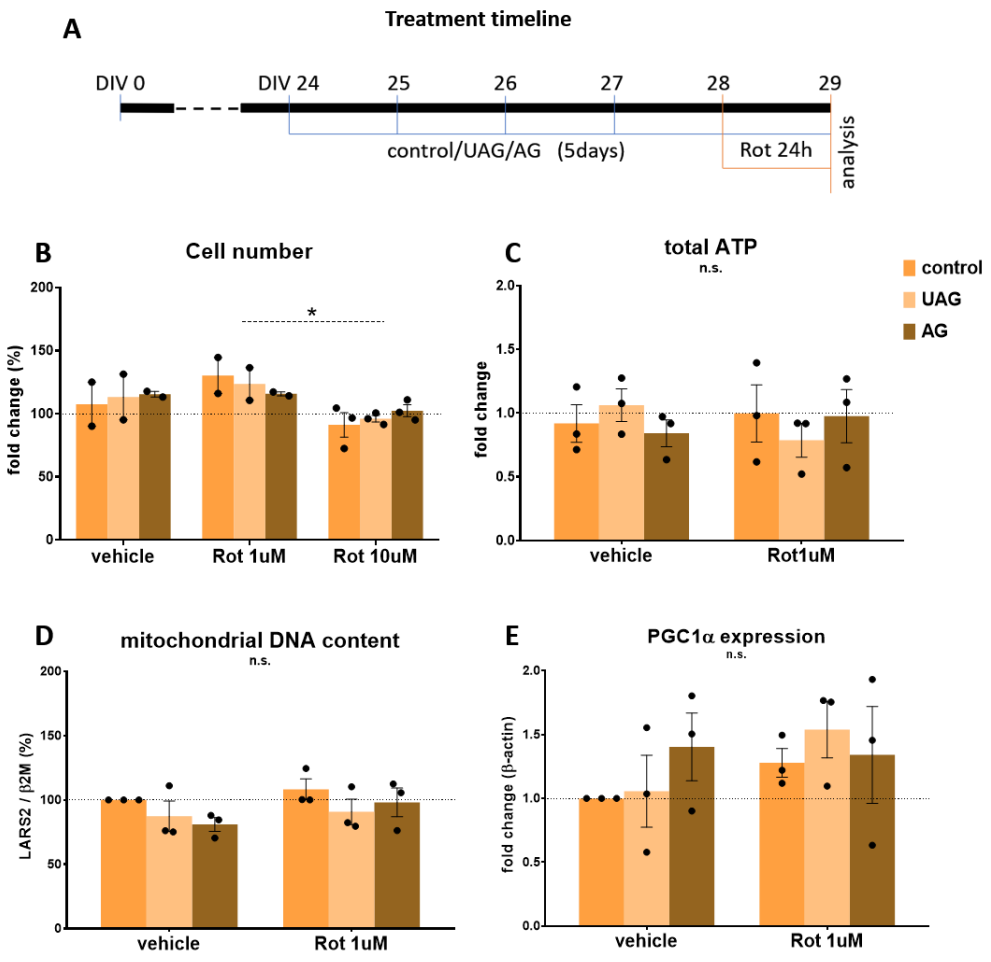
4.3.2 Acylated and unacylated ghrelin exert opposite functions on rotenone-induced mitochondrial network disruption *in vitro*

Rotenone toxicity in ReN neurones was determined by manual cell count (figure 4.5 B); statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparison test. In the vehicle treated group (n=2), neither acyl-ghrelin (115.5 ± 2.24; p=0.9997), nor unacylated ghrelin (113.2 ± 18.08; p>0.9999) significantly affected cell number compared to control (107.5 ± 17.47). Similarly, in cultures treated with 1μM rotenone (n=2) no significant differences were detected between control (130.3 ± 14.28) and acyl-ghrelin (115.7 ± 1.55; p=0.9828) or unacylated ghrelin (123.5 ± 12.93; p>0.9999). The same was reported for cultures treated with 10μM rotenone (n=3): no significant differences were identified between control (91.14 ± 9.64) and acyl-ghrelin (102.4 ± 4.65; p=0.9879) or unacylated ghrelin (96.00 ± 2.64; p>0.9999). It is possible that the small number of replicates (n=2) may have affected the statistical power of the analysis. Therefore, I analysed the overall treatment effects using Tukey's multiple comparison test between vehicle, rotenone 1μM and 10μM, regardless of ghrelin treatment, and found that neither 1μM rotenone (p=0.4379) nor 10μM rotenone (p=0.1670) affected cell viability compared to vehicle treatment. However, a significant difference (*p=0.0150) was detected between 1μM and 10μM rotenone treatments, suggesting that, although these conditions are not lethal for the cells, 10μM rotenone treatment may be detrimental to cell count.

Since mitochondria are responsible for over 90% of the total cellular ATP production (Keilin 1925; Chance and Williams 1956), I used the CellTiter Glo kit, to assess ATP levels (expressed as fold change) in vehicle-treated (n=3) and 1μM rotenone-treated (n=3) ReN neurones (figure 4.5 C). No significant differences were detected between the groups (n=3; p>0.8), regardless of ghrelin treatment [Vehicle group: control (0.918 ± 0.148), acyl-ghrelin (0.842 ± 0.105), unacylated ghrelin (1.06 ± 0.128); rotenone 1μM group: control (0.997 ± 0.225), acyl-ghrelin (0.975 ± 0.208), unacylated ghrelin (0.787 ± 0.133)]. This data suggests that the ATP levels in our system are not affected by rotenone nor ghrelin treatments, although it is also possible that any small difference between the groups may have gone undetected due to the sensitivity of the kit, or the small sample size.

Cell function is affected by the overall health of the mitochondrial network; therefore, the number of active mitochondria may be used as an indirect measure of cell health. With this purpose, I performed RT-qPCR on total DNA extract, to quantify mtDNA copy number in ReN neurones treated with vehicle (n=3) or 1μM rotenone (n=3) (figure 4.5 D). The mitochondria-exclusive housekeeper gene LARS2 (leucyl-tRNA synthetase 2) was used, and the delta Ct values were normalised to nuclear DNA content using the reference gene β2-microglobulin (β2M), as described elsewhere (Zheng et al. 2016). Statistical significance was measured based on the Delta Ct values (figure 4.5 F) with two-way ANOVA and Tukey's multiple comparison test. No significant differences were detected in mitochondrial DNA content ($p>0.3$) between any of the groups. [Vehicle group: control (ΔCt=7.072 ± 0.143), acyl-ghrelin (ΔCt=6.761 ± 0.156), unacylated ghrelin (ΔCt=6.853 ± 0.092); rotenone 1μM group: control (ΔCt=7.179 ± 0.069), acyl-ghrelin (ΔCt=7.024 ± 0.236), unacylated ghrelin (ΔCt=6.915 ± 0.083)], suggesting that mitochondria number is not affected by either rotenone 1μM or ghrelin treatment.

Then, I performed RT-qPCR to assess the PGC1α mRNA expression, the key enzyme involved in mitochondrial biogenesis (figure 4.5 E). Statistical significance was measured with two-way ANOVA and Tukey's multiple comparison test, based on the Delta Ct values (figure 4.5 F), then normalised to β-actin and reported as fold change. No significant differences were detected between groups ($p>0.5$), suggesting that PGC1α gene expression is not affected in this experimental setting. [Vehicle group: control (ΔCt=9.562 ± 0.359), acyl-ghrelin (ΔCt=9.131 ± 0.153), unacylated ghrelin (ΔCt=9.596 ± 0.234); rotenone 1μM group: control (ΔCt=9.218 ± 0,330), acyl-ghrelin (ΔCt=9.284 ± 0.215), unacylated ghrelin (ΔCt=8.973 ± 0.134)].



F

	Delta Ct					
	Mitochondrial LARS2			PGC1α		
	N1	N2	N3	N1	N2	N3
CTR + V	-7.084738	-7.312657	-6.817450	9.804594	8.855501	10.025020
UAG + V	-6.672591	-6.918807	-6.968476	9.168012	9.645800	9.974307
AG + V	-6.578929	-7.070452	-6.632955	8.953338	9.005722	9.435015
CTR + Rot1	-7.088855	-7.314331	-7.133471	9.642657	8.567258	9.444330
UAG + Rot1	-6.754769	-7.032783	-6.958785	8.993256	8.722565	9.203744
AG + Rot1	-6.693683	-7.482382	-6.896367	8.854435	9.514514	9.483356

Figure 4.5 Rotenone effects on cell number and mitochondrial network

(**A**) ReN neurones (DIV24) were treated as shown in the timeline. (**B**) Cell number was quantified using the Cell Profiler software and reported as percentage ± SEM (n=3). (**C**) Total ATP was measured using the CellTiter Glo kit and reported as fold change (n=3). (**D**) Mitochondrial DNA content was quantified by RT-qPCR and normalised per nuclear DNA (n=3) while (**E**) PGC1α mRNA expression was normalised per β-actin (n=3). (**F**) delta Ct values were used for both RT-qPCRs statistical analysis and are reported in the table. Significance was measured by two-way ANOVA and Tukey's multiple comparison test. Data are reported as mean ± SEM. (n.s. not significant; *p≤0,05)

The data presented so far, failed to report significant mitochondrial changes in this cellular model. In order to visualise mitochondria impairment, ReN neurones were treated as shown in the timeline (figure 4.6 A). Briefly, ReN DIV24 were pre-treated with acyl-ghrelin, unacylated ghrelin or control for 5 days, then at DIV28 were treated with vehicle (n=2), rotenone 1μM (n=3) or 10μM (n=3); 24 hours later cells were fixed and stained with MitoTracker – a commercial dye that accumulates inside the mitochondria proportionally to their MMP (figure 4.6 B) (see section 1.3.5). Using the open-source software Cell Profiler, various mitochondrial parameters were measured, including the single mitochondrial fragments – from now on referred to as 'puncta' (figure 4.6 C).

First, I analysed the MMP by measuring total (integrated) intensity of the mitochondrial dye (figure 4.6 D). Using a two-way ANOVA followed by Tukey's multiple comparison test, no significant differences were reported between groups ($p>0.1$). [Vehicle: control (0.934 ± 0.024), acyl-ghrelin (0.886 ± 0.021), unacylated ghrelin (0.775 ± 0.009); rotenone 1μM: control (0.871 ± 0.129), acyl-ghrelin (0.869 ± 0.099), unacylated ghrelin (0.777 ± 0.059); rotenone 10μM: control (1.05 ± 0.067), acyl-ghrelin (1.17 ± 0.097), unacylated ghrelin (1.03 ± 0.109)]. However, when the main treatment effect was analysed as groups, independently of ghrelin, the rotenone 10μM treatment group showed significantly higher total MMP compared to vehicle (*$p=0.037$) and rotenone 1μM (**$p= 0.009$), suggesting that 10μM rotenone treatment affects mitochondria by increasing the MMP.

Subsequently, I analysed the number of puncta per cell, reported as fold change (figure 4.6 E), and no significant changes were detected between the treatments ($p>0.7$). [Vehicle treated: control (1.04 ± 0.062), acyl-ghrelin (1.00 ± 0.064), unacylated ghrelin (0.970 ± 0.084); rotenone 1μM treated: control (0.865 ± 0.058), acyl-ghrelin (0.923 ± 0.0469), unacylated ghrelin (0.851 ± 0.022); rotenone 10μM treated: control (0.990 ± 0.113), acyl-ghrelin (1.07 ± 0.129), unacylated ghrelin (0.968 ± 0.147)]. Lastly, no statistically significant treatment effect was reported between vehicle treated and rotenone 1μM ($p=0.312$) or 10μM ($p=0.999$) groups, nor between 1 and 10μM rotenone ($p=0.225$), suggesting that puncta number is not modulated by rotenone, or that our experimental settings are unable to show a clear difference between groups.

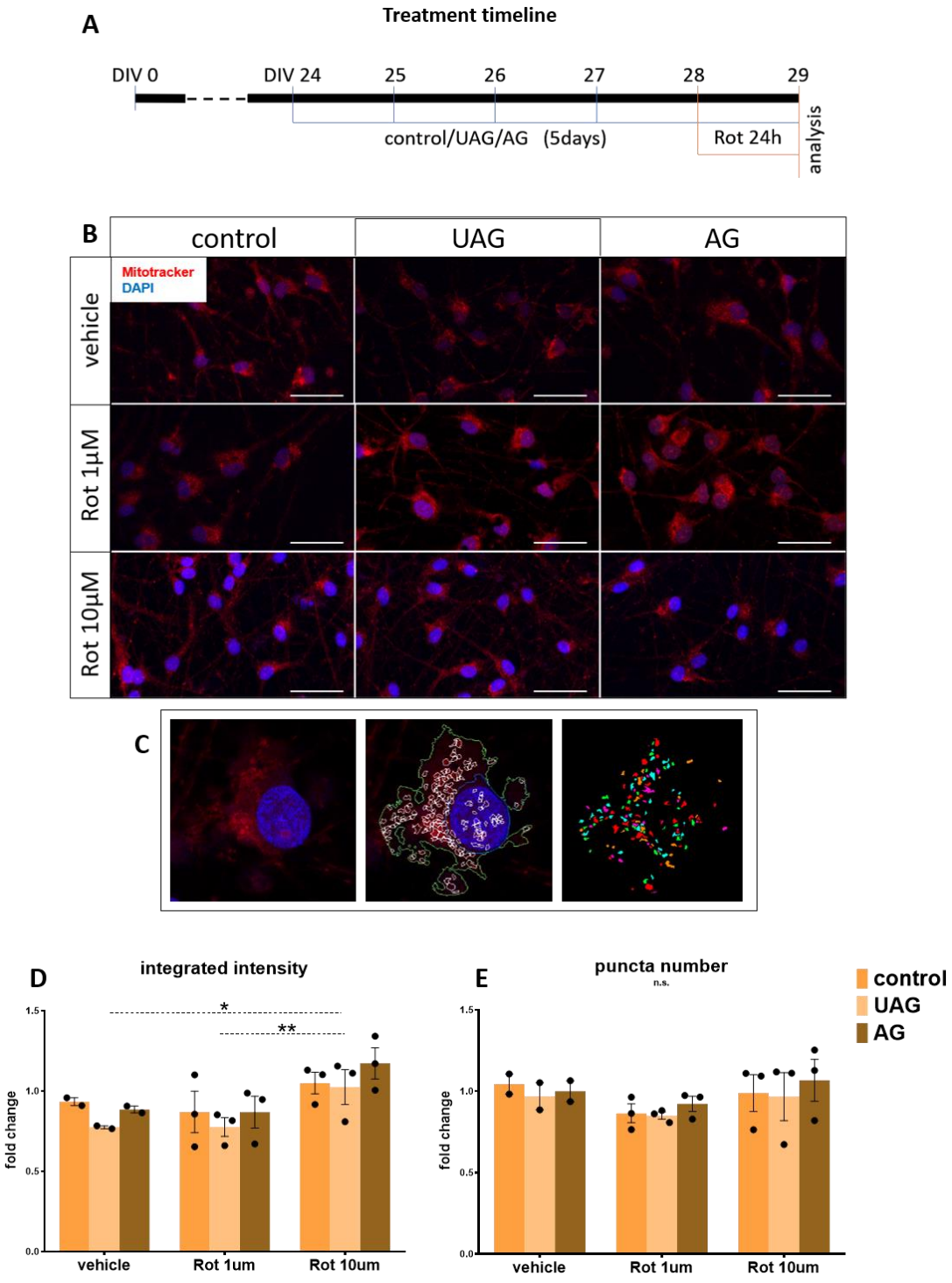


Figure 4.6 Mitochondrial measurements in rotenone treated ReN neurones

(**A**) ReN neurones were treated for five days (DIV24 to DIV28) as shown in the timeline. (**B**) At DIV29, cells were stained with MitoTracker and Hoechst nuclear counterstain (blue). (**C**) Mitochondrial puncta were identified, then (**D**) Total (integrated) intensity and (**E**) puncta number were measured and reported as mean ± SEM (n=3; vehicle n=2). Significance was measured by two-way ANOVA and Tukey's multiple comparison test. (n.s. not significant; **p≤0.005; *p≤0.05).

To identify any trend that may have gone undetected due to the small number of replicates, the integrated intensity and puncta number are presented using a SuperPlot graph (Lord et al. 2020), where the average of each replicate is presented alongside individual cell values (n≥273). D'Agostino-Pearson's normality test was conducted, and the data were found to be non-parametric. Therefore, statistical analysis was performed using Kruskal-Wallis followed by Dunn's multiple comparison test on the expanded dataset, using each cell as a unique replicate. With this approach, I detected a significant reduction of the integrated intensity (figure 4.7 A) between the control of the vehicle group (0.944 ± 0.0224; n=273) and the control of the rotenone 1μM group (0.862 ± 0.0170; n=625; **p=0.0032), suggesting that the low dose 1μM rotenone reduces the MMP at a single cell level, although this effect was not detected in the analysis of the grouped dataset (figure 4.6 D). Interestingly, the control of the 10μM group (1.05 ± 0.022; n=400) showed significantly increased integrated intensity compared to both vehicle group (*p=0.0294) and 1μM group (****p<0.0001), confirming the treatment effect detected previously (figure 4.6 D). Within the vehicle group, integrated intensity was significantly lower after unacylated ghrelin treatment (0.774 ± 0.013; n=347) compared to control (****p=<0.0001) and acyl-ghrelin treatment (0.895 ± 0.021; n=267; **p=0.0057). In the 1μM rotenone group, unacylated ghrelin treated cells (0.770 ± 0.013; n=809) also showed significantly lower intensity compared to control (**p=0.0010) and acyl-ghrelin (0.864 ± 0.016; n=699; ***p=0.0003), suggesting that 5-day treatment with unacylated ghrelin may have a detrimental effect on MMP, when administered on its own or in combination with acute 1μM rotenone treatment. Interestingly, within the 10μM rotenone group, unacylated ghrelin treatment (1.00 ± 0.027; n=318) was not significantly different from control (p>0.999), while acyl-ghrelin treatment (1.17 ± 0.0255; n=345) resulted in significantly higher MMP compared to control (*p=0.0308) and unacylated ghrelin (****p<0.0001). Overall, these data suggest a specific effect of unacylated ghrelin in reducing the MMP and an opposite activity of acyl-ghrelin by either increasing MMP or restoring the baseline (vehicle) level.

Similarly, I analysed cell puncta number (figure 4.7 B). Interestingly, in the vehicle group, neither unacylated ghrelin (0.955 ± 0.018; n=347; p>0.9999), nor acyl-ghrelin (0.975 ± 0.0201; n=267; p>0.9999) significantly affected puncta number compared to control (1.02 ± 0.022; n=273). However, in the rotenone 1μM group, all treatments – control (0.861 ± 0.016; n=625), unacylated ghrelin (0.848 ± 0.015; n=809) and acyl-ghrelin (0.920 ± 0.016; n=699) – were significantly reduced compared to the corresponding treatments in the vehicle group [control (****p=<0.0001), unacylated ghrelin (****p<0.0001) and acyl-ghrelin (*p=0.0289)] suggesting a treatment effect that could not be detected in the previous analysis performed with group means for each replicate (figure 4.6 E). No significant differences (p>0.8) were detected between vehicle-treated and 10μM-treated group (control: 0.993 ± 0.025, n=397; unacylated ghrelin: 0.938 ± 0.030, n=315; acyl-ghrelin: 1.06 ± 0.027, n=342), for any of the treatments, suggesting that the 1μM rotenone dose specifically reduces the number of puncta; however, this effect is lost with treatment at higher doses.

Moreover, in the 1μM rotenone group, acyl-ghrelin significantly increased puncta number compared to unacylated ghrelin (*p=0.0154), but not compared to control (p=0.4967), while unacylated ghrelin did not have a significant effect compared to control (p>0.9999). Lastly, there was no significant difference in the 10μM rotenone group, between control and unacylated ghrelin (p>0.9999), nor acyl-ghrelin (p=0.4124), while acyl-ghrelin significantly increased puncta number compared to unacylated ghrelin (**p=0.006). Similar to what was seen with the MMP analysis, these data suggest that unacylated ghrelin and acyl-ghrelin exert opposite effects, with acyl-ghrelin significantly increasing mitochondria puncta number compared to unacylated ghrelin, regardless of the rotenone treatment.

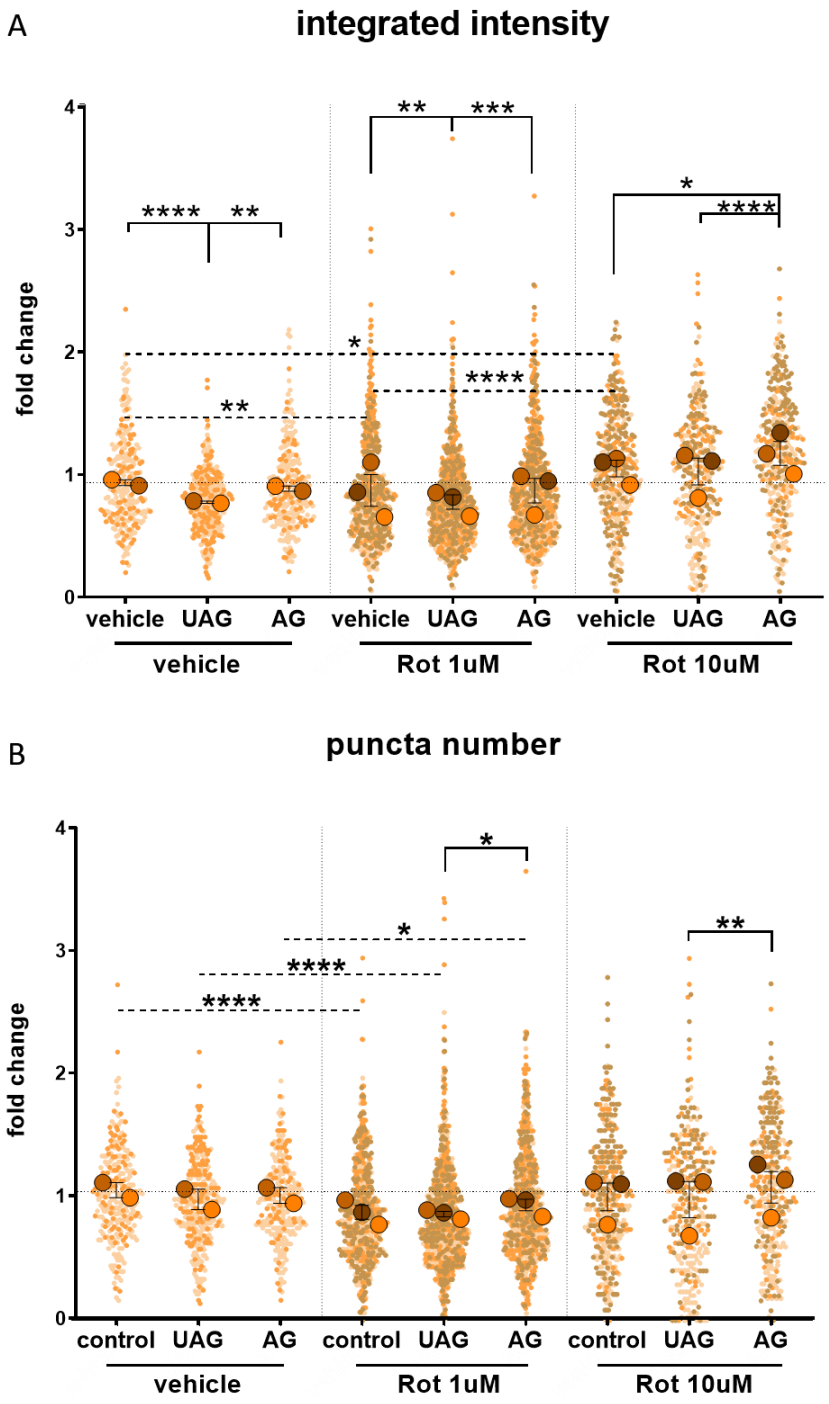


Figure 4.7 Single cells mitochondrial measurements in rotenone treated cells

(**A**) Mitochondria total (integrated) intensity and (**B**) puncta number are shown using a SuperPlot graph, a juxtaposition of the mean ± SEM from figure 4.6 (n=3; vehicle n=2) and the corresponding values per single cells (n≥273). Each large dot represents a group mean, and each colour-coded small dot represents individual cells. Statistical analysis was performed with Kruskal-Wallis and Dunn's multiple comparison on the single cell's values. (****p<0.0001; ***p≤0.0005; **p≤0.005; *p≤0.05)

My data so far showed that acyl-ghrelin may affect mitochondria health, while unacylated ghrelin seems to exert the opposite functions, in a cellular model of PD. To better understand mitochondria network remodelling, I analysed the distributions of the puncta from the datasets in figure 4.7 B. Cells were progressively segregated ('binned') into 13 sub-groups (from now on referred to as 'bins'), according to the number of puncta they contain: bin 1 includes all the cells with 1 to 10 puncta per cell, cells in bin 2 have 11-20 puncta each, and so on, with the final bin having 121-130 puncta per cell. Subsequently, values were reported as percentage of the whole group. The heatmap of the puncta distribution (figure 4.8 A) show that 10μM rotenone treatment increased the percentage of cells with fewer puncta (shift to the left, red arrow) compared to 1μM treatment and vehicle.

To determine whether specific bins of cells are more affected than others, I performed parametric two-way ANOVA followed by Tukey's multiple comparison test (figure 4.8 B). Data are shown as fragmented bars (that add up to 100%), and the numeric values on the top of each bars indicate the highest number of puncta that is found in a single cell within that group. In the vehicle group, acyl-ghrelin significantly increases the percentage of cells in the 21 to 30 puncta per cell bin (21.9 ± 3.99; ~p=0.035) compared to control (8.90 ± 5.24), while there is no significant difference among the other treatments or bins. This suggests that acyl-ghrelin, in the absence of a stressor, increases the number of cells containing less puncta. Indeed, the cell with the most puncta number in the AG/vehicle group has only 82 fragments, compared to the 125 of the control, reflecting the fact that more cells in the AG/vehicle group have less puncta. Interestingly, the cell with most puncta in the UAG/vehicle has only 79 fragments, suggesting that unacylated ghrelin may also reduce the number of puncta per cell, although no significant difference was detected in any of the bins compared to control or acyl-ghrelin.

Both 1μM and 10μM rotenone treatments seem to affect basal puncta content.
The control of the 1μM rotenone treatment showed a significant increase in the percentage of cells containing 21 to 30 puncta per cell (26.4 ± 4.09; ***p=0.0001) compared to the control in the vehicle group (8.90 ± 5.24). The control of the 10μM rotenone treatment, also showed a significant increase in the 21 to 30 puncta per

cell bin (31.1 ± 2.18; ****p<0.0001) compared to the control/vehicle (8.90 ± 5.24), and in the 11 to 20 puncta per cell bin (21.6 ± 1.82; ***p=0.0008) compared to the control/vehicle (5.84 ± 0.964). Moreover, the control of the 10μM rotenone treatment, showed a significant decrease in three groups: the 41 to 50 puncta per cell bin (11.2 ± 3.41; *p=0.0331) compared to control/vehicle (23.19± 2.46), the 51 to 60 puncta per cell bin (2.18 ± 1.38; **p=0.0011) compared to control/vehicle (17.7 ± 0.63) and the 61 to 70 puncta per cell bin (0.201 ± 0.201; *p=0.0165), compared to control/vehicle (13.0 ± 7.75). Accordingly, the highest number of puncta in the control of the 10μM treatment is only 71, compared to the 125 fragments of the control/vehicle.

Analysis of the UAG/10μM rotenone treatment also reported significant differences. The percentage of cells in the 11 to 20 puncta per cell bin was significantly increased (17.7 ± 4.92; *p=0.0345) compared to UAG/vehicle (5.81 ± 3.01); the 41 to 50 puncta per cell bin was significantly decreased (11.4 ± 3.14; **p=0.0049) compared to UAG/vehicle (25.5 ± 2.43). These data are very similar to those reported for the control of the same treatment, suggesting that the effect may be imputable to the rotenone treatment, rather than to the unacylated ghrelin *per se*. However, if the 10μM rotenone treatment is shown to increase the number of cells with less puncta (regardless of unacylated ghrelin treatment), this effect is abrogated by the treatment with acyl-ghrelin. Indeed, AG/10μM rotenone treatment did not significantly affect any bin compared to AG/vehicle, suggesting that acyl-ghrelin is opposing to the shift in puncta number per cell mediated by the 10μM rotenone (and for which unacylated ghrelin is neutral).

Overall, these data suggest that rotenone treatment increases the percentage of cells with less fragments, and the effect is especially evident in the 10μM rotenone group, where both control and unacylated ghrelin significantly increase the 11-20 and 21-30 bins. Interestingly, while control/10μM and UAG/10μM both increase the number of cells with less fragments, AG/10μM does not, suggesting that acyl-ghrelin may have an opposite action, counter effecting the increase seemingly caused by the rotenone 10μM treatment, on the distribution of mitochondrial puncta.

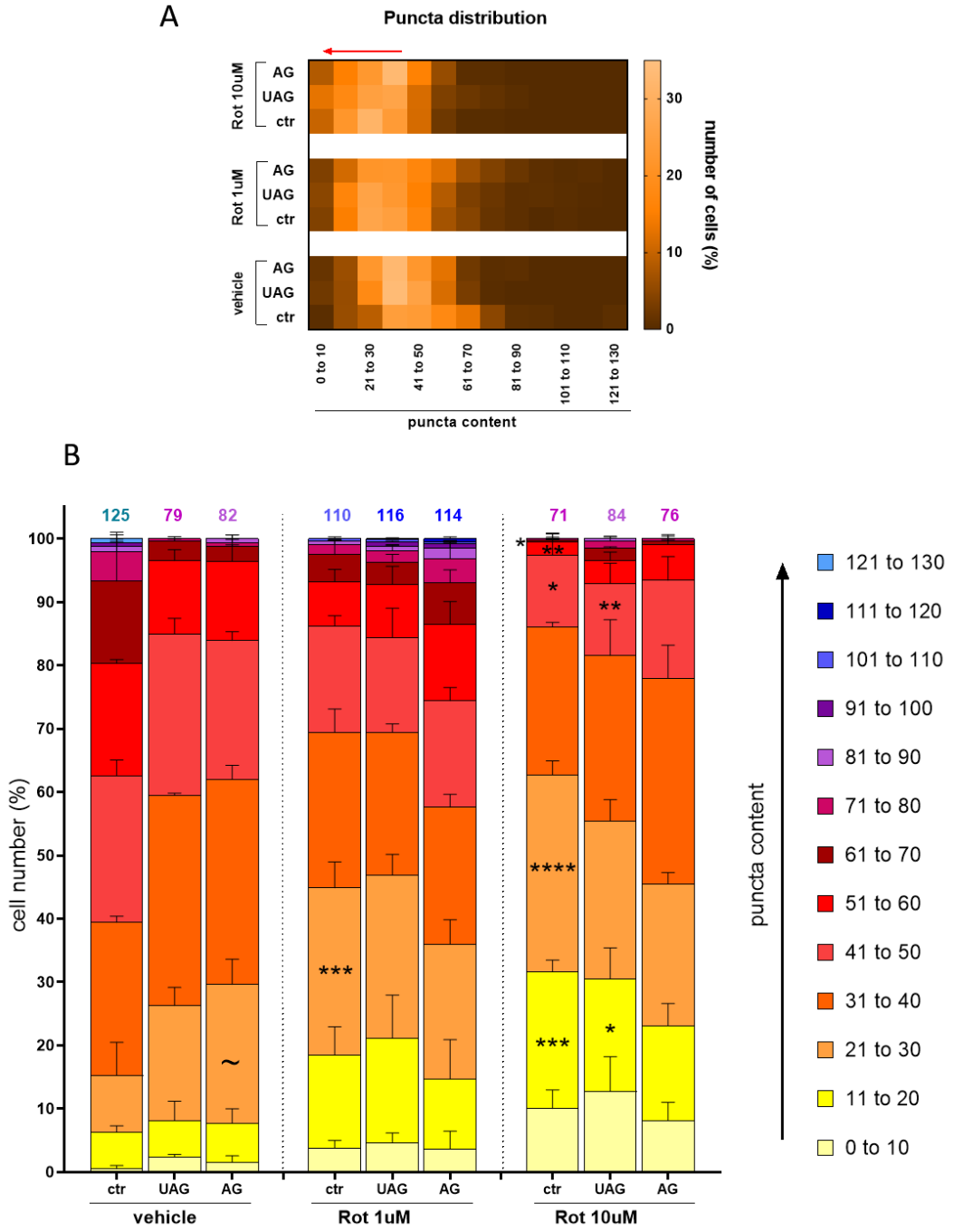


Figure 4.8 Puncta number distribution

(**A**) Heatmap showing the percentage of cells with defined puncta content. (**B**) The same values are represented as stacked bars, in which every colour corresponds to the cells being binned by number of puncta. Parametric two-way ANOVA followed by Tukey's multiple comparison was performed between groups with the same puncta content. (****p<0,0001; ***p≤0,0005; **p≤0,005; *p≤0,05).

Legend: (*) = comparison with the vehicle-treated group; (~) = comparison with the control treatment of the same group.

4.4 Discussion

Calorie restriction is neuroprotective in mice (Duan and Mattson 1999) and primate (Maswood et al. 2004) models of PD. It attenuates age-related decline in dopamine signaling (Roth and Joseph 1994) and prevents cell death in the SN, after excitotoxic or oxidative stress (Bruce-Keller et al. 1999). In the hippocampus, CR reduces neuronal cell death and induces neurogenesis in wild type but not GHS-R KO (Walker et al. 2015), nor in GHRL KO mice (Hornsby et al. 2016), reinforcing the hypothesis that acyl-ghrelin is indeed the main mediator of CR beneficial effects in the brain (Bayliss et al. 2016b; Morgan et al. 2017; Ratcliff et al. 2019). Indeed, acyl-ghrelin has been extensively reported to have a neuroprotective mechanism in mouse models of PD, and circulating ghrelin levels are significantly reduced in the plasma of diseased patients (section 4.1.3), suggesting that the ghrelinergic axis is dysregulated in PD; however, to the best of my knowledge, no study has ever investigated the ghrelinergic system by distinguishing between PD and PDD – the most advanced stage of PD associated to significant cognitive impairment, hence difficult to reproduce in mouse models.

In my investigations for this chapter, I assessed the ghrelinergic system in the brain of PD and PDD patients; furthermore, I investigated the molecular mechanisms behind ghrelin effects on the brain, particularly focusing on mitochondrial health that represent one of the main cellular hallmarks associated to this disease.

4.4.1 GOAT

Ghrelin acylation is essential for binding and activating GHS-R1a (Kojima et al. 1999) and GOAT is the only enzyme known to mediate this reaction (Gutierrez et al. 2008; Kirchner et al. 2009). Interestingly, our group recently showed that the ratio between acyl-ghrelin and unacylated ghrelin (AG:UAG) is significantly reduced in the plasma of PDD patients under fasting and post-prandial conditions, although no significant change was detected in the levels of medium-chain fatty acids (MCFAs) C6, C8, and C10 (Hornsby et al. 2020). Moreover, this reduction significantly correlates with cognitive impairment as assessed by the Montreal Cognitive Assessment (MoCA) – an assay that attributes a numeric score to cognitive function. On the contrary, the cognitively intact PD group exhibits normal

acyl-ghrelin levels, therefore we suggested that the AG:UAG ratio in the plasma may be used as a diagnostic biomarker for dementia in PD (Hornsby et al. 2020).
A dysregulation in AG:UAG ratio suggests an impairment of the acylation or deacylation process. Indeed, I confirmed that GOAT protein expression is significantly impaired in the hippocampal lysates of PDD patients (*p=0.014) (figure 4.2 D and E) and $GOAT^{+}$ cells are significantly reduced in the GCL area (**p=0.0050) (figure 4.2 A and B) (Hornsby et al. 2020). Since GOAT has been shown to re-acylate ghrelin *in situ* in the brain (Murtuza and Isokawa 2018) and peripheral tissues (Hopkins et al. 2017), a diminution of GOAT expression in the human hippocampus may be associated with a reduction in the amount of acyl-ghrelin in the brain. This, in combination with the reduction of peripheral acyl-ghrelin, may contribute to memory deterioration and dementia. Indeed, our group showed that GOAT KO mice exhibit cognitive impairment and memory deficits, that could be restored by treatment with acyl-ghrelin (Hornsby et al. 2020). In light of these findings, we suggest that increasing circulating AG:UAG ratio may be a therapeutic strategy to ameliorate cognitive decline in PDD patients.

My data on MBOAT4 mRNA did not show significant variations in PD or PDD patients compared to age-matched healthy controls (figure 4.2 C). This data may be explained with an impairment in the protein translation and expression: for instance, if MBOAT mRNAs are transcribed at a physiological level but less proteins are translated, it could be hypothesised that the mRNAs are degraded before they could become accessible to the ribosomal system.
Another option could be increased GOAT protein degradation, rather than impairment in production; indeed, it is well known that the two protein degradations pathways in the cell, ALP and UPS, are severely impaired in PD (section 4.1.2) and that early inactivation of one of the two pathways, induces the over-activation of the other, in a compensatory protective mechanism (Ding et al. 2007; Ebrahimi-Fakhari et al. 2011; Shen et al. 2013). To my knowledge, the exact pathway of GOAT degradation has not been investigated yet. Future experiments could study this pathway by using GOAT-expressing cells treated with selective inhibitors of the ALP (Ebrahimi-Fakhari et al. 2011) or UPS (Shen et al. 2013).

Moreover, both degradation pathways require the damaged proteins to be bounded to a poly-ubiquitin chain that acts as a recognition "tag"; indeed, further insight could be obtained by investigating intracellular GOAT ubiquitination, for instance by combining mass spectrometry and co-immunoprecipitation assay (Co-IP) (Free et al. 2009) using anti-GOAT and anti-ubiquitin antibodies. Lastly, PD is associated with DA neurone loss, and PDD patients also exhibit cell death in the hippocampus; indeed, in order to better characterise GOAT loss in PD and PDD, future studies should also focus on identifying which cell type express the enzyme, by determining its co-localisation with markers from different brain cell types.

4.4.2 APT1

The main enzyme known to be involved in ghrelin deacylation is APT1 (Shanado et al. 2004; De Vriese et al. 2004; Satou et al. 2010). In my data, APT1 mRNA and protein levels are not significantly reduced in the hippocampus of brain tissue from PD and PDD patients (figure 4.3). Worthy of note, PDD patients exhibit a 39% reduction on average of APT1 protein level (figure 4.3 D and E), although this value did not reach statistical significance ($p=0.0836$). This data may raise the question of whether APT1 could be somehow affected in PDD, hence paving the way for subsequent studies.

For instance, since APT1 has been detected in numerous peripheral tissues such as circulating macrophages (Hopkins et al. 2017), measuring circulating APT1 extracted from the blood of PD and PDD patients, may give a more comprehensive characterisation of how/if the system may be affected in the disease.

Noteworthy, the quantification performed in this chapter only refers to APT1 expression in the CA4 of the hippocampus. However, as discussed in section 3.4.2, APT1 immunopositivity is also present in other brain areas – such as the rim of tissue surrounding the CP, where it may have a major role in determining acyl-ghrelin access to the brain. Due to technical reasons, the stain in those areas was uncountable, hence further studies will be needed; as a matter of fact, acyl-ghrelin is significantly reduced in the plasma of aged and diseased subject (as shown in sections 3.1.1 and 4.1.3, respectively), hence characterising the expression and activity of APT1 in the cells of the BCSFB, may contribute to understanding the dynamics of ghrelin access in the human diseased brain.

Regardless of its role in the ghrelinergic system, APT1 is mainly known for its activity as de-palmitoylating enzyme, hence it removes palmitic acid from several cytoplasmic and membrane-bound substrates – showing little selectivity for very diverse targets – most of which are still unknown (Lin and Conibear 2015). Notably palmitoylation/de-palmitoylation are key processes for the correct functioning of several GPCRs, including the serotonin receptor 5-HT1A (Renner et al. 2007; Kobe et al. 2008), although the responsible enzymes have not been identified yet. Ultimately, the possible role of APT1 in diverse biological processes in the brain is an intriguing open question that needs further studies.

4.4.3 GHS-R1a and total ghrelin

My data show no significant difference in GHS-R1a protein expression or mRNA levels in hippocampal lysates from PD or PDD patients, compared to the control cohort (figure 4.4 A to D), suggesting that the diseased brain may still be receptive to acyl-ghrelin. This data is particularly interesting in consideration of a potential ghrelin-based therapeutic intervention aiming at increasing AG:UAG ratio. Indeed, acyl-ghrelin would require an intact GHS-R1a signalling system in order to exert its beneficial therapeutic functions. However, although I was able to confirm GHS-R1a expression, my data does not give any information about its activity. Moreover, the receptor isoform GHS-R1b, has been shown to interfere with GHS-R1a activity via several mechanisms, as will be better described in section 6.

My data also showed a significant reduction of total ghrelin protein expression in hippocampal lysates from PDD patients, compared to the control cohort (figure 4.4 E and F). Interestingly, previous data from our group showed that, while AG:UAG ratio is impaired, total ghrelin levels are not different in the plasma of PD or PDD patients compared to the control (Hornsby et al. 2020). This discrepancy may be explained by the different nature of the samples analysed – brain lysates versus plasma. Indeed, since acyl ghrelin is a soluble protein, its concentration in tissue lysates may have been underestimated. Moreover, circulating acyl-ghrelin has been shown to interact with large plasmatic proteins, that may influence its isolation and purification from blood, as seen in section 1.2.2 (Beaumont et al. 2003; De Vriese et al. 2007; Lufrano et al. 2016).

Lastly, it is also possible that, although total ghrelin level is not impaired in the plasma of PD and PDD patients, there may be an alteration in the mechanisms that regulates its access to the brain, hence determining a reduction of its concentration in the brain parenchyma, as detected in our brain tissue lysates. Consistently, several studies report dysfunction in the blood-brain permeability of aged healthy subjects (Simpson et al. 2010), as well as PD patients (Kortekaas et al. 2005; Wada et al. 2006), and poor permeability to drugs represent one of the major obstacle to finding a cure for PD (Begley 2004).

4.4.4 Mitochondrial health

The second aim of this study, was to determine ghrelin mechanisms of action *in vitro*, using differentiated human neurones treated with acylated or unacylated ghrelin, and rotenone 1μM or 10μM to model PD, as shown in figure 4.5 A.
My experimental conditions did not cause cell death within the timeline of my experiments; however, a treatment effect was detected with 10μM rotenone, that significantly reduced cell number compared to the 1μM treatment group (*p=0.0150) (figure 4.5 B). This data suggests that 10μM may be detrimental for the cells; yet none of the treatment groups were significantly different compared to the vehicle, suggesting that the differences between the groups may be too small to be detected with this assay. To better investigate cell toxicity, future studies should incorporate cell viability assays such as MTT or other commercially available kits, while also increasing rotenone dosage and exposure time.

The following experiments were designed so that rotenone treatment would induce a mild mitochondrial oxidative stress, simulating the early stages of the disease and without causing extensive cell death; hence 1μM rotenone concentration was deemed appropriate for my experimental aims. However, using these settings, neither total ATP production (figure 4.5 C) nor mtDNA copy number (figure 4.5 D) showed any significant changes between groups. Similarly, no significant difference was detected in PGC1α mRNA levels (figure 4.5 E).
These results are in contrast with numerous findings showing that acyl-ghrelin increases PGC1α via the AMPK pathway, as shown in section 1.4.4. Very recently, a study used the human neuroblastoma cell line SH-SY5Y – one of the most used cell models – pre-treated with acyl-ghrelin 1μM for 2 hours, then incubated with

rotenone 0.5μM for 24 hours. Wang and colleagues showed that the activation of the pAMPK/SIRT1/PGC1α pathway in the acyl-ghrelin pre-treated cells was associated with increased cell viability, TOM20 expression and autophagy, along with reduced ROS, mitophagy, cytC release and α-synuclein accumulation (Wang et al. 2021). This discrepancy with my data, may be due to differences in the experimental settings such as timing, reagent concentrations, and assays used to analyse mitochondrial health. Indeed, countless techniques have been used in different models, as described in section 1.3.5; for instance, to model PD *in vitro*, rotenone doses have been ranging from 50nM up to 1mM (Wang et al. 2002; Jiang et al. 2004; Hsuan et al. 2006; Pan et al. 2009; Ruan et al. 2010). Furthermore, different cell lines show different responses to the same stressors; for instance, ReN neurones may have a different response threshold from SH-SY5Y cells, hence they may require higher doses or longer incubation times.

For all the reasons above, standardization of mitochondrial analysis, such as MMP quantification, is challenging. Currently, one of the gold standard techniques used to characterize mitochondrial activity is the Seahorse XF Analyser that measures the oxygen consumption rate (OCR) *in vitro* (section 1.3.5). The assay requires the cells to be incubated a few hours in advance in a standardised cell media, supplied by the company, with the purpose of maintaining constant pH values during the whole length of the experiment (few hours in total). Unfortunately, ReN neurones did not respond well to the media composition and died as quickly as thirty minutes after the media change (appendix 3). Indeed, the Seahorse media contains low amount of nutrients and is therefore supplemented with serum to prevent cell death. However, early optimisation studies that I performed at the beginning of my Ph.D. (not shown), reported that serum-containing media is detrimental for neuronal differentiation of the ReN cells. Indeed, to my knowledge, most published studies performed the Seahorse assay on ReN stem cells (Velichkovska et al. 2019), or between 7 and 14 days into the differentiation process (Ghosh et al. 2020; Wong et al. 2021), due to the frailty of more mature ReN neurones in the seahorse media; only recently one study used them at DIV21 (Pamies et al. 2021). Since ReN cells do not express GHS-R1a receptor until DIV22-24 of the differentiation process (figure 3.8), these technical difficulties have prevented me to use the assay for the purposes of this thesis. Future investigations may consider different assays, such

as commercial kits to detect intracellular ROS (Ranzato et al. 2012), or may use a different cell lines, whose compatibility with the Seahorse media has been previously established, such as the SH-SY5Y cells (Alvarez-Fischer et al. 2013) or hiPS-derived neurones (Zheng et al. 2016).

Since my data so far failed to show any significant change of mitochondrial health, I used the MitoTracker, described in section 1.3.5, in order to visualise and quantify MMP and mitochondrial number, or "puncta" (figure 4.6 B and C). Moreover, I included the 10μM rotenone treatment in this analysis. Although no effect was detected in most of the groups, the 10μM rotenone treatment induced a significant reduction of the MMP – regardless of the ghrelin pre-treatment – compared to both vehicle and 1μM (figure 4.6 D), although no effect was detected on puncta number (figure 4.6 E).

Due to the low sensitivity of this assay, and the limited number of biological replicates (n=3), I also performed the statistical analysis on the expanded datasets (figure 4.7; n>200). Using this approach, I was able to pick up differences that could not be detected with a smaller dataset; however, this also increased the statistical power of the analysis. Hence – although some of these results are significant ($p<0.05$) – we should be cautious to draw definitive conclusions on their biological relevance. Indeed, in order to improve the accuracy and replicability of these analysis, future experiments should rather increase the number of biological replicates to be included in the study.

Statistical analysis of the expanded dataset showed that rotenone treatments affect the MMP (figure 4.7 A). In the vehicle-treated group, unacylated ghrelin reduced the MMP compared to the control, while acyl-ghrelin did not, suggesting that unacylated ghrelin may have a detrimental effect *per se*. The 1μM rotenone treatment significantly decreased the MMP compared to the vehicle; interestingly, unacylated ghrelin decreased the MMP even more, while acyl-ghrelin did not exert any effect as it was not significantly different from the control. On the contrary, 10μM rotenone treatment significantly increased the MMP compared to both vehicle and 1μM treatment groups. In this context, unacylated ghrelin did not seem to induce any changes compared to the control, while, surprisingly, acyl-ghrelin exacerbated the significant increase in MMP.

Variations of the MMP are a way for these organelles to regulate their physiological functions. For instance, it is well known that a significant drop of the MMP, induces the accumulation of PINK1 on the OMM and the activation of the PINK1/Parkin mitophagic cascade (section 1.3.3). On the contrary, the MMP increases when the mitochondria are particularly active; for instance, in activated lymphocytes during cell division (Darzynkiewicz et al. 1982), or epithelial cells during wound healing (Johnson et al. 1981). More in general MMP increases when cells undergo a complex process that requires a lot of energy, such as replication and motility. However, MMP is also known to increase drastically just before mitophagy, when the organelles try their last resources to restore respiration by combining and increasing their activity. In this sense, the significant MMP increase observed in my samples after 10μM rotenone treatment, may represent a feedback effect, driving energy production to counteract rotenone-induced complex I inhibition. On the contrary, the slight decrease of MMP observed in my samples after 1μM treatment, is more difficult to interpret, as it may either represent a reduction in the number of mitochondria, or just represent natural MMP fluctuations. A possible way to overcome this technical issue, would be to perform live cell imaging in order to track mitochondria fate with time (Xu et al. 2021). Another way would be to support these data with the analysis of other markers, such as PGC1α to determine variations of mitochondrial biogenesis, or indirect markers such as TOM20 and mtDNA copy number, to establish the number of functional organelles.

Analysis of the puncta number expanded dataset (figure 4.7 B), showed that neither acylated nor unacylated ghrelin exerted any effect in the vehicle-treated group. However, 1μM rotenone treatment significantly decreased puncta number compared to vehicle, while acyl-ghrelin somehow counteracted this reduction by restoring puncta number to the vehicle-treated level. Interestingly, 10μM did not affect puncta number compared to vehicle nor 1μM, however acyl-ghrelin seemed to increase puncta number compared to unacylated ghrelin.
Overall, these data show that rotenone treatments affect mitochondrial health in our system, although different doses showed different patterns of action, suggesting a different level of toxicity. Future studies should include a dose-response curve, in order to determine rotenone's window of action on these cells.

Interestingly, in most treatment groups unacylated ghrelin seemed to have a detrimental effect on mitochondrial health, while acyl-ghrelin showed the opposite trend. Wang and colleagues, in their study on acyl-ghrelin neuroprotective activity on rotenone-treated SH-SY5Y cells, used MitoTracker and JC-1 – another commonly used MMP-sensitive probe – and reported that acyl-ghrelin alleviated rotenone-induced MMP loss, restoring the control levels (Wang et al. 2021). Although our data were not consistent with this report, the opposite pattern of action shown by acylated and unacylated ghrelin is promising. Indeed, our group recently showed that unacylated ghrelin exerts a detrimental effect on AHN in rodents and that acyl-ghrelin is able to reverse the effect and to rescue memory function (Hornsby et al. 2020).

Lastly, when looking in more details at puncta number, my data showed that rotenone 1μM and 10μM significantly increased the number of cells with less puncta (figure 4.8), suggesting either increased mitochondrial fusion or increased mitophagy. In the vehicle group, acylated and unacylated ghrelin both seemed to increase the number of cells with less puncta, although only acyl-ghrelin showed a significant difference. Interestingly, In the 10μM treatment group, unacylated ghrelin significantly reduced the number of cells with less puncta, while acyl-ghrelin treatment was not significantly different from the control. This effect again suggests that acyl-ghrelin acts toward a restoration of the condition in the vehicle and this effect is opposite to unacylated ghrelin.

Although these results further contributed to the investigation of mitochondrial health, they also open a number of questions that will need to be addressed in future studies. Firstly, a reduction in the number of puncta, or mitochondrial fragments, may have two very different causes: on one side, it may be associated to a shift in the fission/fusion balance, towards increased fusion; on the other side it may be interpreted as an increase in mitophagy – or rather a shift in the balance between mitophagy and mitochondrial biogenesis. To add another layer of complexity, mitochondrial fusion may also have a dual function: on one side, fusion and mitochondrial biogenesis traditionally occur when mitochondria are healthy and very active, however fusion is also a protective mechanism that may occur when the intracellular levels of oxidative stress significantly increase, hence mitochondria join forces to increase the overall respiration rate (Gomes et al.

2011). Indeed, a change in the number of fragments does not provide a clear picture of the mitochondrial health; however, it may still be useful if combined to other elements. For instance, future study could investigate the size and area of the puncta, to determine whether a change in their number may correlate to a change in the size: indeed, larger puncta may represent a healthier network, while smaller fragments may underlie increased fragmentation and degradation. Concerning mitochondria size and shape, numerous reports have successfully investigated mitochondrial health by analysing their 3D structure by live electron microscopy, during cell apoptosis (Sun et al. 2007a; Oztel et al. 2017). Ultimately, these evidences highlight the difficulties in quantifying mitochondrial health via standardised protocol; therefore, future studies should include a more comprehensive set of assays that include molecular, biochemical and microscopic approaches.

4.4.5 Conclusions

Ultimately, the data from this chapter highlight an impairment in the ghrelinergic system in the hippocampus of PD patients, and more evidently in PDD, suggesting a correlation with memory and cognitive function.

ReN neurones were not sufficiently capable of replicating PD oxidative stress *in vitro*, however all data point to a detrimental effect of unacylated ghrelin on mitochondrial health, and an opposite activity of acylated ghrelin.

4.5 Key findings

- GOAT protein expression, but not mRNA, is significantly reduced in the hippocampus of PD patients, and more evidently in samples from PDD patients, suggesting a correlation with cognitive decline (figure 4.2).

- APT1 mRNA and protein levels do not vary in the hippocampus of PD, nor PDD patients (figure 4.3).

- GHS-R1a mRNA level does not vary in the hippocampus of PD not PDD patients (figure 4.4).

- Total ghrelin protein levels are significantly reduced in hippocampal lysates from PDD patients, compared to PD (figure 4.4).

- Rotenone affects MMP and puncta number in a dose-dependent way.
- Acylated and unacylated ghrelin exert opposite functions on mitochondrial health, with unacylated ghrelin acting detrimental towards both MMP and puncta number.

Chapter 5

Ghrelinergic system in Alzheimer's Disease

5.1 Introduction

5.1.1 Onset, progression, and classification

Alzheimer's disease (AD) is a chronic neurodegenerative disorder characterised by progressive memory loss, cognitive decline and reduced lifespan and quality. Although AD is a medically distinct pathology, its symptoms are commonly associated with normal ageing, probably because 95% of the cases are diagnosed between the age of 80 and 90 (Prince et al. 2015). Indeed, it accounts for 60-80% of the cases of dementia in the world (Barnes and Yaffe 2011) and affects 1-8% of the European population over 65 years of age and 25% over 85 years (Ferri et al. 2005). In 2016 dementia was reported to be one of the major causes of death, accounting for 11.6% of all deaths in England and Wales (Vital Statistics Office of National Statistics 2017).

Despite increasing efforts in the field, there is currently no cure that can stop the neurodegeneration and the only existing treatments are limited to improve the quality of life for the affected patients. Due to improved health care, AD patients can survive up to 10 years after the diagnosis, during which they often develop co-morbidities, such as heart and lung diseases, that ultimately represent the major cause of death (Alzheimer's disease facts and figures. 2020). Moreover, affected patients need to rely on others for assistance, making it one of the most high-cost diseases for the national health system (Meek et al. 1998).

The disease develops over three distinct phases (Dubois et al. 2010). The pre-clinical, or pre-symptomatic phase occurs when the neuropathological changes appear, although the patients maintain normal cognition and show no symptoms (Patel et al. 2019). AD has a very long pre-clinical phase, as it has been estimated that the biochemical hallmarks of the disease may appear up to 20 years before the onset of clinical AD (Bateman et al. 2012; Fagan et al. 2014).

The second phase is the early symptomatic phase, also referred to as "prodromal AD", characterized by mild cognitive impairment with some evidence of neurodegeneration. In these individuals, the cognitive decline is greater than expected at their age, although not yet debilitating (Dubois 2000). Interestingly, not all pre-symptomatic and prodromal AD will proceed into the last stage of the disease. Indeed, there are several evidences of non-demented elderly subjects whose brain present extensive lesions, despite intact cognitive functions, suggesting that the pathological processes might or might not become symptomatic during life (Price and Morris 1999; Aizenstein et al. 2008). It has been suggested to use the general term "Alzheimer's pathology" to refer to the not-clinically manifested AD (Dubois et al. 2010).

The third phase is clinical AD, first described in 1907 by Alois Alzheimer (Alzheimer 1907), and characterised with dementia and cognitive symptoms sufficiently severe to interfere with social functioning and daily activities. This phase can be identified in the *post-mortem* brain, from the presence of specific lesions: the senile plaques, also known as amyloid-β (Aβ) plaques, caused by the extracellular deposition of insoluble Aβ protein (Glenner and Wong 1984), and the neurofibrillary tangles (NFTs) constituted by hyper-phosphorylated tau protein (p-Tau) (Grundke-Iqbal et al. 1986). Traditionally, the loss of acetylcholine (Ach)-producing cortical neurones was considered the main cause of the memory loss, although it is now widely accepted that AD most likely originates from a multi-systems failure, and also involves other cells types such as DA neurones, astrocytes and microglia (reviewed in Hampel et al. 2019). Other changes that occur in the brain include neuronal dystrophy, synaptic loss, astrogliosis and vascular alteration (Wisniewski and Silverman 1997).

AD severity is described using the Braak staging method (figure 5.1 A), that attributes a numeric value to the *post-mortem* brain, based on the extension and localisation of the lesions (Braak and Braak 1991). The pre-clinical stage (phases I and II) is entirely asymptomatic; the "early AD" (phases III and IV) is characterised by an extensive Aβ deposition in the frontal and temporal lobes and a limited NFT accumulation in the medial temporal lobes and the hippocampus; lastly, during the "late AD" (phases V and VI), Aβ and NFT progressively spreads to other areas of the neocortex (Braak et al. 1993) (figure 5.1 A). Ultimately, the most affected areas are the frontal and temporal lobes, the limbic system, and the hippocampus, that is also one of the earliest areas to be affected (Fox et al. 1996). Indeed, impairments of spatial and episodic memory – both mainly dependent on the hippocampus – are often the first symptoms experienced by patients (Bature et al. 2017).

Finding an effective treatment for the disease has become a priority for all developed countries, but the lack of knowledge about the pathogenic molecular mechanisms is a great obstacle for the development of new treatments.

5.1.2 Pathogenesis and mitochondrial impairment

Like PD, most AD cases occur sporadically and the exact etiopathogenesis is unknown. However, approximately 1% of the cases are inherited in an autosomal dominant fashion and occur between 30 (early onset) and 60 (late onset) years of age. Familial AD (fAD) has been associated with more than 200 mutations, mainly involving three genes implicated in the production of Aβ peptides: amyloid precursor protein (APP), presenilin 1 (PSEN1) and presenilin 2 (PSEN2) (Games et al. 1995; Sherrington et al. 1995; De Strooper et al. 1998; Younkin 1998).

APP is a large integral membrane glycoprotein of unknown function (figure 5.1, point 1) (Kang et al. 1987), that is cleaved to generate a variety of fragments with contrasting effects on neural function (Turner et al. 2003). Physiologically, the majority of the APP is cleaved in the non-amyloidogenic pathway (Kojro and Fahrenholz 2005), mediated by the α-secretase enzyme located on the plasma membrane (Epis et al. 2012). First, an 83-aminoacid-long N-terminal intermediate is generated, then, this is further processed and secreted as a small soluble peptide known as APP-α (Haass and Selkoe 1993). APP-α has been shown to have neuroprotective and neurotrophic functions, and to regulate cell excitability and synaptic plasticity (Milosch et al. 2014).

Less common is the amyloidogenic cleavage pathway, that occurs after APP is internalised in endosomes (figure 5.1, point 2) (Selkoe et al. 1996). Inside the vesicles, APP is first cleaved by the β-secretase b-site APP-cleaving enzyme 1 (BACE1), generating a membrane-bound C-terminal fragment, C99, and a 99-amino acid-long N-terminal fragment, APP-β (Vassar et al. 1999). C99 is further cleaved to produce the APP intracellular domain (AICD) peptide, a transcription factor involved in regulating expression of numerous genes, including APP and BACE 1 (figure 5.1, point 3) (von Rotz et al. 2004). The APP-β peptide is 13 amino-acids longer than its α isomer, and it contains a short hydrophobic region known as Aβ region. Inside the endosome, APP-β is further processed by the γ-secretase complex, that cleaves the hydrophobic domain at different sites, generating Aβ peptides of variable length, between 36 and 43 amino acidic residues (Mundy 1994). Therefore, the exact site of cleavage is critical for the development of AD: the most common variant produced, Aβ40, is released outside the cell where it maintains its monomeric form, while the two longer isoforms, Aβ42 and Aβ43, less frequent in physiological conditions, display high self-aggregating activity (Burdick et al. 1992). In the brain of AD patients, Aβ42 monomers rapidly aggregate to form Aβ oligomers (AβOs), that induce other monomers to aggregate, acting as "seeds" (figure 5.1, point 4), ultimately leading to the formation of long Aβ fibrils and senile plaques (Sowade and Jahn 2017). The extracellular Aβ plaques are toxic to neurones as they are responsible for the disruption of cellular communication and the induction of microglia-mediated inflammatory response (figure 5.1, point 5) (Eng et al. 2004). Pathogenic mutations in PSEN1 and PSEN2, two components of the γ-secretase complex, favour the production of the longer peptides (Le Guennec et al. 2017; Szaruga et al. 2017), causing an increase in the Aβ42/Aβ40 ratio, that has been recently suggested as an early biomarker of AD (Vergallo et al. 2019).

The other main hallmark of AD are the intracellular inclusions of NFTs, composed of the microtubule-associated protein Tau (figure 5.1, point 6). Tau's role is to assemble tubulin dimers into microtubules and to regulate their dynamics and activity during axonal transport (Cleveland et al. 1977). The protein has 79 serine and threonine residues that could be differentially phosphorylated, allowing a fine space-time regulation of microtubule function and stability (Kishi et al. 2005; Zempel et al. 2010). However, in AD Tau is three-four times more phosphorylated

than normal, causing aberrant polymerisation into toxic fibrillary structures (Rossor et al. 1996). p-Tau, trapped inside the NFTs, cannot efficiently bind the microtubule networks, that collapses (Mandelkow et al. 1995; Liu et al. 2005). This results in impaired axonal transport of organelles, such as peroxisomes and mitochondria, making the cell more vulnerable to oxidative stress, ultimately leading to cellular dysfunction (Stamer et al. 2002; Mandelkow et al. 2003). Moreover, the accumulation of NFTs in the cytoplasm triggers the ER response (figure 5.1, point 7), and vice-versa, causing an increase in oxidative stress and inducing cell death via apoptosis (figure 5.1, point 8) (Ho et al. 2012).
AD has not been linked to any mutation in the Tau gene (MAPT), however p-Tau accumulation positively correlate with cognitive decline in AD patients (Ghoshal et al. 2002) and their localisation in the brain serve as diagnostic criteria for staging disease progression (Braak and Braak 1991).

In the past decade, growing interest has been dedicated to mitochondrial dysfunction, that is also considered to be one of the main hallmarks of AD (recently reviewed in Butterfield and Halliwell 2019). AD patients often display impaired mitochondrial morphology (Johnson and Blum 1970) and number (Hirai et al. 2001), defective oxidative phosphorylation and ROS hyper-production (Pamplona et al. 2005), increased mtDNA oxidation and mutations (Wang et al. 2006).
Several Tau isoforms bind the mitochondrial membranes or enter the lumen (Amadoro et al. 2011; Cieri et al. 2018); mitochondrial p-Tau has been shown to impair ATP production (Atlante et al. 2008), calcium flux and communication with the ER (Cieri et al. 2018). Aβ has also been shown to enter the cell and directly induce mitochondrial impairment (figure 5.1, point 9). Indeed AβOs are formed in the extracellular compartment from secreted monomers, however they can re-enter the cell via numerous uptake mechanisms (Bates et al. 2009). The main entry pathway is mediated by lipid rafts (figure 5.1, point 10), highly specialised areas in the cellular membrane, rich in cholesterol and proteins, whose function is to favour cellular communication and signal transduction (Vetrivel and Thinakaran 2010). This is consistent with reports showing that cholesterol interacts with both soluble and fibrillar Aβ (Wood et al. 2002). Moreover, high cholesterol-diets increase the risk to develop AD (Kivipelto et al. 2001) while cholesterol depletion in the culture media decreases Aβ production in competent neurones (Simons et al. 1998).

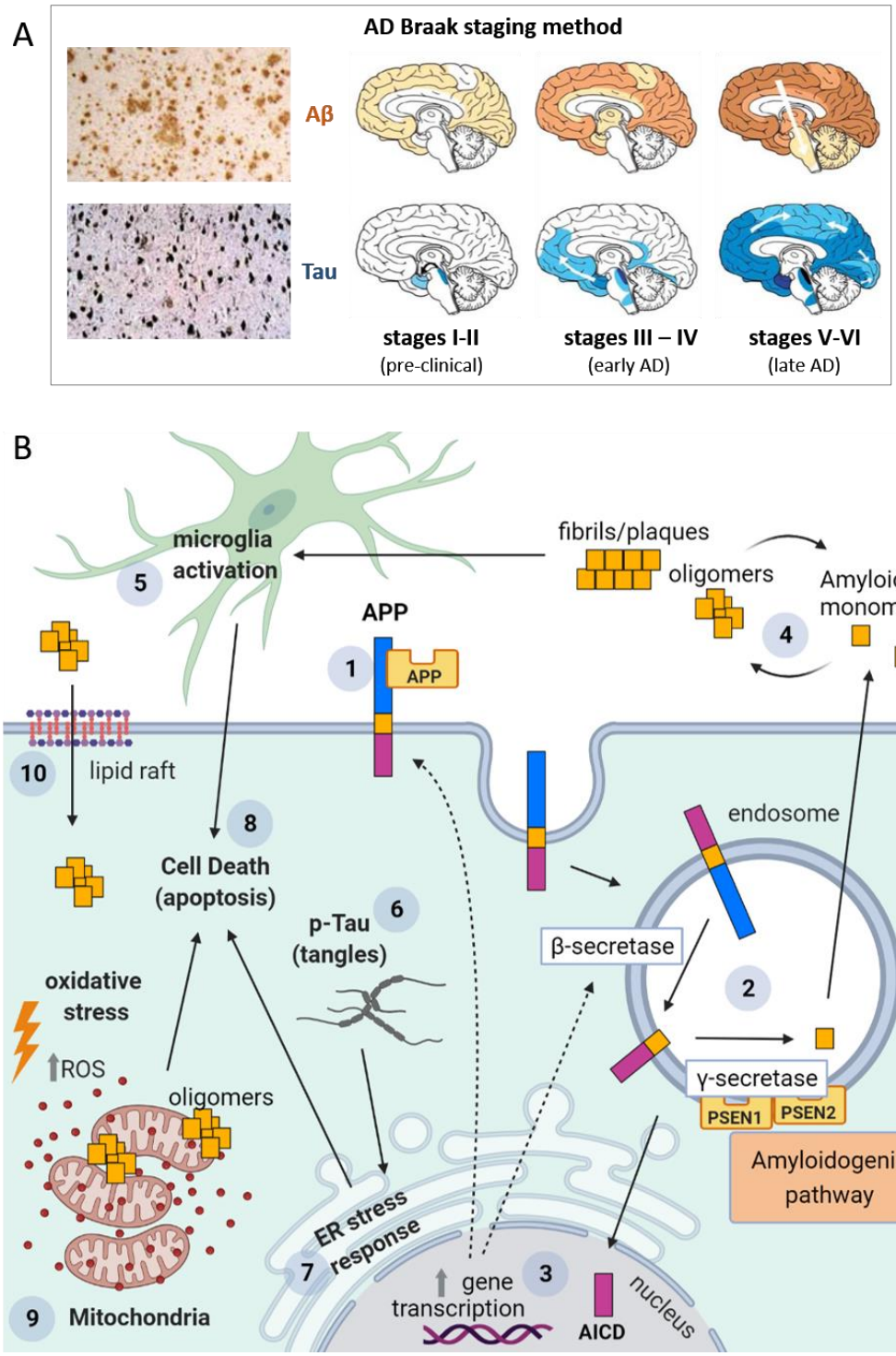


Figure 5.1 AD staging and molecular pathways

A Aβ and p-Tau deposition during AD, according to the Braak staging method (reprinted with permission from Jouanne et al. 2017, Elsevier licence number 5007761374603). **B** AD molecular pathways: **1** APP protein; **2** amyloidogenic pathway; **3** increased gene transcription; **4** Aβ seeding effect; **5** activation of the immune response; **6** p-Tau NFTs; **8** induction of apoptosis; **9** mitochondrial impairment; **10** lipid rafts (*created with BioRender.com*).

Another possible access pathway is endocytosis mediated by receptors such as the N-methyl-D-aspartate (NMDA) glutamate receptor (Snyder et al. 2005) or the α7 nicotinic acetylcholine receptor (α7nChR) (Nagele et al. 2002), although the precise mechanisms are still unclear. Either way, a study performed on cultured neurones showed that only the small oligomers can enter the cells, while fibrils and filaments remain in the media for 24 hours after incubation (Chafekar et al. 2008). This may be explained by the different 3D conformation of the aggregates: AβOs are spherical structures with an average diameter (z-height) of 5-10 nm (Chromy et al. 2003), while fibrils are rod-shaped aggregates with a diameter of 7-15 nm and over 1μm in length (Hill et al. 2011), and thus their structural features is not compatible with internalization.

Once internalised by the cell, AβOs accumulate in a time-dependent manner inside the mitochondrial membrane (Caspersen et al. 2005) and their presence has been confirmed in the mitochondria of affected brain cells in both AD patients and transgenic mice (Brunetti et al. 2020). The two main mitochondrial transmembrane channels, TOM and TIM, form complexes with AβOs, allowing them to go through the highly selective mitochondrial membranes and to accumulate in the lumen (Hansson Petersen et al. 2008; Pagani and Eckert 2011). Ultimately, both extracellular and intracellular AβOs-mediated toxicity promote mitochondrial fragmentation through the mitochondrial pro-fission protein DRP1 (Barsoum et al. 2006; Manczak et al. 2011).

Interestingly, AβOs made from the Aβ42 peptide are more toxic than fibrils both *in vivo* (He et al. 2012) and *in vitro* (Nimmrich et al. 2008). However, Aβ42 peptide has very high self-aggregating activity as it quickly polymerises when exposed to aqueous solutions – such as cell media – producing insoluble aggregates and long fibrils. These irregular structures are difficult to quantify, since the aggregation process occurs randomly, hence the exact conditions of each experiment are not reproducible. To avoid compromising the accuracy of my data, I used a protocol created by Dr. Klein's lab (Lambert et al. 2001), described in chapter 2, that consists of resuspending the lyophilised peptide through a series of decreasingly hydrophobic solvents, before being diluted in cell culture media, in order to prevent disordered aggregation.

This protocol has been extensively used in literature because of its simplicity – although it requires two days of preparation – and because of the numerous advantages of using oligomers over fibrils. Notably, oligomers enter the cells, as previously shown, therefore are more efficient in quickly inducing oxidative stress, making them the gold standard in reproducing AD molecular stress *in vitro*.

5.1.3 Ghrelin and AD

In humans, ghrelin plasma levels decrease with age (Rigamonti et al. 2002) and this reduction mediates the appetite loss, slower gastric emptying and reduced gastrointestinal hormone secretion typically found in aged subjects, as shown in chapter 3. AD patients also commonly exhibit significant weight and appetite loss (Gillette-Guyonnet et al. 2000), although a study reported that ghrelin plasma level in AD patients was not significantly different from age-matched healthy subjects (Proto et al. 2006). Notably, other components of the ghrelinergic system have been found to be affected in AD. In the temporal lobe of AD patients, GOAT and GHS-R1a mRNA were shown to be decreased compared to controls, while GHS-R1b mRNA was increased (Gahete et al. 2010a), suggesting that the ghrelinergic axis may be altered in the brain of AD patients, possibly contributing to the severe cognitive deficit observed in this pathology.

To the best of my knowledge, the work mentioned above from Gahete and colleagues, is the only one linking the ghrelinergic system to AD using human brain samples. Indeed, ghrelin has never been administered to AD patients, despite the numerous reports about its beneficial effects on memory and cognition in animal models of AD. For instance, intra-hippocampal injections of ghrelin protect against neurones loss, prevent synaptic degeneration, and rescue memory deficits in mice injected with Aβ42 and in the APPSwDI transgenic model of AD (Moon et al. 2011; Kunath et al. 2015; Eslami et al. 2018). Administrations of ghrelin or other GHS-R1a agonists, reduce Aβ accumulation and plaque deposition in the hippocampus (Dhurandhar et al. 2013; Kang et al. 2015), decrease tau hyperphosphorylation (Chen et al. 2010; Kang et al. 2015), and delay the onset of early AD events such as microgliosis and inflammation (Santos et al. 2017; Jeong et al. 2018). Notably, ghrelin treatment in hippocampal primary cells has been shown to reduce AβOs-induced mitochondrial dysfunction and oxidative stress, in a GHS-R1a-dependent manner (Martins et al. 2013; Gomes et al. 2014).

As previously mentioned in chapter 4, ghrelin-mediated neuroprotective effect in PD occurs through the rapid activation of AMPK/UCP2 pathway, that enhances mitochondrial respiration and proliferation; hence, several studies have been investigating chemicals aimed at increasing AMPK tenor in the brain.

Interestingly, two of these compounds, Resveratrol and Metformin, have also been investigated in the context of AD (Guo et al. 2013; Rotermund et al. 2018). Genetic mouse models of AD, display reduced expression levels of AMPK in the hippocampus, as well some of its downstream signals such as reduced PGC1α and NRF (Pedro et al. 2014).

Considering all evidence, the AMPK pathway and any compound that activates it, such as acyl-ghrelin, may represent an effective strategy against both PD and AD. Alas, the number of studies investigating the ghrelinergic system in the brain of AD patients is inadequate and pose a large limitation to the possibility of a ghrelinergic-based therapy.

5.2 Aims

My work for this chapter will attempt to address the lack of knowledge about the ghrelinergic system in the brain of AD patients; hence, I will investigate the ghrelinergic axis in human tissue and cellular models of AD.

The aims of this chapter are:

- To quantify the expression levels of key enzymes involved in ghrelinergic signalling axis, such as GOAT, APT1 and GHS-R1a, in the human hippocampus of AD patients at different stages of the disease.
- To analyse the effect of ghrelin in an *in vitro* model of human neurones, pre-treated with acylated or unacylated ghrelin for 5 days, then treated with AβOs at different concentrations. Several parameters of mitochondrial health will be taken into consideration, such as MMP and biogenesis.

5.3 Results

5.3.1 The ghrelinergic axis is deregulated in the hippocampus of AD patients

GOAT immunopositivity in the GCL was identified as before (figure 5.2 A, black arrowheads). Immunopositive cells were counted to be 433.3 ± 160.8 cells per mm^2 of GCL in controls (n=4), 444.7 ± 71.52 cells per mm^2 of GCL in early AD (n=6), and 664.9 ± 91.38 cells per mm^2 of GCL in late AD (n=6), with no significant differences reported relative to the control (p=0.9136 for early AD; p=0.2328 for late AD), nor between disease groups (p=0.2253) (figure 5.2 B). Western blot analysis, reported as fold change to GAPDH, showed no significant changes between controls (0.9616 ± 0.09621) (n=5) and early AD (0.6775 ± 0.1646) (n=5) (p=0.1306), or late AD (0.8753 ± 0.1617) (n=4) (p=0.7350) (figure 5.2 D and E), nor between early and late AD (p=0.2771). On the contrary, MBOAT4 gene expression from tissue lysates, reported as fold change to β-actin, was significantly increased (lower ΔCt) in late AD (ΔCt=6.023 ± 0.2255) (n=5), compared to controls (ΔCt=7.025 ± 0.2016) (n=5) (**p=0.0058). No significant difference was detected between controls and early AD (ΔCt=6.553 ± 0.1848) (n=5) (p=0.2031), nor between early and late AD (p=0.1376) (figure 5.2 C). These data suggest a dysregulation in MBOAT4 gene transcription, although they are not supported by an alteration at the protein level.

APT1 immunopositive cells in the CA4 (figure 5.3 A, black arrowheads) were quantified to be 134.3 ± 56.62 cells per mm^2 of CA4 in control brains (n=4), 107.3 ± 15.38 cells per mm^2 of CA4 in early AD brains (n=6), and 157.3 ± 25.00 cells per mm^2 of CA4 in late AD brains (n=5), with no significant differences reported between the groups (control vs early AD p=0.8625; control vs late AD p=0.1936; early vs late AD p=0.2093) (figure 5.3 B). Similarly, western blot protein quantification, reported as fold change to GAPDH, showed no significant changes of APT1 expression between control (1.019 ± 0.2594) (n=5) and early AD (1.172 ± 0.1516) (n=5) (p=0.6714), or late AD (1.038 ± 0.2441) (n=5) (p=0.8320), nor between disease groups (p=0.5245) (figure 5.3 D and E). Interestingly, LYPLA1 gene expression from tissue lysates, reported as fold change to β-actin, showed a trend, close to significance, between controls (ΔCt=5.780 ± 0.1409) (n=5) and late

AD (ΔCt=5.279 ± 0.1457) (n=5) (+p=0.0660), but no significance with early AD (ΔCt=5.295 ± 0.2920) (n=5) (p=0.0771), nor between early and late AD (p=0.9436) (figure 5.3 C), suggesting that APT1 expression may also be impaired in late AD, although not quite to a significant level.

Because of technical difficulties in performing the BaseScope assay in FFPE tissues, GHS-R1a mRNA expression was analysed via RT-qPCR. Gene expression in tissue lysates, reported as fold change to β-actin, showed no differences between controls (ΔCt=6.447 ± 0.6679) (n=5) and early AD (ΔCt=6.980 ± 0.7244) (n=5) (p=0.4963), or late AD (ΔCt=6.368 ± 0.1607) (n=4) (p=0.8586), as well as between early and late AD (p=0.4124) (figure 5.4 A).

Lastly, western blot quantification of total ghrelin in tissue lysates, reported as fold change to GAPDH, also showed no changes between controls (0.5543 ± 0.03914) (n=2) and early AD (0.6833 ± 0.08998) (n=4) (p=0.5423), or late AD (0.6924 ± 0.1978) (n=5) (p=0.9139), and no difference between early and late AD (p=0.3568) (figure 5.4 B and C), although the small number of replicates in the control group (n=2) may have negatively influenced the statistical power of the analysis.

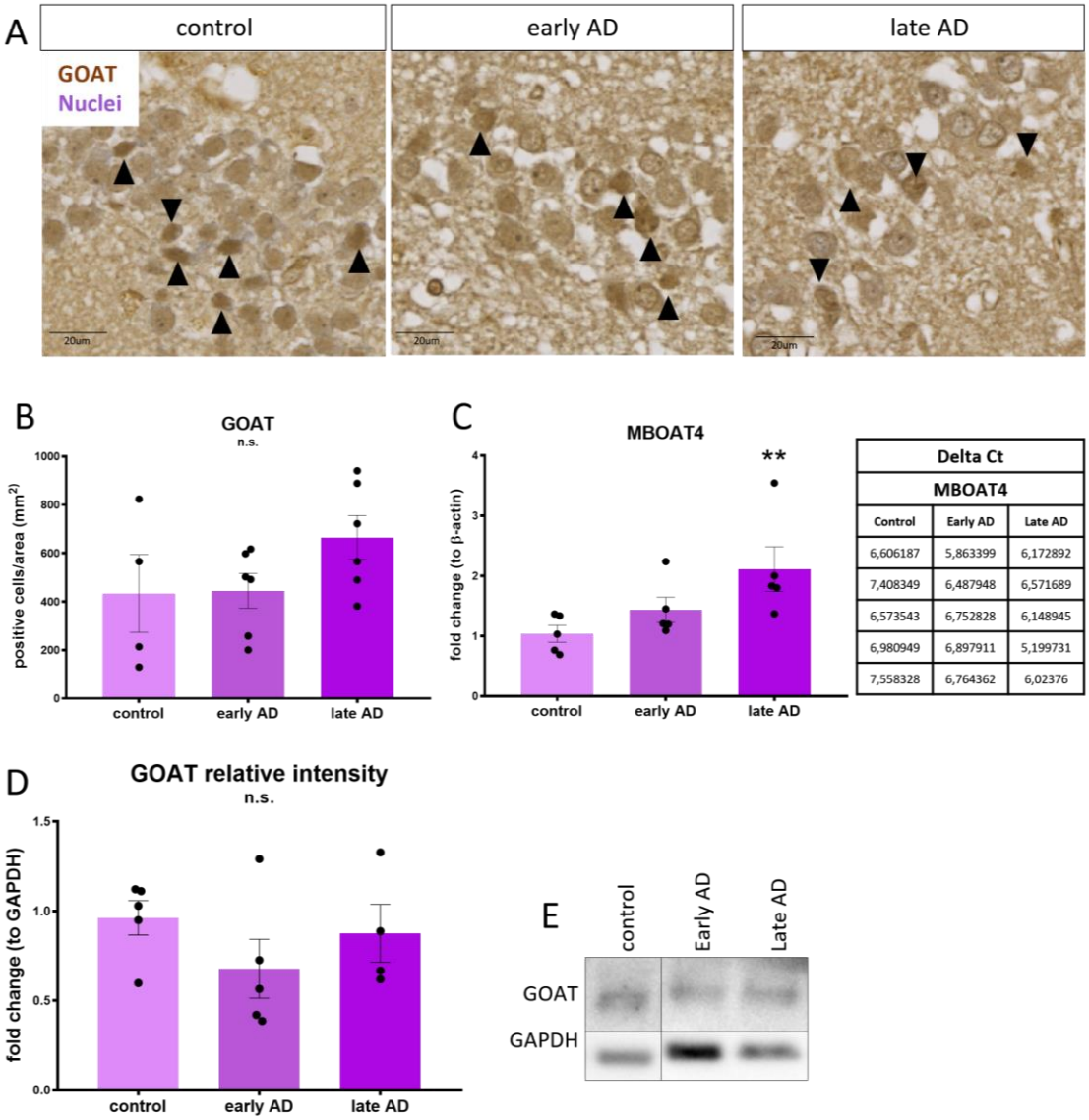
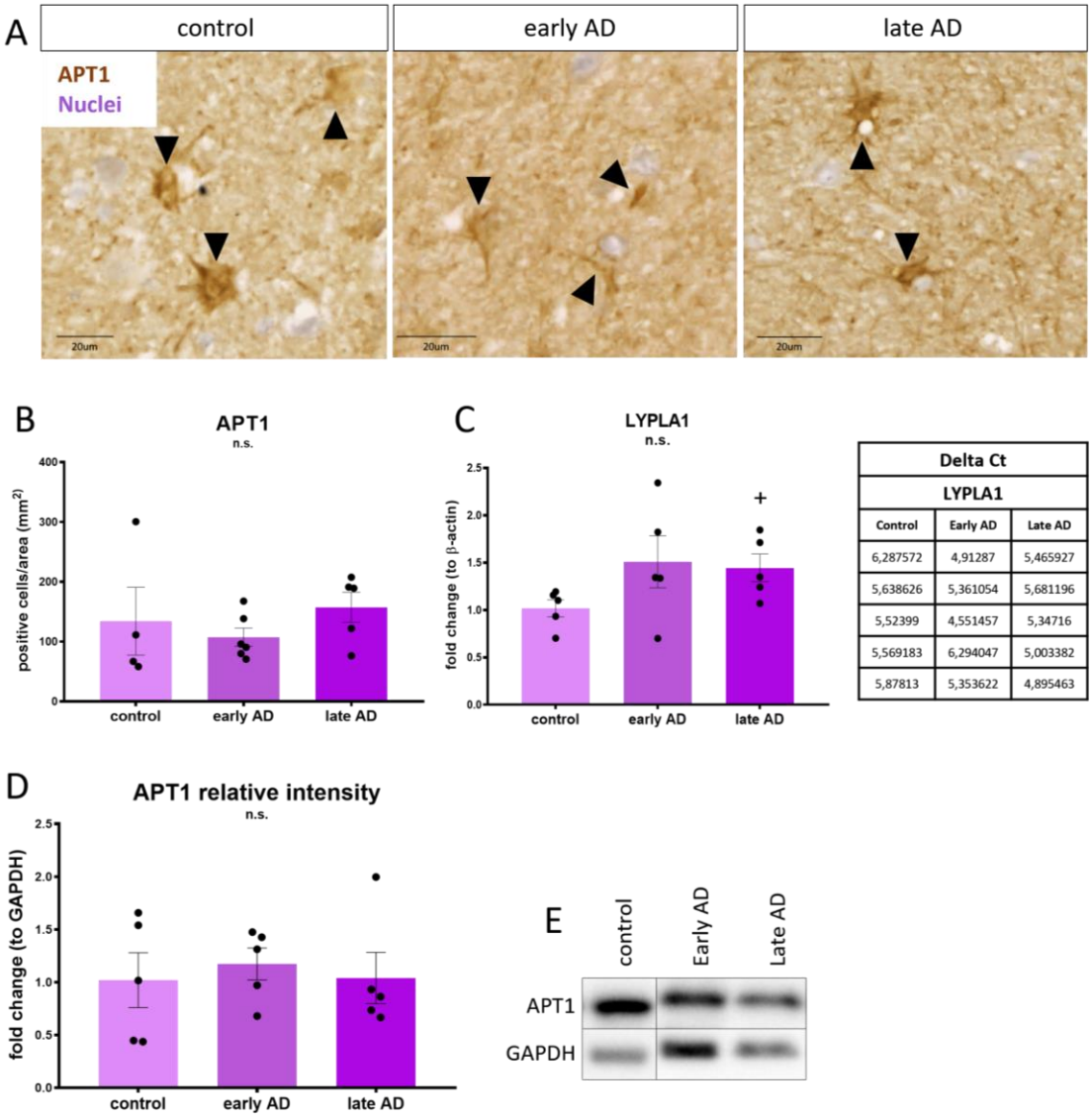


Figure 5.2 GOAT expression in AD

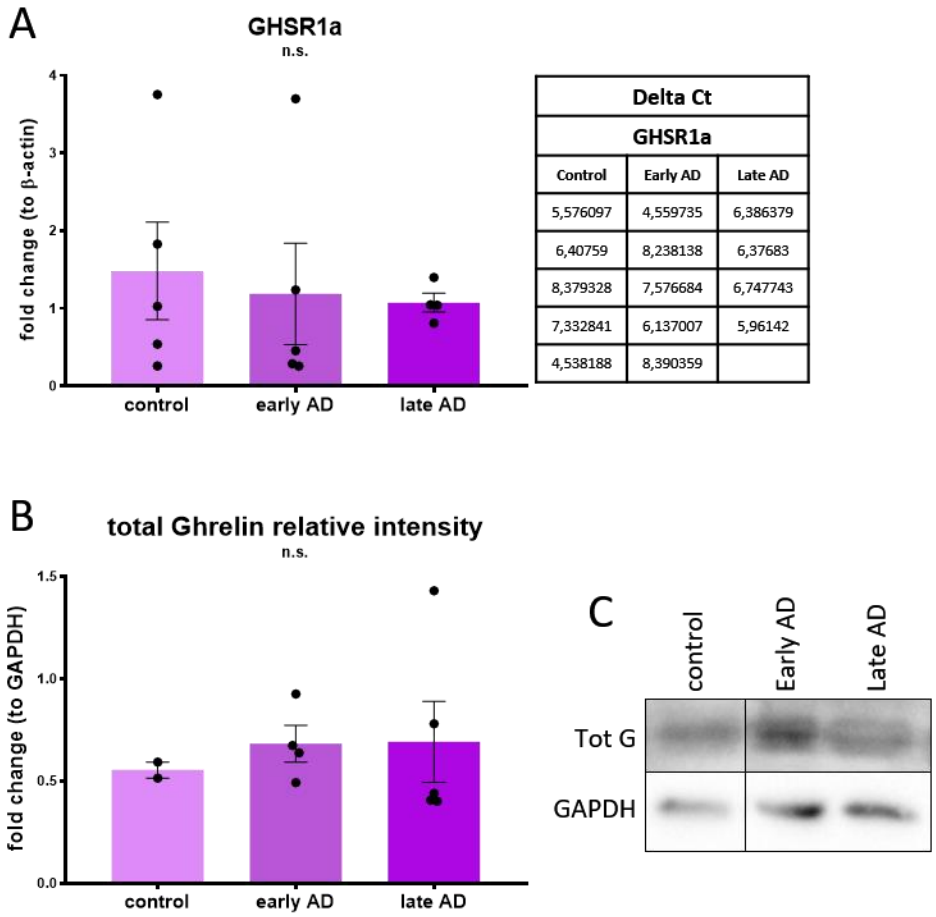
(**A**) GOAT immunoreactivity (in brown, black arrowheads) was identified in the GCL of controls (left), early AD (centre) and late AD (right), (**B**) quantified and normalised per the area of the GCL (in mm^2). (**C**) MBOAT4 mRNA expression was quantified via RT-qPCR while (**D**, **E**), GOAT relative protein expression was quantified by WB. ROUT analysis was performed to remove any outliers from the groups. Significance was measured by Kruskal-Wallis test with Dunn's multiple comparison. Data are reported as mean ± SEM. (n.s. not significant; **p≤0,005)



Delta Ct		
LYPLA1		
Control	Early AD	Late AD
6,287572	4,91287	5,465927
5,638626	5,361054	5,681196
5,52399	4,551457	5,34716
5,569183	6,294047	5,003382
5,87813	5,353622	4,895463

Figure 5.3 APT1 expression in AD

(**A**) APT1 immunoreactivity (in brown, black arrowheads) was identified in the CA4 of controls (left), early AD (centre) and late AD (right) and (**B**) quantified as positive cells per mm^2 of CA4. (**C**) LYPLA1 mRNA expression was quantified via RT-qPCR while (**D**, **E**) APT1 relative protein expression was quantified by WB. ROUT analysis was performed to remove any outliers from the groups. Significance was measured by Kruskal-Wallis test with Dunn's multiple comparison. Data are reported as mean ± SEM. (n.s. not significant; **+**p=0,0660)



Delta Ct		
GHSR1a		
Control	Early AD	Late AD
5,576097	4,559735	6,386379
6,40759	8,238138	6,37683
8,379328	7,576684	6,747743
7,332841	6,137007	5,96142
4,538188	8,390359	

Figure 5.4 GHSR1a and tot ghrelin expression in AD

(**A**) GHS-R1a mRNA expression was quantified via RT-qPCR. (**B**, **C**) Total ghrelin protein expression was quantified by WB. ROUT analysis was performed to remove any outliers from the groups. Significance was measured by Kruskal-Wallis test with Dunn's multiple comparison. Data are reported as mean ± SEM. (n.s. not significant)

5.3.2 Amyloid affects cell viability and mitochondria homeostasis

For each batch of Aβ peptide that was resuspended, structure and size validation was performed by AFM with the help of our collaborator, Dr. Andrea Gazze (Swansea University, Medical school) (Gazze et al. 2021).

As a result of using Klein's lab protocol, ordered oligomeric structures were generated, appearing at the AFM as spheroids of uniform size, with diameter of approximately 10nm (figure 5.5 A). As a negative control for fibril formation, the Aβ peptide-HFIP stock solution was diluted straight in distilled water for 24 hours at 4°C, generating heterogeneous ("disordered") particles (figure 5.5 B). Lastly, when the peptide was left in water for one week at 4°C, oligomers polymerised in a disorderly fashion generating small and medium-length fibrils – such as the one shown in figure 5.5 C, consisting in the aggregation of 4 oligomers (numbered 1 to 4). For the purpose of this thesis, only ordered AβOs such as those shown in figure 5.5 A, were used for cell treatments.

ReN neurones at day *in vitro* 28 (DIV28), were treated for 24 hours with AβOs, then assessed for immunoreactivity to anti-Aβ42 antibody (6E10). Mostly of the cells appear immunopositive (figure 5.5 D), indicating they internalised the AβOs preparation. Interestingly, some cells presented granularity in the cytoplasm (figure 5.5 D, white arrowheads), suggesting a compartmentalised distribution of the peptide inside the cell. Furthermore, some large aggregates of variable size (approximately 20-80μm) were identified in the areas between the cells (figure 5.5 E and F), consistently with a process of extracellular plaque aggregation.

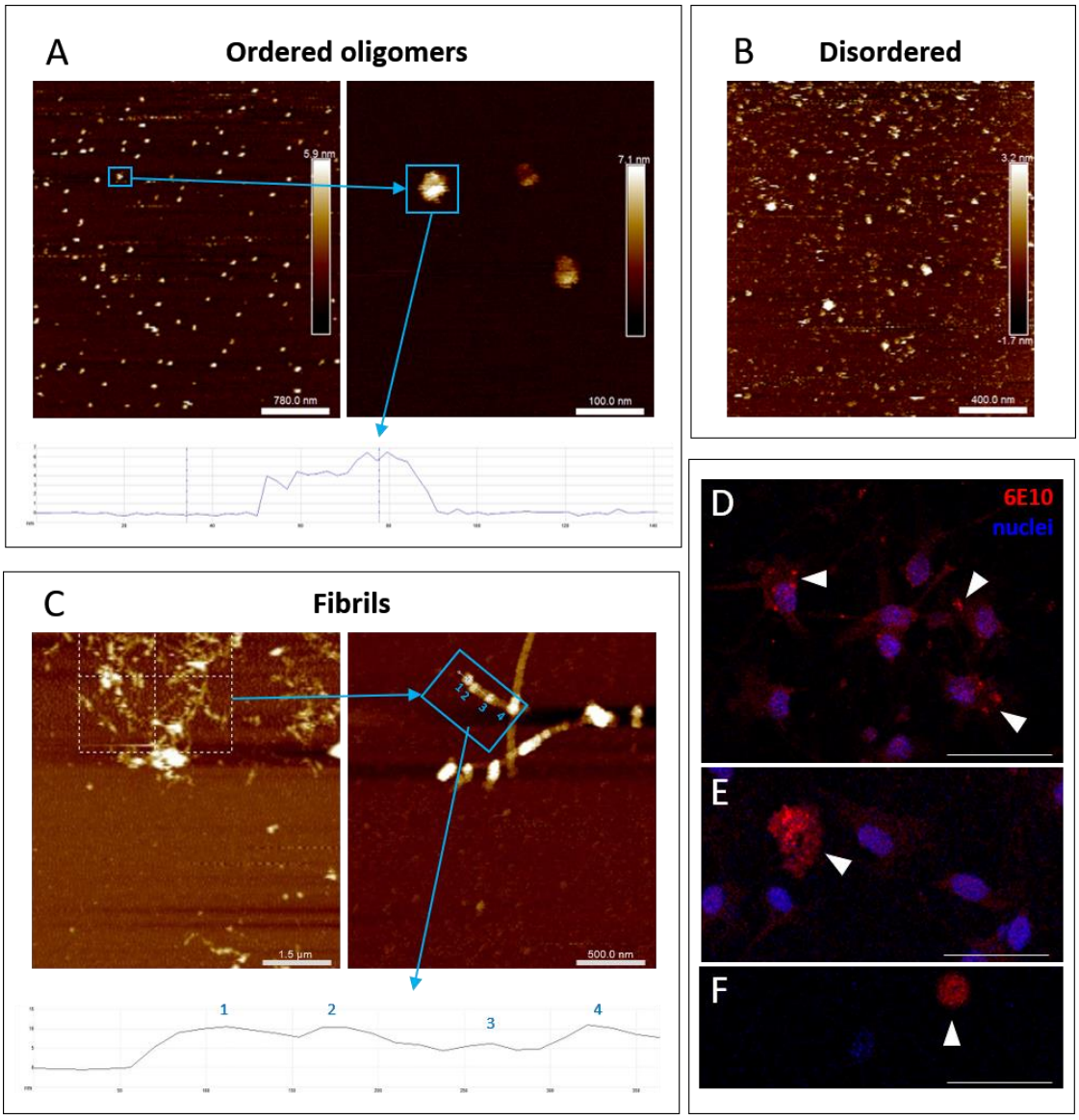


Figure 5.5 AFM validation of the amyloid-β oligomers preparation

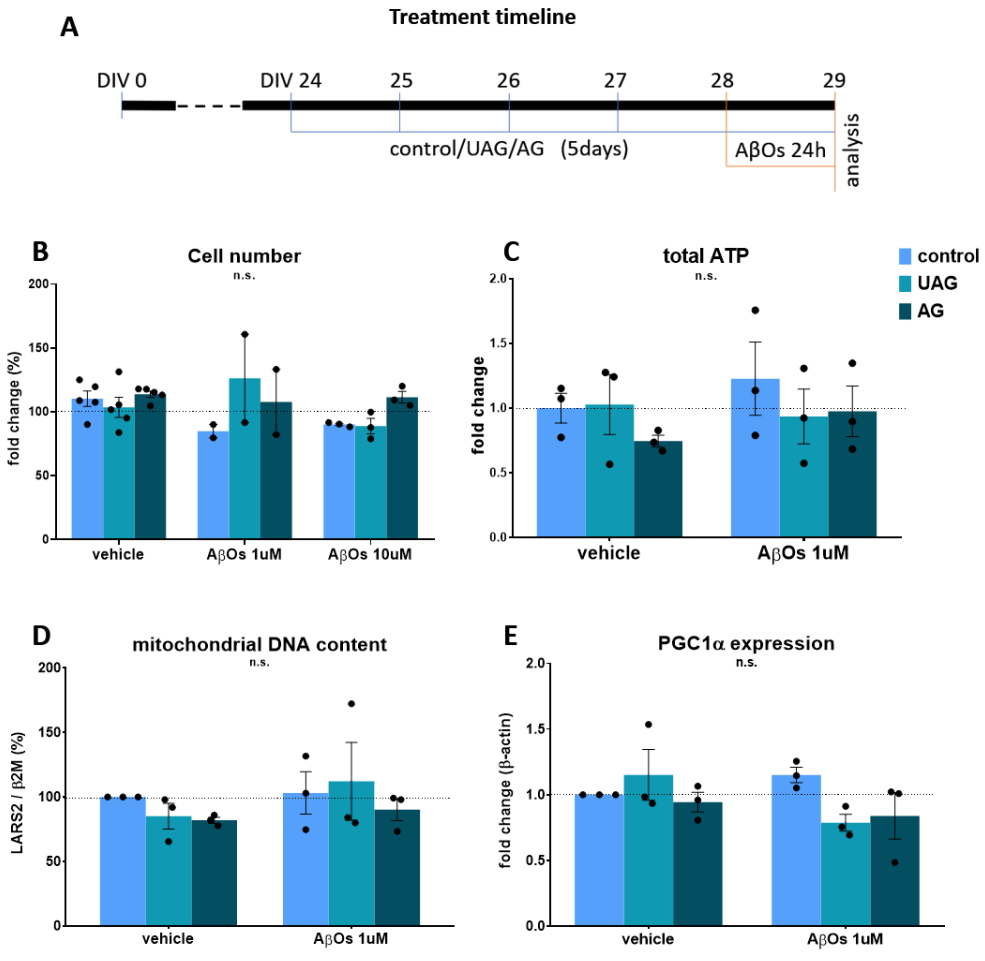
(**A**) The homogeneity of AβOs obtained with the protocol from Klein group, was verified by AFM and compared to preparations obtained using different protocols, such as (**B**) disordered oligomers or (**C**) fibrils. (**D**) ReN neurones (DIV28) treated with AβOs for 24 hours, were assessed for immunopositivity to anti-6E10 antibody. (**E**, **F**) Possible extra-cellular large AβOs aggregations. Scale bar 50um.

ReN neurones treated with vehicle, AβOs 1μM and 10μM, were stained with Hoechst and analysed (figure 5.6 B), as shown previously. In the vehicle treated group (n=5), neither acyl-ghrelin (114 ± 2.44; p>0.9999), nor unacylated ghrelin (103 ± 7.87; p>0.9999) significantly affected cell number compared to control (110 ± 6.01). Similarly, in cells treated with 1μM AβOs (n=2) no significant differences were detected between control (84.8 ± 5.22) and acyl-ghrelin (108 ± 25.5; p=0.9103) or unacylated ghrelin (126 ± 34.5; p=0.3291). Cultures treated with 10μM AβOs (n=3) were not significantly different [control (90.1 ± 0.986); acyl-ghrelin (111 ± 4.49; p=0.8316); unacylated ghrelin (88.8 ± 6.06; p>0.9999)]. When analysing the overall treatment effects, no significant differences were detected between vehicle, AβOs 1μM and 10μM, regardless of AG/UAG treatment (p>0.2), suggesting that these treatment conditions are not detrimental to the cells.

I then investigated total cellular ATP production using CellTiter Glo kit (figure 5.6 C), as shown in chapter 4. No significant differences were detected between any of the groups (n=3; p>0.5), regardless of AG/UAG treatment, suggesting that ATP levels are unaffected by AβOs treatment. [Vehicle group: control (1.00 ± 0.115), acyl-ghrelin (0.745 ± 0.0457), unacylated ghrelin (1.03 ± 0.231); AβOs 1μM group: control (1.23 ± 0.283), acyl-ghrelin (0.976 ± 0.196), unacylated ghrelin (0.936 ± 0.212)].

To further investigate mitochondrial health, I quantified mtDNA copy number in ReN neurones treated with vehicle (n=3) or AβOs 1μM (n=3), and normalised to DNA content (figure 5.6 D and F). No significant differences were detected (p>0.8) between any of the groups, suggesting that AβOs treatment does not affect mitochondria number. [Vehicle group: control (ΔCt=7.60 ± 0.123), acyl-ghrelin (ΔCt=7.31 ± 0.111), unacylated ghrelin (ΔCt=7.35 ± 0.111); AβOs 1μM group: control (ΔCt=7.61 ± 0.193), acyl-ghrelin (ΔCt=7.44 ± 0.187), unacylated ghrelin (ΔCt=7.67 ± 0.382)].

Lastly, I assessed the expression of PGC1α mRNA (figure 5.6 E and F) from neurones treated with vehicle (n=3), AβOs 1μM (n=3), or 10μM (n=3). No significant differences were detected between groups (p>0.5), suggesting that PGC1α gene expression is not affected in these experimental settings. [Vehicle group: control (ΔCt=10.6 ± 0.131), acyl-ghrelin (ΔCt=10.7 ± 0.172), unacylated ghrelin (ΔCt=10.4 ± 0.0963); AβOs 1μM group: control (ΔCt=10.4 ± 0.200), acyl-ghrelin (ΔCt=10.9 ± 0.486), unacylated ghrelin (ΔCt=11.0 ± 0.052)]



F

	Delta Ct					
	Mitochondrial LARS2			PGC1α		
	N1	N2	N3	N1	N2	N3
CTR + V	-7,59459	-7,38931	-7,81637	10,50108	10,86988	10,45508
UAG + V	-7,56455	-7,26876	-7,20568	10,52429	10,2509	10,55328
AG + V	-7,2316	-7,17087	-7,53153	10,81203	10,92863	10,36329
CTR + AβOs1	-7,9912	-7,43072	-7,39643	10,30434	10,79585	10,12728
UAG + AβOs1	-8,37864	-7,06636	-7,56659	11,0285	11,00202	10,86104
AG + AβOs1	-7,14812	-7,37645	-7,78674	11,0285	11,00202	10,86104

Figure 5.6 AβOs affect ReN neurones cell number and mitochondrial network

ReN neurones (DIV24), were treated as shown in the timeline. (**B**) Cell number was quantified using the Cell Profiler software and reported as percentage ± SEM (n=3). (**C**) Total ATP was measured using the CellTiter Glo kit and reported as fold change (n=3). (**D**) Mitochondrial DNA content was quantified by RT-qPCR and normalised per nuclear DNA (n=3) while (**E**) PGC1α mRNA expression was normalised per β-actin (n=3). (**F**) delta Ct values were used for both RT-qPCRs statistical analysis and are reported in the table. Significance was measured by two-way ANOVA and Tukey's multiple comparison test. Data are reported as mean ± SEM. (n.s. not significant).

Similar to rotenone treatment, the experimental settings used in the first part of the experiment failed to report a significant difference between treatments for all the mitochondrial parameters analysed. In order to visualise any significance that may have been undetected, I used MitoTracker to stain ReN neurones treated with either vehicle (n=6), AβOs 1μM (n=3) or 10μM (n=3), as shown in the timeline (figure 5.7 A and B).

First, I measured the total (integrated) intensity of the MMP stain (figure 5.7 C). Using a two-way ANOVA followed by Tukey's multiple comparison test, no significant differences were reported between groups ($p>0.3$). [Vehicle: control (0.966 ± 0.0893), acyl-ghrelin (1.00 ± 0.0830), unacylated ghrelin (0.993 ± 0.132); AβOs 1μM: control (0.696 ± 0.0576), acyl-ghrelin (0.782 ± 0.0561), unacylated ghrelin (0.654 ± 0.0919); AβOs 10μM: control (0.985 ± 0.0416), acyl-ghrelin (0.987 ± 0.0563), unacylated ghrelin (0.917 ± 0.0708)]. Interestingly, a strong treatment effect was detected. The group of cells treated with AβOs 1μM (regardless of AG/UAG) exhibit significantly reduced MMP (integrated intensity) compared to either the control group (**$p=0.0068$) or the 10 μM group (*$p=0.0361$), while no difference was detected between the 10μM and control group ($p=0.9521$). These data show that AβOs treatments may affect mitochondria health differently, according on the peptide concentration, therefore suggesting the existence of multiple regulatory mechanisms.

Subsequently, I analysed the number of puncta per cell reported as fold change (figure 5.7 D) and did not detect any significant changes between the groups ($p>0.4$). [Vehicle treated: control (1.01 ± 0.0439), acyl-ghrelin (1.03 ± 0.0445), unacylated ghrelin (1.03 ± 0.0542); AβOs 1μM treated: control (0.866 ± 0.105), acyl-ghrelin (0.953 ± 0.0271), unacylated ghrelin (0.836 ± 0.0899); AβOs 10μM treated: control (0.982 ± 0.0420), acyl-ghrelin (0.979 ± 0.0507), unacylated ghrelin (0.899 ± 0.101)]. Interestingly, a significant treatment effect was detected, with puncta number being reduced in the AβOs 1μM group compared to control (*$p=0.0299$), but not to the 10μM ($p=0.3823$), nor between 1 and 10μM AβOs ($p=0.4739$). This data confirms the previous evidence that AβOs 1μM treatment affects mitochondrial network, and that this effect is somehow abrogated at 10μM, suggesting a potential compensatory mechanism.

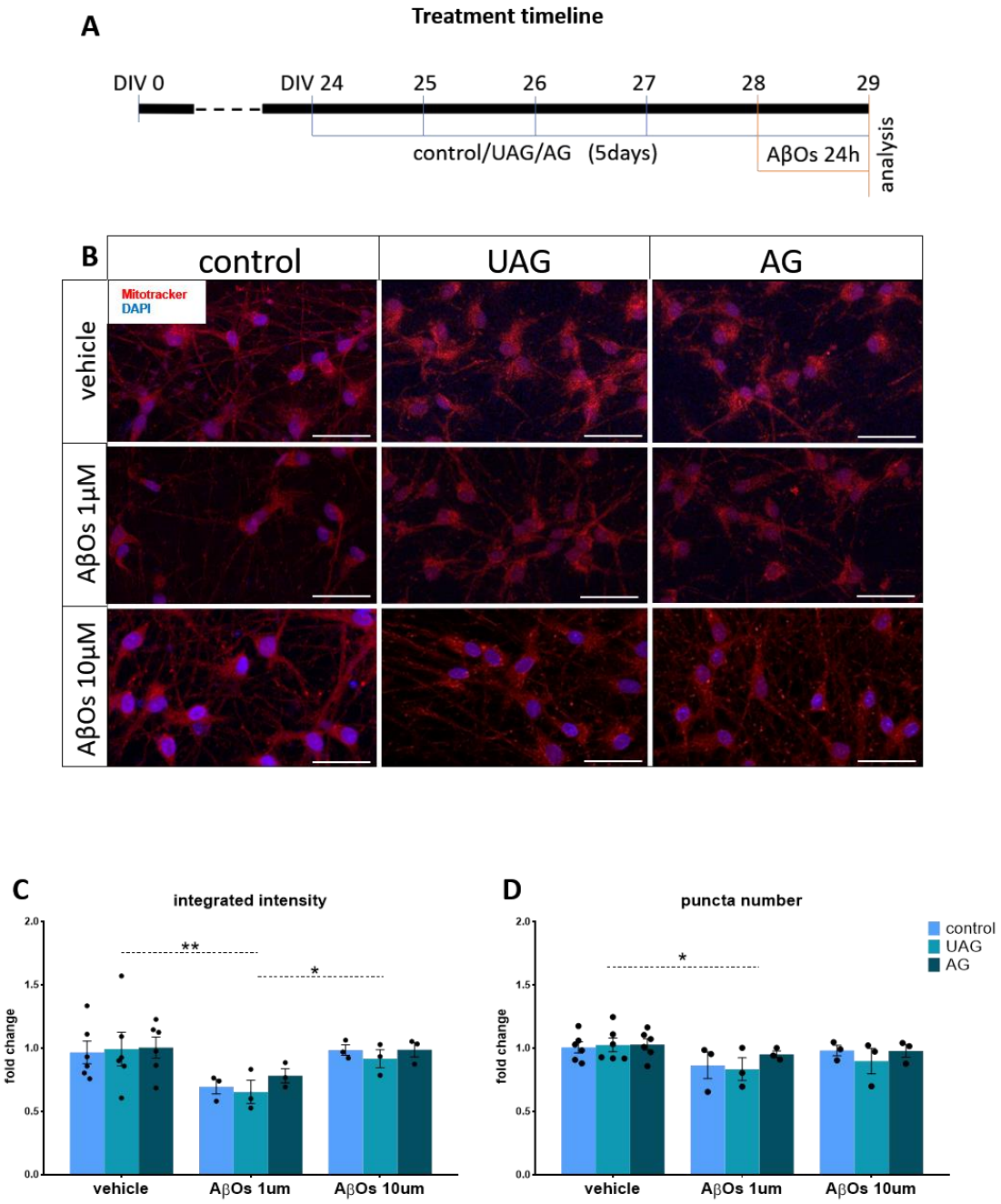


Figure 5.7 Mitochondrial measurements in AβOs treated cells

(**A**) ReN neurones (DIV24) were treated as shown in the timeline. (**B**) Cells were stained with MitoTracker and Hoechst nuclear counterstain (blue). Mitochondrial "puncta" were identified using Cell Profiler software. (**C**) Total (integrated) intensity and (**D**) puncta number were measured and reported as mean ± SEM (n=3; vehicle n=6). Significance was measured by two-way ANOVA and Tukey's multiple comparison test. (n.s. not significant; **p≤0.005; *p≤0.05).

The SuperPlot graph was used to present the single cell data (n≥222), as shown in chapter 4. Data distribution was found to be non-parametric after D'Agostino-Pearson's normality test; hence, analysis was performed using Kruskal-Wallis and Dunn's multiple comparison test on the expanded dataset. Using these settings, I detected a significant reduction of the MMP (figure 5.8 A) between each treatment of the vehicle group (control:0.926 ± 0.0148, n=1071; unacylated ghrelin:0.925 ± 0.0142, n=1247; acyl-ghrelin:0.998 ± 0.0153, n=1065) and the corresponding treatments of the AβOs 1μM group (control:0.720 ± 0.0152, n=498, ****p<0.0001; unacylated ghrelin:0.685 ± 0.0151, n=492, ****p<0.0001; acyl-ghrelin:0.778 ± 0.0146, n=550, ****p <0.0001) (dashed lines). Similarly, the AβOs 1μM group was significantly reduced compared to the corresponding 10μM treatment (control:0.967 ± 0.0289, n=248, ****p<0.0001; unacylated ghrelin:0.885 ± 0.0330, n=222, ****p<0.0001; acyl-ghrelin:0.950 ± 0.0260, n=260, ****p<0.0001) (dotted lines), consistently with the treatment effect detected earlier (figure 5.7 C). On the contrary, no difference was detected between vehicle and AβOs 10μM (p>0.5), reinforcing the hypothesis that 1μM and 10μM doses exert different effects on mitochondria. Acyl-ghrelin significantly increased the MMP in the vehicle group, compared to control (**p=0.0024) and unacylated ghrelin (****p<0.0001), and in the 1μM groups compared to unacylated ghrelin (***p=0.0004). No other significant differences were detected within the 1μM nor the 10μM treatment.

Subsequently, I analysed puncta number per cell (figure 5.8 B). In the vehicle group, neither unacylated ghrelin (1.03 ± 0.014; n=1247; p>0.9999), nor acyl-ghrelin (1.07 ± 0.0161; n=1065; p=0.1668) significantly affected puncta number compared to control (0.997 ± 0.0147; n=1071). However, all three treatments were significantly higher compared to the corresponding treatments in the AβOs 1μM group [control (0.909 ± 0.0201; n=498; **p=0.0025), unacylated ghrelin (0.872 ± 0.0193; n=492; ****p<0.0001) and acyl-ghrelin (0.951 ± 0.0174; n=550; **p=0.0011)], supporting the treatment effect reported previously (figure 5.7 B). Interestingly, within the 1μM treatment group, acyl-ghrelin significantly increases puncta number compared to unacylated ghrelin (*p=0.0355. Lastly, no significant difference (p>0.2) was detected between any of the treatments in the 10μM group [control (0.975 ± 0.0274; n=317), unacylated ghrelin (0.866 ± 0.0309; n=291) and acyl-ghrelin (0.970 ± 0.0257; n=328)], reinforcing the hypothesis of a dose-dependent effect of AβOs treatments.

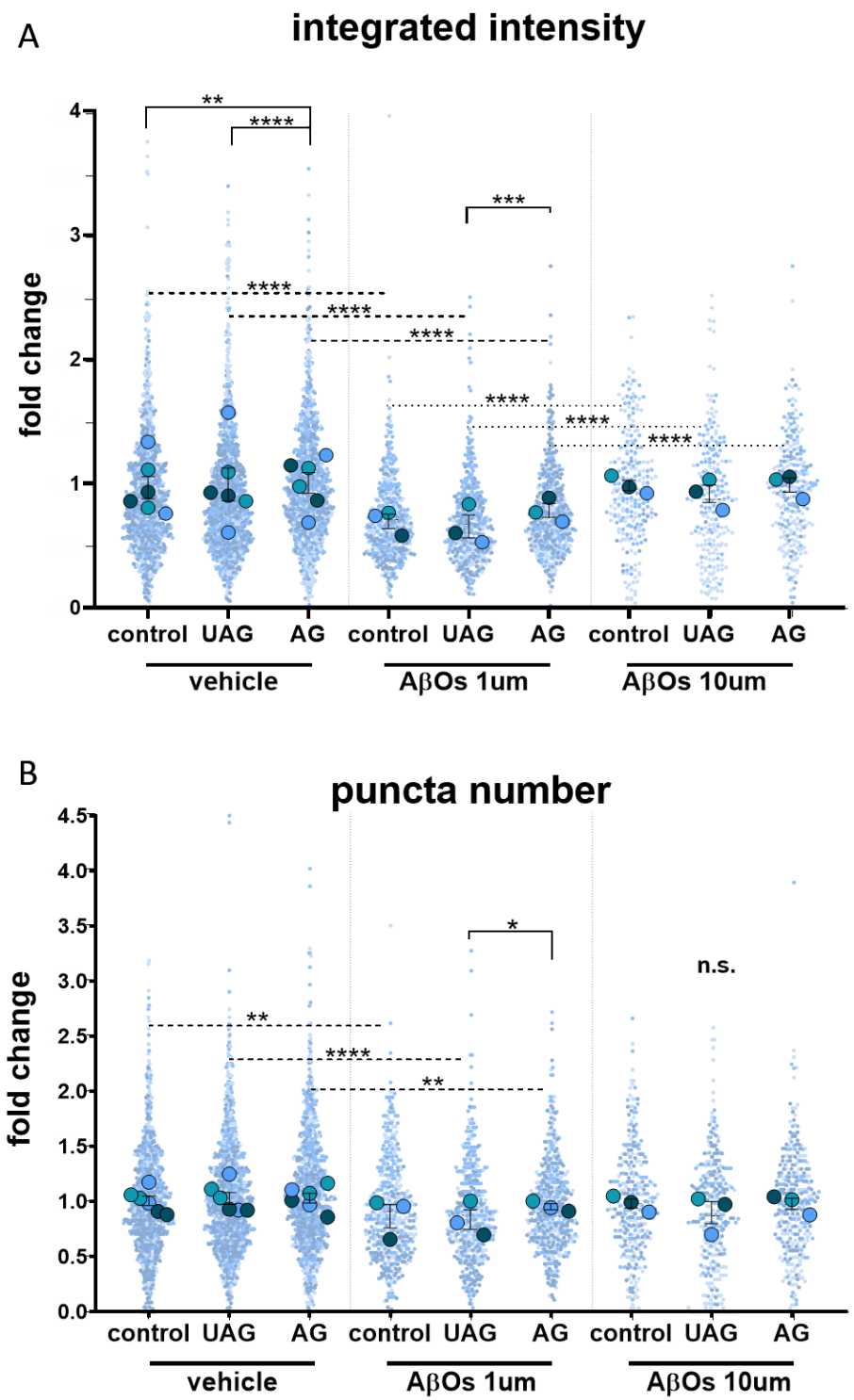


Figure 5.8 Single cells mitochondrial analysis in AβOs treated ReN neurones

(**A**) Mitochondria integrated intensity and (**B**) puncta number are shown using a SuperPlot graph, combining the mean ± SEM from figure 5.7 (n=3; vehicle n=6) and the corresponding values per single cells (n≥222). Each large dot represents a group mean, and each colour-coded small dot represent individual cells. Statistical analysis was performed with Kruskal-Wallis and Dunn's multiple comparison on the single cell's values. (****p<0.0001; ***p≤0.0005; **p≤0.005; *p≤0.05).

5.4 Discussion

Memory loss and cognitive decline are the most typical symptoms of AD. CR has been shown to improve memory performance in elderly humans and to rescue memory loss in transgenic AD models, as shown in section 1.1.2. Notably, acyl-ghrelin and ghrelin agonists have been shown to improve AD-related symptoms and pathogenesis in several mice model, reducing tau hyperphosphorylation (Chen et al. 2010), rescuing cell loss by inducing AHN (Moon et al. 2014), reducing Aβ plaques and preventing synaptic loss and tissue inflammation (Santos et al. 2017; Jeong et al. 2018). Most importantly, acyl-ghrelin has been shown to significantly rescue cognitive function and memory impairment (Kang et al. 2015; Kunath et al. 2015; Santos et al. 2017; Eslami et al. 2018). Despite the growing number of evidence linking acyl-ghrelin axis to neuroprotection *in vitro* and *in vivo*, its role in the human brain has been poorly considered; hence, to the best of my knowledge this is the first study investigating the ghrelinergic system enzymes in the AD brain.

5.4.1 GOAT, APT1, GHS-R1a and total ghrelin

My experiments show no significant difference of GOAT protein expression in the hippocampus, whilst MBOAT4 mRNA level was significantly increased in late AD patients compared to age-matched healthy controls (figure 5.3, **p=0.0058). Consistently, $GOAT^+$ cells in the DG of late AD patients were approximately 44% higher than control, although this value did not reach statistical significance (figure 5.3 B, p=0.2328); moreover, no changes in GOAT protein level were detectable in whole-hippocampus tissue lysates (figure 5.3 D and E).

A poor correlation between mRNA and protein expression may have the most diverse causes, such as post-translational modification, ribosome occupancy, protein degradation, and more. However, there are several factors that are known to specifically upregulate MBOAT4 mRNA, such as CR (Hatef et al. 2015) or acyl-ghrelin administration (Gahete et al. 2010b). Interestingly, a recent study showed that MBOAT4 gene may also be transactivated by mTOR, via the gene enhancer ERM transcription factor variant 5 (ETV5), in murine GOAT-producing cells (Mao et al. 2019). mTOR is traditionally considered an endogenous antagonist of the pAMPK pathway; indeed, pAMPK inhibits mTOR signalling by phosphorylating its deactivator Raptor (section 1.2.9), while mTOR pathway is responsible for the

leptin-induced post-prandial inhibition in AMPK phosphorylation (Dagon et al. 2012). In animal models of AD, upregulation of mTOR signaling has been shown to drive the pathological progression of the disease, while several mTOR inhibitors, including rapamycin, rescue the cognitive deficits (Spilman et al. 2010; Wang et al. 2014a). In light of this, the upregulation of MBOAT4 in late AD patients reported in this thesis, may be associated to increased mTOR signalling and may serve as compensatory mechanism against oxidative stress and metabolic impairment. Indeed, since GOAT can re-acylate ghrelin *in situ* (section 1.4.4), its overexpression could potentially increase acyl-ghrelin tenor in the hippocampus of these patients. Nevertheless, more studies are needed to characterise the role of GOAT in memory and cognition; for instance, animal models of AD could be treated with either GOAT inhibitors or agonists, to study how it may affect memory. Another approach could be to use GOAT KO mice (Hornsby et al. 2020) treated with Aβ or other toxins to simulate AD, to determine the role of the enzyme in the pathogenesis of the disease. Subsequently, animals could be treated with acylated or unacylated ghrelin, to investigate whether the treatment may potentially rescue the effect.

In my data, APT1 expression was not significantly different in early or late AD, compared to age-matched healthy subjects. Although, LYPLA1 in late AD showed a slight increase close to significance (+p=0.0660), this trend did not match a change in protein levels and suggests that APT1 is not impaired in the brain of AD subjects. Consistently, neither GHS-R1a mRNA, nor total ghrelin protein expressions were found altered in the brain of AD patients.

Overall, these data are consistent with previous reports showing that ghrelin levels are not impaired in the plasma of AD patients (Proto et al. 2006), however, they seem to stand against the hypothesis previously postulated (chapter 4), that GOAT level correlates with dementia in the brain of patients affected with PD and PDD. This apparent contradiction is easily explained when looking at the main characteristics of PD and AD. Indeed, despite the numerous shared molecular and pathological pathways, the two diseases affect different areas of the brain. More specifically, even if both diseases are associated with memory loss and cognitive impairment, the specific symptoms are different enough to suggest the activation of diverse molecular mechanisms, as will be better described in section 6.

5.4.2 Mitochondrial health

The second aim of this study was to induce Aβ toxicity and to characterise the role of ghrelin in maintaining cell homeostasis *in vitro*. AβOs were produced as described in section 2, and their internalisation in ReN neurones was successfully verified 24 hours after treatment (figure 5.5). Then, ReN neurones were treated as shown in figure 5.6, and analysed to assess mitochondrial health.

Analogously to what seen in chapter 4, *in vitro* results from this section are contradictory. 1μM AβOs treatment negatively affected both MMP and puncta number, while the 10μM treatment did not induce any change in any of the mitochondrial aspects analysed in this chapter. The reason for that may be found in the technical conditions of the experiments. As previously mentioned, AβOs are quite challenging to prepare, because of the high self-aggregation tendency when in contact with aqueous solutions. Indeed, the last step of the AβOs preparation, requires for the compounds to rest at 4^{o}C for 24 hours, in a very small volume of cell media. As the 10μM preparation is highly concentrated, it is plausible that, in this last step, some of the amyloid peptide may have formed small proto-fibrils or fibrils in solution; in this scenario, some of the peptide could have been 'sequestered' inside the fibrils, hence the final concentration of the oligomers could have been significantly lower than expected.

Moreover, as previously mentioned, fibrils have been shown to be less toxic than AβOs both *in vivo* (He et al. 2012) and *in vitro* (Nimmrich et al. 2008), therefore it is possible that 24 hours treatment with the oligomer/fibril mix, may have not been sufficient to exert any detectable effect.

Furthermore, since the cells have been growing as a monolayer, any proto-fibril or fibril that could have formed, would have then been washed away during the fixation steps at the end of the treatment, hence remaining undetected. To test this hypothesis, future studies should use live confocal microscopy to investigate AβOs internalisation in ReN neurones, and more specifically whether the peptides would be able to directly interact with mitochondria.

Lastly, the protocol elaborated from Dr. Klein's group was originally designed for low concentration-treatments (100-500nM); although I carefully calculated each quantity and volume in a way that would have maintained the concentration of each solution consistent with the original protocol, it is possible that the presence

of a more concentrated amount of peptide may have negatively affected the oligomerisation process. Hence, future studies should better characterise AβOs at different concentrations, and design experiments that would utilise less concentrated peptide for longer incubation periods.

Notably, the analysis of the extended mitochondria dataset (figure 5.8) reported that unacylated ghrelin exerts a detrimental effect on both MMP and puncta number, while acyl-ghrelin produces a statistically significant counteracting effect. Interestingly neither unacylated nor acylated ghrelin exerted any effect in the group treated with 10μM AβOs, and the overall observed effects were in general less evident than the one produced in chapter 4 (figure 4,7). For this reason, I did not perform any further analysis on the puncta distribution.

Overall, these data suggest a scarce reproducibility of the experimental conditions used in this study. To overcome these technical issues and obtain more consistent results in the future, a different cell type may be adopted such as the SH-SY5Y, for which decades of previous researches have contributed establishing a vast number of standardised protocols.

5.4.3 Conclusions

In summary, this chapter shows that some components of the ghrelinergic system may be affected in the hippocampus of late AD patients, although the data are circumstantial and will need further studies. ReN neurones did not recapitulated AD mitochondrial stress *in vitro*, however the data confirmed that unacylated ghrelin exerts a detrimental activity on mitochondrial health, to which acyl-ghrelin display an opposite effect.

5.5 Key findings

- GOAT mRNA expression is significantly increased in the hippocampus of late AD patients, whilst protein level is not affected (figure 5.2).

- APT1 mRNA and protein, GHS-R1a mRNA and total ghrelin protein expression levels do not vary in the hippocampus of early AD, nor late AD patients (figure 5.3 and 5.4).

- Orderly-structured AβOs can be successfully produced using Dr. Klein's protocol and are internalised by ReN neurones *in vitro* (figure 5.5).
- 1μM, but not 10μM AβOs, affects MMP and puncta number independently of ghrelin treatment (figure 5.7 and 5.8).
- Acylated and unacylated ghrelin exert opposite functions on mitochondrial health, with unacylated ghrelin acting detrimentally towards both MMP and puncta number.

Chapter 6

General discussion and conclusions

6.1 Discussion

6.1.1 Neurodegenerative diseases and the ghrelinergic system

In the past fifty years, age-related neurodegenerative diseases have become one of largest health care problems in the developed countries. The available therapeutic treatments significantly improve survival rate and life quality, so patients can have a near-normal life expectancy; however, since the physical and mental deteriorations cannot be stopped, with time patients become gradually more dependent on others, increasing the economic burden on the healthcare system and patient families, calling for a quick identification of a new, definitive cure.

PD and AD are the two most common neurodegenerative disorders and, together, represent most of the cases of dementia in the world. PD affects DA neurones of the SN and the striatum, hence is mostly associated to motor impairment, tremors, and postural instability (chapter 4). AD mostly affects the cholinergic system in the hippocampus and entorhinal cortex, and is responsible for gradual memory loss, also affecting the capability of forming of new ones (chapter 5). The two diseases are likely to have different causes – or possibly different combinations of similar factors – and this is especially evident from the diverse brain areas involved. However, these two pathologies share some common ground; they are both characterised by age-related, irreversible, accumulation of intracellular damage, such as misfolded proteins and oxidative stress (reviewed in Umeno et al. 2017). At the molecular level, both diseases exhibit ER stress (Ho et al. 2012; Mazzulli et al. 2016), ALP and UPS failure (Bates et al. 2009; Ebrahimi-Fakhari et al. 2011), inflammation and microglia activation (Bartels et al. 2020), and mitochondrial

impairment (Schapira et al. 1990; Bates et al. 2009) (figures 4.1 and 5.1); indeed, in both pathologies oxidative stress and impaired energy metabolism affect neuronal plasticity and cognition, ultimately resulting in failure of the quality control systems and accumulation of damage in the brain.

There are also numerous biochemical players that are known to intervene in both diseases: Aβ plaques accumulate in the brain of PD patients and trigger inflammation (Masliah et al. 2001); p-tau co-localises with α-synuclein in Lewy bodies (Arima et al. 1999) and α-synuclein accumulates in the brain of AD patients (Larson et al. 2012). Moreover, although PD was originally thought to be a motor disease, it is now more evident that non-motor deficits are prominent features. *Post-mortem* brain tissue of patients affected by PD, exhibit extensive cell loss in the hippocampus and the entorhinal cortex (Hu et al. 2001) and memory impairment affects 67% of patients with PD at a later stage (Portin and Rinne 1987; Aarsland et al. 2003; Noe et al. 2004; Hely et al. 2005) – thereby renamed PD with dementia, PDD (Dubois et al. 2007; Emre et al. 2007). Memory loss in PDD is less severe than in AD (Paolo et al. 1995; Aarsland et al. 2003) and it is generally agreed that memory deficit in PDD affects memory retrieval, rather than encoding and storage as typically seen in AD. Nonetheless, PDD patients exhibit impairment in object recognition, non-verbal memory and visual recognition memory, comparable to what observed in subjects with AD (Noe et al. 2004); verbal fluency and concept formation are also compromised (Paolo et al. 1995; Starkstem et al. 1996; Aarsland et al. 2003; Noe et al. 2004). Lastly, spatial memory is impaired (Starkstem et al. 1996), but in PD this is also associated to a worse perception of the environment, therefore suggesting a distinct mechanism to AD (Mosimann et al. 2004).

Several therapeutic approaches that have been clinically assessed in rodents for one disease, have also proven effective against other disease models; this is the case of the AMPK activators Resveratrol (Guo et al. 2013), Metformin (Rotermund et al. 2018), and CR (Srivastava and C. Haigis 2011). Indeed, CR has been extensively reported to extend longevity in rodents and non-human primates, and to prevent age-associated cellular stress, by activating AMPK pathway and reducing mitochondrial impairment (section 1.1.2).

Acyl-ghrelin is the main mediator of the beneficial effects of CR in the brain, acting as a feedback signal of negative energy balance and contributing to maintaining physiological and neurological function during low energy state (section 1.2.2).
This thesis was designed to compensate for the lack of studies about the ghrelinergic system in the human brain, especially in the context of neurodegenerative diseases. Therefore, I investigated the expression of key regulatory proteins in *post-mortem* brain tissue from PD and AD subjects, and the effects of ghrelin on mitochondria in human neurones.

6.1.2 GOAT and APT1 are affected in the diseased brain

The experiments performed in this thesis showed that the three main proteins of the ghrelinergic system – GOAT, APT1 and GHS-R1a – are expressed in the human brain and their levels are unchanged at both protein and mRNA level in the hippocampus of aged healthy subjects (chapter 3).
However, GOAT protein expression is significantly reduced in the hippocampus of PDD patients (chapter 4), suggesting a reduced ability to produce acyl-ghrelin in the brain. Indeed, our group recently reported that circulating AG:UAG is reduced in the plasma of PDD patients, and correlates significantly with the cognitive ability of the patients, measured by the MoCA score (Hornsby et al. 2020). These data, in combination with my findings, point to the possibility that the impairment of the ghrelinergic system may have a role in exacerbating cognitive dysfunction in these patients.
Late AD patients also show prominent cognitive impairment, however in our investigations MBOAT mRNA levels were significantly increased in these patients, compared to early AD and controls (chapter 5). The discrepancy between late AD and PDD patients – suggesting that the ghrelinergic system is differently regulated in those two diseases – does not come entirely as a surprise since AD and PD have very distinct pathogenesis, despite them sharing some characteristics. Interestingly, the increase observed at mRNA level did not match with an increase in protein levels, probably due a general impairment of the protein translation machinery, as described in chapter 5.
To my knowledge, the ratio between acylated and unacylated ghrelin has never been measured in the plasma of AD patients – despite total ghrelin levels having been reported to be unaffected (Proto et al. 2006). If AG:UAG ratio is found to be

impaired in late AD – the same way we found it impaired in PDD (Hornsby et al. 2020) – this could explain the increase in MBOAT mRNA levels, aiming at compensating the lack of acylated-ghrelin. Future studies should investigate the ghrelinergic system in AD patients at different stages of the disease, in order to confirm or refute this hypothesis and shed further light on the role of acyl-ghrelin in dementia and cognitive impairment.

Another key element that is missing from our knowledge of the ghrelinergic system in the human brain, is which cell type express the ghrelin-related enzymes and whether this may change with age or during disease progression. For instance, as mentioned in chapter 3, the newly found localisation of APT1 in the cells of the BCSFB could point to a local regulation of acyl-ghrelin access to the brain. Similarly, since GOAT has been found to re-acylate ghrelin *in situ* (Hopkins et al. 2017; Murtuza and Isokawa 2018), it is of high priority to investigate its activity and role in determining acyl-ghrelin availability in the brain.
Interestingly, full length monomeric and dimeric GOAT was detected in the circulation of fasted rodents (Stengel et al. 2010), and in human plasma (Goebel-Stengel et al. 2013), suggesting that the enzyme may be able to re-acylate ghrelin in the circulation. Future studies should investigate the transport mechanism of GOAT enzyme in plasma, since transmembrane proteins would evidently need a specific delivery system, such as albumin or small vesicles. Moreover, several ELISA kits are commercially available, that could allow precise measurement of GOAT protein level in the plasma of patients affected with PD and AD. Lastly, in order to determine whether circulating GOAT extracted from patient's plasma is fully functional, its enzymatic activity should be investigated for instance using the enzyme-linked click-chemistry (cat-ELCCA), a fluorescent assay that was previously used to assess GOAT activity *in vitro* (Garner and Janda 2010).

Opposite to GOAT, both LYPLA1 mRNA and APT1 protein levels were found to be unaffected in our investigations. Since ghrelin is the only substrate of GOAT (Darling et al. 2015) and GOAT is the only acylating enzyme of ghrelin (Gutierrez et al. 2008; Kirchner et al. 2009), an impairment in GOAT expression may be directly connected to defects in the ghrelinergic axis. On the contrary, APT1 shows very little specificity for its ligands, therefore a variation in its expression and/or

activity, may involve a much greater number of systems. For instance, APT1 is responsible for the de-palmitoylation of PSD-95, an important synaptic protein implicated in neurodegenerative disease (Virlogeux et al. 2021). Hence, our findings that APT1 were not affected in the diseases brain, do not undermine the original hypothesis of a ghrelinergic system impairment.

Notably, numerous enzymes have been shown to deacylate ghrelin, such as APT2 (Satou et al. 2010), α2-macroglobulin (Eubanks et al. 2011), butyl-cholinesterase (BChE) (Schopfer et al. 2015), and notum (Zhao et al. 2021), while the involvement of others, like the platelet activating factor (PAF), has been hypothesised (Shanado et al. 2004). Satou et al. showed that both APT1 and APT2 can catalyse acyl-ghrelin deacylation in a ghrelin-expressing cell line (Satou et al. 2010). This study also reported that APT1 was profusely present in the cell supernatant, while APT2 was not detectable, suggesting that APT1 may be released into the circulation. Based on these evidences, the authors suggested that APT1 deacylating ability *in vivo* is more relevant than that of APT2 – since APT1 would have access to the circulating hormone, that is indeed the largest percentage of the hormone in the body. A few years later, others advocated a role for both enzymes in ghrelin deacylation (Won et al. 2018), although this hypothesis was never experimentally confirmed. Notably, the original authors later claimed that APT2 is not able to deacylate ghrelin at all (Satou et al. 2011), although, to the best of my knowledge, no data is available to either support or refute this hypothesis.

The only other research published on the matter, from De Vriese and colleagues, suggested that ghrelin deacylation is mediated by distinct enzymes in rodents compared to humans (De Vriese et al. 2004). In particular, they showed that ghrelin deacylation was significantly reduced by a pan-serine protease inhibitor, in a ghrelin-producing human cell line; interestingly, both APT1 and BChE belong to the same group of enzymes that would have been inhibited in this experiment. However, the study did not mention APT1; the authors hypothesised that – among the many enzymes inhibited in the assay – BChE may be the main one involved in ghrelin deacylation. In order to confirm this theory, they treated the same cell line with purified human BChE and observed a significant increase of ghrelin deacylation, proportional to the dose of the enzyme used. These results were later replicated in other studies (De Vriese et al. 2007; Dantas et al. 2011); yet, none of

these researches ever reported the total inhibition of ghrelin deacylation process, suggesting that it may occur via a combination of enzymes.
Nevertheless, BChE is an interesting enzyme, since it increases during AD and BChE inhibitors have been tested for several decades on numerous animal models of the disease (Darvesh 2016; Macdonald et al. 2017). Indeed, as mentioned in chapter 5, one of the main biochemical changes that occur in the brain of AD patients, is the loss of cholinergic innervation in the cortex, and BChE is mostly known for its activity in degrading excess Ach (Li et al. 2000). Two BChE inhibitors, rivastigmine and galantamine, originally developed against mild cognitive impairment in early AD, are now routinely administered in PDD patients and in subjects with other forms of memory impairment (Tricco et al. 2013).
Ultimately, more studies are needed to characterise the enzymes mentioned above, the extent of their involvement in ghrelin de-acylation process, and their expression in the healthy and diseases human brain.

Ghrelin acylation is essential for its activity and binding with GHS-R1a, as described in section 1.2.8. Indeed, stabilization of the acyl-group – hence preventing the conversion of acyl-ghrelin to unacylated ghrelin – in order to increase its central bioavailability, could represent a potential therapeutic approach to improve memory and cognition. A pharmaceutical strategy to achieve this, could be to use compounds preventing its deacylation. For instance, since APT1 has been shown to be involved in some cancer types (Wang et al. 2015b), small inhibitory compounds have been extensively used in cancer research. Moreover, with the availability of the APT1 and APT2 crystal structures (Devedjiev et al. 2000; Won et al. 2017), different studies have been focusing on synthetising more effective and well-tolerated small molecules that could effectively reduce APT1 de-acylation activity in the brain. One of this compounds, Palmostatin B, that inhibits both APT1 and 2, has been tested for neuroprotective effects in rodent models of neurodegenerative disease (Dekker et al. 2010), confirming the potential therapeutic relevance of repristinating AG:UAG ratio by inhibiting its de-acylation.

6.1.3 The human hippocampus is receptive to acyl-ghrelin signalling in ageing and diseases

GHS-R1a expression is intact in the brain of aged healthy subjects, as well as in both PD (chapter 4) and AD patients (chapter 5), regardless of the progression of the disease. These data are very promising, since the presence of a ghrelin-receptive system would favour any therapeutical strategy aimed at increasing the ghrelinergic tenor in the brain.

However, GHS-R gene originates two variants of the receptor (section 1.2.8, figure 1.3). The less known isoform, GHS-R1b, lacks the intracellular sequence: although it can still bind acyl-ghrelin, it is not able to induce any intracellular signalling pathways. Yet, GHS-R1b has been shown to interfere with GHS-R1a activity via several mechanisms, such as acting as a dominant negative and sequestrating acyl-ghrelin from the extracellular space (Leung et al. 2007), increasing GHS-R1a internalization (Chow et al. 2012), or dimerising with GHS-R1a and directly modulating its signal transduction (Mary et al. 2013; Navarro et al. 2016). Notably, GHS-R1b mRNA is overexpressed in diseased human tissue, for instance in certain tumours (Arnaldi et al. 2003; Barzon et al. 2005), suggesting a disease-specific regulation of the ghrelinergic system.

Although my data show GHS-R1a mRNA expression to be unchanged with ageing and in diseases, it cannot be excluded that variation of GHS-R1b expression (either increase or reduction) may still influence acyl-ghrelin signalling in the aged brain. It is of high priority to identify a strategy aimed at accurately quantify GHS-R1b in the human brain.

GHS-R1b originates from alternative splicing, in which the splice site between exon1 and intron1 is skipped and a short intronic sequence is included in the final transcript – before an early stop codon terminates transcription. Because of this, targeting the mRNA sequence of the 1a isoform is easy, as long as the oligonucleotide probe is designed to recognise the sequence spanning the exon1-exon2 region (see section 1.2.8, figure 1.3, yellow boxes). However, to detect the mRNA of the 1b isoform, the probe should be designed to recognise the exon1-intron sequence, but this region is not exclusively present in GHS-R1b transcript. Indeed, the GHS-R mRNA precursors will also include the exon1-intron segment in

their sequence. Consequently, any probe that would be able to recognise GHS-R1b mRNA, would also target any other transcript originating from the GHSR gene, except for the GHS-R1a. For this reason, it is not possible to specifically identify GHS-R1b mRNA presence and activity in the human brain tissue. On the contrary, to investigate GHS-R1b protein, there are few commercially available antibodies that specifically detect the short intronic sequence typical of the 1b splice variant. However, the close similarity between the 1a and 1b isoform – that share 267 amino acids, and only differ for a short 24-amino acid-long sequence – significantly increase the chance of cross-reaction with the other variant, therefore reducing the specificity of the antibody.

6.1.4 Antibody limitations in the staining of human tissues

The use of antibody-based protein-staining in human tissue can be quite challenging. The first problem is that all proteins have both a 2D and a 3D structure. The term "2D structure" refers to the sequence of consecutive amino acids, corresponding to the codon sequences in the original mRNAs. This structure is very rarely detectable inside the cell; indeed, during protein translation, the newly formed proteins, emerging from the ribosomes, are immediately bound to specific chaperon enzymes, and quickly folded into what will ultimately represent their functional "3D" structure. Some commercially available antibodies are able to detect both the native (2D) and the final (3D) protein structures; however, some of them can only detect one of the two forms. This is the case of the two anti-GOAT antibodies used in this thesis (reported in section 2, table 8): one is specifically able to detect the 3D protein structure – by binding amino acids that are present on different part of the native proteins, but are adjacent after protein folding – and therefore can be only used to detect *in situ* immunoreactivity (i.e., immunohistochemistry), while the other antibody only recognises the 2D sequence, therefore can only be used with those techniques that require protein denaturation (unfolding) (i.e., Western blot).

The second problem is antibody specificity against its target. Indeed, proteins that have multiple isoforms (mostly splicing variants), such as GHS-R and ghrelin, are more likely to be erroneously detected. Our group has previously shown that one anti-GHS-R1a antibody commercially available is not specific for the 1a isoform, as claimed, since its immunoreactivity is unaffected in GHS-R-ablated mice (Ratcliff et

al. 2019). Interestingly, a new antibody has been recently produced and commercialised, that seem to show increased specificity against the 1a isoform, although more studies will be needed to confirm its efficiency. Similarly, it has been extensively reported that the most used anti-ghrelin antibodies cannot distinguish between the numerous splice variants produced from the GHRL gene (Wortley et al. 2004; Furness et al. 2011). Accordingly, in this thesis neither anti-GHSR1a nor anti-ghrelin antibodies have been used in immunohistochemistry; however, both antibodies have been used in Western blot, because this technique provide an additional checkpoint, the protein molecular weight, that should reduce the chance of quantifying non-specific proteins.

6.1.5 Acyl-ghrelin and unacylated ghrelin have opposite effects on rotenone-induced mitochondrial health *in vitro*

The second aim of this study, was to characterise the mechanisms *in vitro* of acyl-ghrelin neuroprotection, using human stem cell-derived mature neurones. More specifically, I pre-treated ReN neurones with acylated or unacylated ghrelin for 5 days, then treated them for 24h with either rotenone or AβOs. Finally, I quantified cell number, total ATP levels, mitochondrial DNA content and other mitochondrial health markers such as PGC1α mRNA expression, MMP and mitochondria "puncta" number. My data showed a very low sensitivity of ReN neurones to the stressor used, since a treatment effect between 1 and 10μM doses was barely detectable. When analysing the extended dataset (n>220) some effects started to appear more clearly. First of all, in both rotenone and AβOs treatment, 1μM and 10μM exert very different effects in mitochondria health. Moreover, the extended group showed a large spread of the data, suggesting there is a lot of variability in the same experiment.

In order to better characterise the precise molecular mechanisms – and to exclude the possibility that these data only show random fluctuations – future study should combine live microscopy with mitochondrial tracking. Moreover, diverse mitochondrial markers, such as TOM20 or DPR1, or assays, such as Seahorse, should be combined to obtain more relatable data. Another possible strategy would consist in increasing acylated and unacylated ghrelin concentration, extend the

timeline of the experiments, or even inhibit serine hydrolases to prevent acyl ghrelin from being prematurely degraded in the media.

An interesting element from the experiments in this thesis, is that acyl- and unacylated ghrelin treatment seems to consistently exert an opposite effect on mitochondrial health. Future studies should investigate whether the double treatment AG+UAG may rescue unacylated ghrelin detrimental effect on mitochondria health. However, since ReN neurones express both protein and mRNA of APT1, as well as GOAT mRNA (figure 3.7), these data need to be considered cautiously. Indeed, GOAT and APT1 – if functioning – may interfere with acylated and unacylated ghrelin concentration and influence the outcome of the experiment. Furthermore, although GHS-R1a mRNA was present on ReN neurones at DIV 22-24 (figure 3.8), I could not investigate its protein level nor its activity.

Notably, a number of technical noises may also have contributed to such variability. For instance, DMSO and other chemicals commonly used to prepare and dissolve reagents may have an effect by themselves, altering cellular response and affecting the final data (Sanmartín-Suárez et al. 2011). Moreover, Phenol red, one of the main components of the cell media, has been shown to interact with Aβ at high concentration (Wu et al. 2006), hence it could reduce the efficacy of the treatment. Indeed, both DMSO and Phenol red were also present in the vehicle treatment, therefore it was not possible to determine whether they may have influenced the culture. Although we can exclude that neither DMSO nor Phenol red may be responsible for the variations detected between the well – since both components were present in all the wells – it still cannot be ruled out that they may have influenced the ability of the cells to respond to the stressors. Further studies should determine in advance the best media condition in order to exclude any interference with the treatments.

For what concern the disease model, several groups reported that longer treatments at lower doses better recapitulate the complexity of these diseases. For instance, chronic low-dosage of rotenone treatments in cells, are able to induce the formation of α-synuclein intracellular occlusions and neuritic changes, such as synapse loss, hence better recapitulating the molecular features of PD (Borland et

al. 2008). Since my aim was to induce mild oxidative stress to simulate the early stage of the diseases, I did not consider longer treatments in my experimental settings. However, future studies should determine whether incorporating longer treatments may be more appropriate for investigating the ghrelinergic axis in this context.

Lastly, human stem-cell neurones, although being the closest systems currently available to model neurodegenerative diseases, have flaws in their capability of recapitulating the complexity of the adult human brain, whose maturation occurs through several decades. One of the main issues is generating authentically "aged" neuronal cells *in vitro*, since primary cells would have accumulated unique features over several decades, such as epigenetic modifications, that would be difficult to accurately reproduce in cell culture (Studer et al. 2015). For instance, the type and position of epigenetic modifications in the DNA of "mature" stem cell-derived neurones, correspond to those found in immature (embryonal) neuronal progenitor cells (NPC) (Miller et al. 2013).

6.2 Conclusions

PD and AD are two different – yet similar – neurodegenerative disorders, affecting millions of people worldwide and for which no cure has been identified yet. Due to the numerous common elements between the two diseases, an increasingly number of studies are now focusing on identifying a "common cure", aimed at increasing mitochondria homeostasis, reducing oxidative stress, and restoring impaired metabolism. Acyl-ghrelin is a promising therapeutic tool due to its ability to reach the brain and to exert numerous beneficial effects via the activation of its receptor GHS-R1a.

My thesis reports for the first time that the expression levels of key enzymes of the ghrelinergic axis are impaired in the brain of patients with PDD, and may also be affected in late AD, paving the way for future studies investigating a potential correlation between the ghrelinergic axis and cognitive functions in human. Moreover, my data also show that unacylated ghrelin exerts a detrimental effect on mitochondrial health *in vitro*, opposite to its acylated counterpart. In combination with previous studies, these data suggest the therapeutic potential of a new pharmacological approach, in which AG:UAG ratio is maintained or increased in the brain of aged and diseased patients, in order to counteract the oxidative stress and metabolic impairment that characterise these two diseases.

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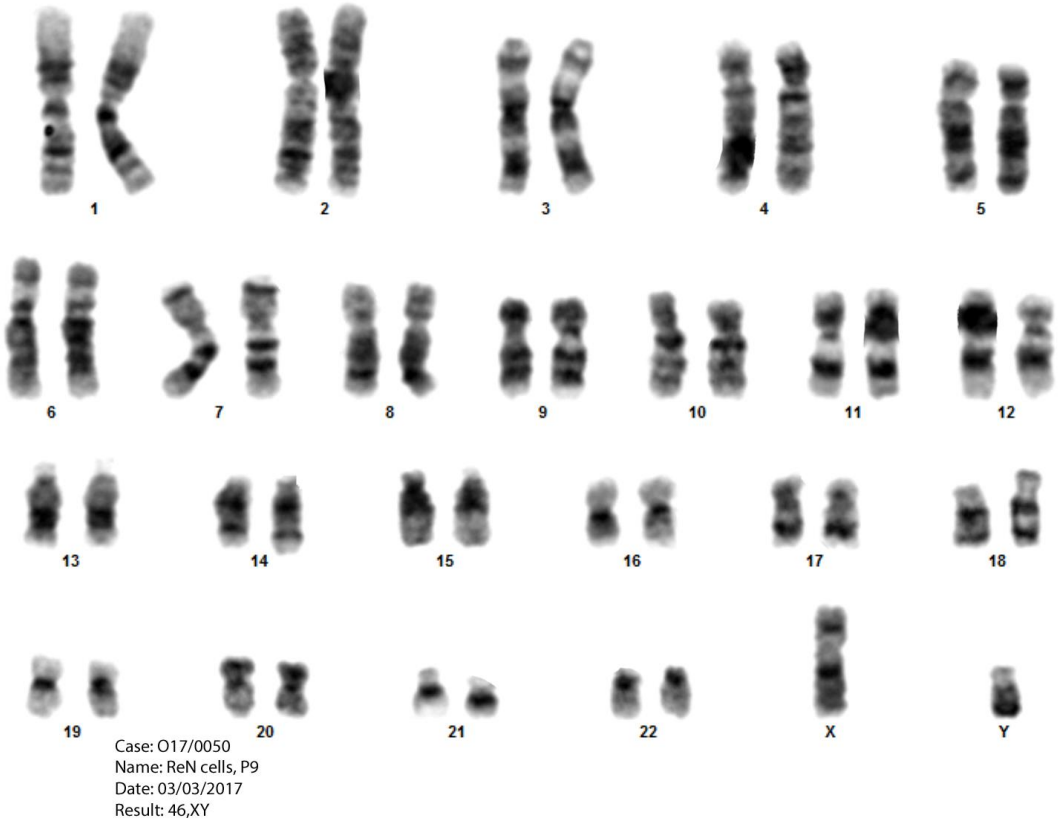
Appendices

A.1. ReN cells karyotipisation

ReN stem cells at passage 9, were karyotyped using the CellGS karyotype service (*cellgs.com/products/karyotype-service-for-live-cells*).



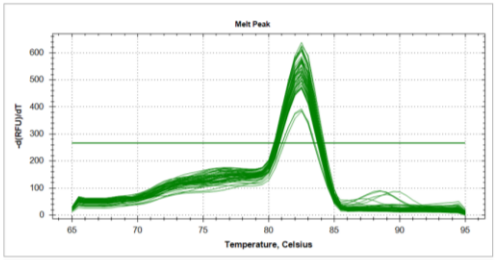
A.2. RT-qPCR primer sequences and optimisation

The following table shows the oligonucleotide sequences of all human primers used in this thesis. All primers were determined to be optimal at 60 °C. Melt curves are shown in the next page.

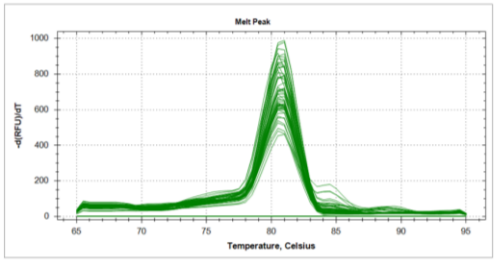
	forward	reverse	source
MBOAT4	TCTTTGTCTGAGCATGTGTAA	AAGCACTGGACCCTTGAACA	Primer design (NM_001100916)
LYPLA1	AGAAACTGGCAGGTGTCAC	GTGGCACTGGAGAATAGAAATATC	Primer design (NM_006330)
GHS-R1a	AAATGCTGGCTGTAGTGG	AGGCTCAAAGGATTTGGAAA	our design
mLARS2	CACCCAAGAACAGGGTTTGT	TGGCCATGGGTATGTTGTTA	Venegas et al. 2011
β2M	TGCTGTCTCCATGTTTGATGTATC T	TCTCTGCTCCCCACCTCTAAGT	Venegas et al. 2011
PGC1α	AGC CTC TTT GCC CAG ATC TT	GGC AAT CCG TCT TCA TCC AC	Silvennoinen et al. 2015
βactin	GATGGCCACGGCTGCTTC	TGCCTCAGGGCAGCGGAA	Churm et al. 2019

qPCR primers - melt curves

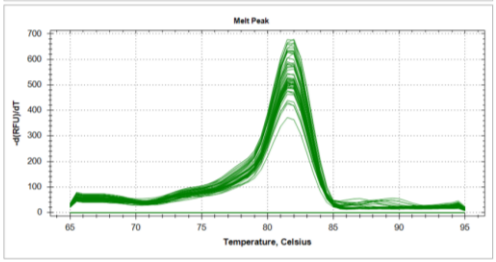
GOAT



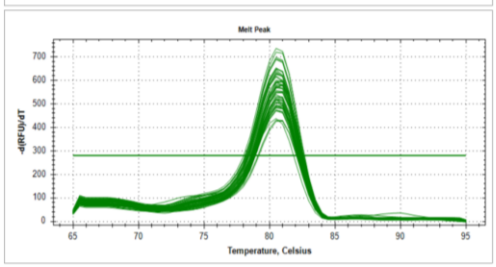
PGC1α



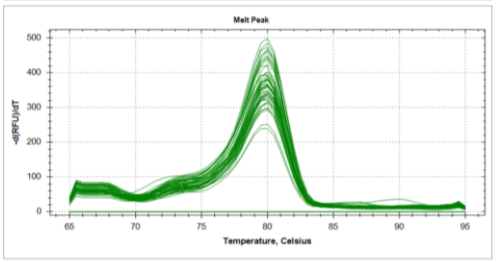
APT1



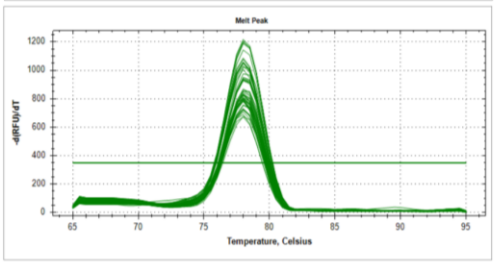
β2M



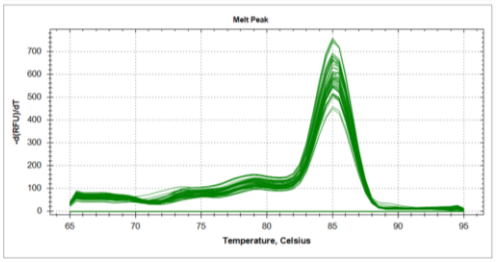
GHS-R1a



mLARS2

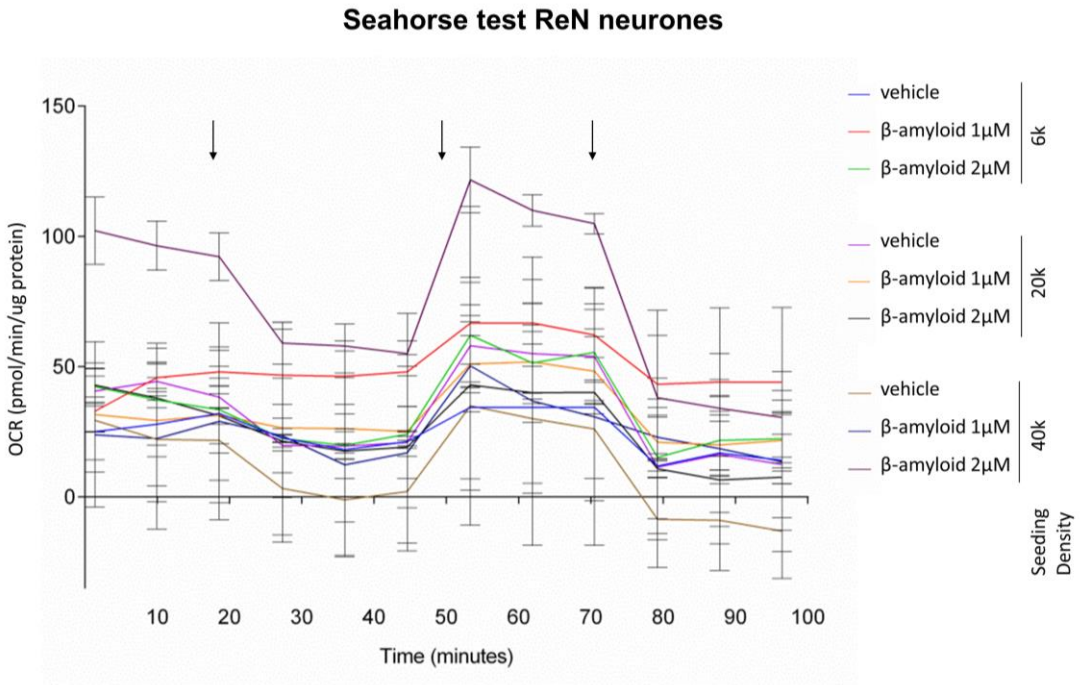


Bactin



A.3. ReN neurones seahorse protocol

The following figure summarises diverse Seahorse protocols performed on ReN neurones (DIV24) (n=1) in separate days. Stem cells (DIV0) were seeded in seahorse 96well plates at various cell densities, then allowed to differentiate as described in section 2.2. At DIV24, cells were treated with either vehicle, AβOs 1µM or 2µM for 24 hours, then media was changed, and cells were processed following the specific protocol from the supplier. More specifically, pH-controlled media was added to the cells for 1 hour, then plates were inserted in the Agilent Seahorse XFe96 Analyzer that performed three consecutive injections indicated by the black arrows: 1µM of Oligomycin A was injected after approximately 20 seconds, 1µM of FCCP was injected after approximately 50 seconds, and a combination of 1µM of rotenone + 1µM of antimycin A were injected after approximately 70 seconds. During the whole procedure, ROS released in the media were measured; error bars indicate the SEM of three consecutive readings from a single biological replicate.



legend: 60k = 60'000 cells/well; 200k = 200'000 cells/well; 400k = 400'000 cells/well

A.4. GHS-R1a signalling, full figure

The following figure summarises the most known GHS-R1a-mediated intracellular pathways, as described in section 1.2.9 (figure 1.4) and section 1.3.6 (figure 1.6).