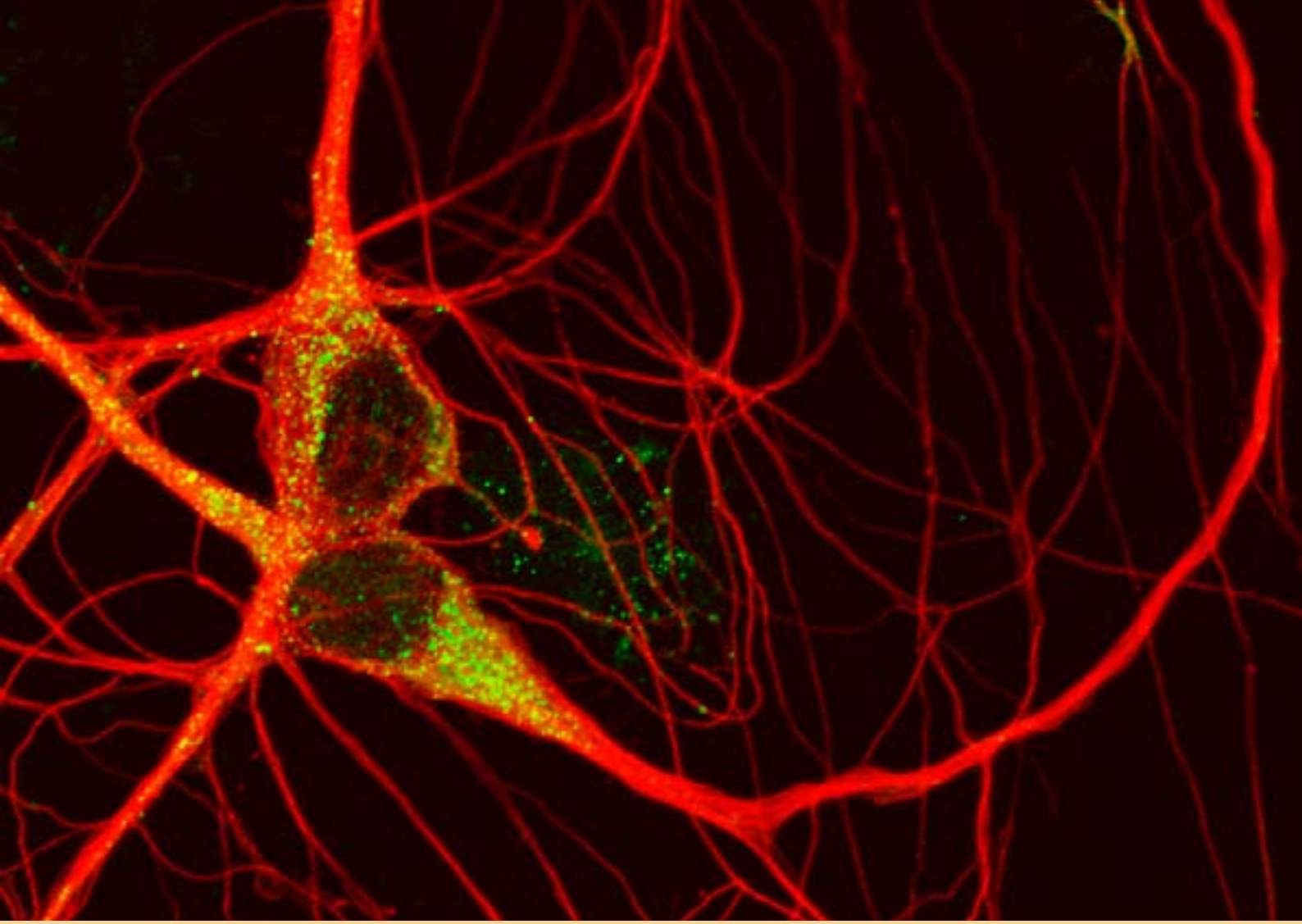

Mariana Botelho da Rocha

Autophagy in the hypothalamus: role of Neuropeptide Y and impact on Synaptic Plasticity

Doctoral Thesis in Pharmaceutical Sciences, with specialization in Pharmacology and Pharmacotherapy, supervised by Professor Cláudia Margarida Gonçalves Cavadas and Professor Luís Pereira de Almeida, and presented to the Faculty of Pharmacy of the University of Coimbra

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Universidade de Coimbra

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Autofagia no hipotálamo: o papel do Neuropeptídeo Y e o impacto na Plasticidade Sináptica

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Dissertation presented to Faculty of Pharmacy of the University of Coimbra in fulfillment of the requirements for a Doctoral degree in Pharmaceutical Sciences, speciality Pharmacology and Pharmacotherapy.

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Cover note: Microscopy confocal image of rat hypothalamic neurons co-labeled with the neuronal marker beta III Tubulin (red) and autophagy marker (LC3B, green).

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Table of Contents

List of abbreviations

3-MA	3-methyladenine
5-HT7	5-hydroxytryptamine 7
AAV	Adeno-associated virus
ABC	Avidin-biotin complex
AC	Adenylate cyclase
ACTH	Adrenocorticotropin
AD	Alzheimer's disease
AgRP	Agouti related peptide
Ambra1	Bcl-2-interacting protein-regulated autophagy
AMP	Adenosine monophosphate
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	AMPA receptor
AMPK	AMP-dependent protein kinase
ANOVA	Analysis of variance
AP2	Clathrin adaptor protein 2
AP-P	Aminopeptidase P
ARC	Arcuate nucleus
ATG	Autophagy related genes
ATP	Adenosine triphosphate
AV	Autophagic vacuoles
BCA	Bicinchoninic acid
Bcl-2	B cell lymphoma 2
Beclin-1	Bcl-2-interacting protein
BSA	Bovine serum albumin
CaMKII	Calcium/calmodulin-dependent protein kinase II
CaMKK	Calcium-activated calmodulin-dependent kinase kinase-
cAMP	Cycle adenosine monophosphatase
CART	Cocaine amphetamine-regulated-transcript
cDNA	Complementar deoxyribonucleic acid
CMA	Chaperone-mediated autophagy
CNS	Central nervous system
CPB	Carboxypeptidase B
CPON	C-terminal flanking peptide of NPY
CR	Caloric restriction
CRH	Corticotropin-releasing hormone
CRP	C-reactive protein
DAB	Diaminobenzidine
DAKO	Dako fluorescence mounting medium
DMEM	Dulbeco's modified eagle medium
DMH	Dorsomedial hypothalamus
DNA	Deoxyribonucleic acid
DPP	Dipeptidyl peptidase
DTT	Dithiothreitol
Dyrk1a	Dual specificity tyrosine-phosporylation-regulated kinase 1a
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-carrier enzyme
ECF	Enhanced chemiofluorescence

EGF	Epidermal growth factor
EGFP	Enhanced green fluorescence protein
EM	Electron microscopy
Epac	cAMP-exchange protein activated
EPSC	Excitatory postsynaptic current
ER	Endoplasmic Reticulum
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor-2
FIP200	Focal adhesion kinase family-interacting protein of 200 kDa
FOXO	Forkhead box O
GABA	γ-aminobutyric acid
$GABA_AR$	GABA type A receptor
$GABA_BR$	GABA type B receptor
GAIP	G-protein subunit alpha-interacting protein
GAP	GTPase activating protein
GDP	Guanosine diphosphate
GFAP	Glial fibrillary acidic protein
GH	Growth Hormone
GHSR	Growth hormone secretagogue receptor
GnRH	Gonadotropin releasing hormone
GPCR	G protein-coupled receptor
GS	Goat serum
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
h	Hour
HAP1	Huntingtin-associated protein 1
HD	Huntington's disease
HIF	Hypoxia-inducible factor
hPAX-P2	Human placental antigen X-P2
HSC 70	Heat shock cognate protein 70 kDa
HSP	Heat-shock protein
IGF-1	Insulin-like growth factor 1
IgG	Immunoglobulin G
iGluR	Ionotropic glutamate receptor
IL	Interleukin
IM	Isolation membrane
IMPase	Inositol monophosphatase
IP_3	Inositol triphosphates
IRS	Insulin receptor substrates
LAMP-2	Lysosome-associated membrane protein type 2
LC3B	Microtubule associated protein-1 light chain-3B
LDL	Low density lipoprotein
LH	Lateral hypothalamic area
LKB-1	Liver kinase B-1
MAP	Mitogen-activated protein
MAP4K2	Mitogen-activated protein kinase kinase kinase kinase-2
MAPK	Mitogen-activated protein kinase
MC3R	Melanocortin 3 receptor
MC4R	Melanocortin 4 receptor

MCH	Melanin-concentrating hormone
MEK	Mitogen-activated protein kinase kinase
mGluR	Metabotropic glutamate receptor
min	Minutes
MJD	Machado-Joseph's disease
mRNA	Messenger ribonucleic acid
MSH	Melanocyte-stimulating hormone
MTOR	Mechanistic target of rapamycin
mTORC	MTOR complex
NF-κB	Factor nuclear kappa B
NF-κBiα	Nuclear factor kappa B inhibitor alpha
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
NPY	Neuropeptide Y
NSCs	Neural stem cells
NTS	Nucleus of solitary tract
PAM	Peptidylglycine alpha-amidating monoxygenase
PAS	Phagophore assembly site
PBS	Phosphate buffered saline
PC	Phohormone convertase
PD	Parkinson's disease
PDK1	Phosphoinositide-dependent protein kinase 1
PE	Phosphatidylethanolamine
PFR	Perifornical region
PGC-1α	Proliferation activated receptor 1 alpha
$PI(3,4,5)P_3$	Phosphatidylinositides (3,4,5)-trisphosphate
$PI(4,5)P_3$	Phosphatidylinositides (4,5)-trisphosphate
PI3K	Phosphatidylinositol-3-kinase
PI3KC1	Phosphatidylinositol-3-kinase class I
PI3KC3	Phosphatidylinositol-3-kinase class III
PI3P	Phosphatidyl-inositol-3-phosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLCε	cAMP-phospholipase C-ε
PMSF	Phenylmethylsulfonyl fluoride
POMC	Pro-opiomelanocortin
PP	Pancreatic peptide
PP2B	Protein phosphatase 2B
PSC	Postsynaptic current
PSD	Postsynaptic density
PSP	Postsynaptic potential
PTEN	Phosphatase and tensin homologue
PVDF	Polyvinylidene fluoride
PVN	Paraventricular nucleus
PYY	Peptide YY
Rag	Ras-related small GTPases
Rapa	Rapamycin
REDD1	Regulated in development and DNA damage 1
Rheb	Ras homologue enriched brain

RIPA	Radio-immuno precipitation assay
ROS	Reactive oxygen species
rpm	Rotations per minutes
RSK1	Ribosomal S6 protein kinase alpha1
S6K1	Ribosomal S6 protein-kinase
SAP-102	Synapse-associated protein-120
SCN	Suprachiasmatic nucleus
SDS	Sodium dodecysulphate
SEM	Standard error of the mean
Sirt	Sirtuins
SNS	Sympathetic nervous system
SQSTM1	Sequestosome 1
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline and Tween20
TGF-1β	Transforming growth factor beta 1
TNF-α	Tumor necrosis factor-alpha
TRH	Thyrotropin-releasing hormone
TSC	Tuberous sclerosis complex
TX-100	Triton X-100
UBA	Ubiquitin-associated
UCP	Uncoupling protein
ULK	UNC-51-like kinase
UPS	Uniquitin-proteosome system
VEGF	Vascular endothelial growth factor
vGlut	Glutamate vesicular transport
VMH	Ventromedial hypothalamus

Resumo

O hipotálamo é uma região do cérebro que regula o desenvolvimento, o crescimento e o metabolismo. Recentemente, foi também demonstrado que o hipotálamo desempenha um papel chave no desenvolvimento generalizado do envelhecimento. A autofagia é um processo intracelular envolvido na reciclagem dos constituintes da célula e na manutenção da homeostase celular. Durante o envelhecimento e em doenças associadas ao envelhecimento ocorre diminuição da autofagia.

Por outro lado, em diversas espécies de animais, a restrição calórica (RC) é uma robusta intervenção anti-envelhecimento, aumentando o tempo de vida e diminuindo a incidência de doenças associadas à idade. A RC estimula a autofagia e também aumenta os níveis do neuropeptídeo Y (NPY) no hipotálamo. Diversos trabalhos mostram que o NPY tem um papel neuroprotector, aumenta a resistência ao stress. Contudo, o papel do NPY na autofagia nunca foi investigado.

Desta forma, o primeiro objectivo deste trabalho foi estudar o papel do NPY na autofagia em neurónios do hipotálamo. Os resultados mostraram que o NPY estimula a autofagia numa linha de neurónios hipotalâmicos de murganho (mHypo-N42) e também em culturas primárias de células hipotalâmicas neurais diferenciadas de rato. O NPY aumentou o fluxo autofágico em neurónios do hipotálamo através da activação dos receptores Y_1 ou Y_5, e que activam as vias de sinalização intracelular PI3K, ERK e PKA. O efeito do NPY na autofagia num modelo *in vivo* também foi avaliado, através da sobre-expressão do NPY no núcleo arqueado do hipotálamo de murganhos C57BL/6, pela tecnologia de transferência génica usando vírus adenoassociados. Os resultados mostraram que o NPY também estimula a autofagia no hipotálamo *in vivo*.

O núcleo arqueado do hipotálamo, responsável pela homeostase energética, é composto por duas populações neuronais distintas – neurónios que expressam POMC e CART, e neurónios que expressam NPY e AgRP. Estas duas populações regulam o anabolismo e catabolismo, recebendo e integrando sinais nutricionais e hormonais da periferia. Estudos recentes sugerem que a plasticidade sináptica dos circuitos hipotalâmicos envolvidos na ingestão alimentar também tem um papel na regulação da homeostase energética. Contudo, o papel da autofagia na plasticidade dos circuitos hipotalâmicos nunca foi investigado.

Desta forma, o segundo objectivo deste trabalho foi investigar o papel da inibição específica de uma proteína fundamental do processo de autofagia, a Atg7, na organização sináptica, com uma dieta normal e em privação de alimentos. Os murganhos com inibição específica da proteína Atg7 nos neurónios POMC (POMC-Cre; $Atg7^{loxP/loxP}$) foram usados como modelo

animal de estudo e os murganhos com expressão inalterada de Atg7 (Atg7$^{loxP/loxP}$ mice) como controlos. Nestes animais avaliou-se a organização sináptica dos neurónios POMC. Os animais foram mantidos durante cerca de 10 semanas com acesso livre a uma dieta padrão ou sem acesso a comida durante uma noite. O núcleo arqueado do hipotálamo destes animais foi analisado por microscopia electrónica, microscopia de fluorescência e por microscopia óptica de luz visível.

Os neurónios hipotalâmicos POMC dos murganhos POMC-Cre; Atg7$^{loxP/loxP}$, com ausência de Atg7 nos neurónios hipotalâmicos POMC, apresentaram um aumento da área e do perímetro desses neurónios, e apresentaram acumulação de nematossomas. Além disso, os neurónios hipotalâmicos POMC dos murganhos POMC-Cre; Atg7$^{loxP/loxP}$ apresentaram mais contactos sinápticos, que se traduzem num aumento dos contactos simétricos inibitórios. Depois de uma noite sem acesso a comida, os neurónios do núcleo arqueado do hipotálamo dos murganhos POMC-Cre; Atg7$^{loxP/loxP}$ apresentaram menor imunorreactividade para c-Fos, que sugere menor activação neuronal.

Em conclusão, os resultados desta tese mostram que o NPY induz o fluxo autofágico em neurónios do hipotálamo, e que a autofagia desempenha um papel na regulação da plasticidade sináptica dos neurónios POMC. Uma vez que a autofagia no hipotálamo e os níveis do NPY diminuem com o envelhecimento, a modulação do NPY pode ser um mecanismo protector contra a disfunção hipotalâmica associada ao aumento da idade. Por outro lado, a modulação da autofagia, através de um mecanismo sináptico subjacente, pode oferecer estratégias para a regulação do peso corporal.

Palavras-chave: Neuropeptídeo Y, NPY, receptores do NPY, Hipotálamo, Autofagia, Neurónios POMC, Plasticidade Sináptica

Abstract

The hypothalamus is the brain region that regulates development, growth and metabolism, and has gained increased attention for its key role in the progression of whole body aging. Additionally, autophagy, a highly regulated intracellular process involved in the turnover of most cellular constituents and in the maintenance of cellular homeostasis, is impaired in aging, contributing to the aging phenotype and to the aggravation of age-related diseases.

On the other hand, caloric restriction (CR) is a robust anti-aging intervention, increasing lifespan and decreasing the incidence of age-related diseases. CR increases autophagy in different brain areas and increases neuropeptide Y (NPY) levels in the hypothalamus. Moroever, NPY has neuroprotective effects and increases resistance to stress and mean lifespan. However, the role of NPY on autophagy has never investigated before.

Therefore, the first aim of this study was to investigate the role of NPY on autophagy in hypothalamic neurons. The results show that NPY stimulated autophagy in mouse hypothalamic cell line N42 (mHypo-N42) and also in rat differentiated hypothalamic neural cell cultures. Moreover, NPY stimulated the autophagic flux in hypothalamic neurons by activating NPY Y_1 or Y_5 receptors, through PI3K, ERK and PKA intracellular signaling pathways. We also evaluated the role of NPY on autophagy *in vivo*, by overexpressing NPY in the arcute nucleus (ARC) of hypothalamus of C57BL/6 mice, using adenoassociated viral (AAV) gene transfer technology. The results show that NPY also stimulated autophagy in hypothalamus *in vivo.*

The hypothalamic ARC, responsible for energy homeostasis, is composed by two major neuronal populations – cocaine- and amphetamine-regulated transcript (CART)/Pro-opiomelanocortin (POMC) expressing neurons and agouti-related peptide (AgRP)/ neuropeptide Y (NPY) expressing neurons. These two neuronal populations regulate anabolic and catabolic state, receiving and integrating peripheral nutritional and hormonal signals. Recent observations suggest that synaptic plasticity in the hypothalamic feeding circuits has also a critical role in regulation of energy homeostasis, since the neuronal synaptic input organization in the hypothalamus is able to adapt and rearrange rapidly in response to metabolic hormones. In addition, autophagy in the hypothalamus was identified as a player in metabolic regulation. However, a role for autophagy in plasticity of hypothalamic feeding circuits has not been explored.

Therefore, the second aim of this study was to investigate the role of Atg7 deletion in POMC neurons in the synaptic organization in mice under standard diet and food deprivation. POMC-specific Atg7 knockout mice (POMC-Cre; $Atg7^{loxP/loxP}$) were used as animal model and Cre-

negative Atg7$^{loxP/loxP}$ mice as controls, to evaluate the synaptic organization of the hypothalamic POMC neurons and neuronal activation in hypothalamic ARC. Animals were maintained during 10 weeks with standard diet or overnight fasting, and then brains slices containing arcuate nucleus of the hypothalamus were stained for electron microscopy and for fluorescence and light microscopy.

The specific Atg7 deletion in POMC neurons resulted in an increased cell area and perimeter, and nematosomes accumulation. Moreover, we observed that POMC-Cre; Atg7$^{loxP/loxP}$ neurons have more synaptic inputs and more symmetric, putatively inhibitory inputs. After an overnight fasting, POMC-specific Atg7 knockout mice show no normal adaptation to food deprivation, with an impaired neuronal activation in hypothalamic ARC.

Overall, these results show that NPY induces autophagic flux in hypothalamic neurons, and that autophagy has a role in the control of synaptic plasticity of POMC neurons. Since both hypothalamic autophagy and NPY levels decrease with age, modulation of NPY may act as a protective mechanism against impaired hypothalamic dysfunction associated with age. Moreover, autophagy modulation, through underlying synaptic mechanism, might offer strategies to the body weight regulation.

Keywords: Neuropeptide Y, NPY, NPY receptors, Hypothalamus, Autophagy, POMC neurons, Synaptic Plasticity

Glossary

Hypothalamus: brain area localized in bottom that contains a number of small nuclei with a variety of functions.

Arcuate nucleus: a region of the hypothalamus where the nutrient sensing neurons are located.

Neuropeptide Y (NPY): a 36 amino-acid peptide abundant in mammalian brain, acting as neurotransmitter and with a widespread distribution in body.

Pro-opiomelanocortin (POMC) neurons: a group of neurons located in the ARC that express POMC; neuropeptides resulting from processing and cleavage of the POMC gene product have anorexigenic actions and are increased in situations of energy excess.

Caloric Restriction: a dietary regimen with reduction of 20-60% of calorie intake.

Autophagy: a highly regulated self-degradative process involved in the turnover of most cellular constituents and in the maintenance of cellular homeostasis.

Autophagosome: the central organelle in the macroautophagic pathway is a double membrane-bound vacuole formed from a membrane, known as the phagophore, which sequesters cytoplasmic material.

Anorexigenic: appetite suppressant that decreases the food intake.

Orexigenic: appetite stimulant that increases food intake.

Synapse: specialized structures on neuronal cell membrane that mediate rapid and highly efficient information transmission by electrical or chemical signals from a neuron to its target cells in highly plastic manner.

Synaptic Plasticity: the cellular phenomenon by which synapses are able to strengthen or weaken over time, in response to increases or decreases in their activity.

Excitatory Synapse: a synapse in which the nerve impulse in a presynaptic cell tend to increase the probability of the postsynaptic cell to fire an action potential.

Inhibitory Synapse: a synapse in which the nerve impulse in a presynaptic cell results in a reduced likelihood for a postsynaptic cell to fire an action potential.

CHAPTER 1

General Introduction

1.1 Aging

Population aging was the most distinguishing demographic event observed in the previous century and it will remain an important population issue throughout the 21st century. In 2050, it is predicted that the number of people over the age of 60 will be 21 per cent of the global population, almost double of the current 12 per cent (United Nations) (Figure 1.1).

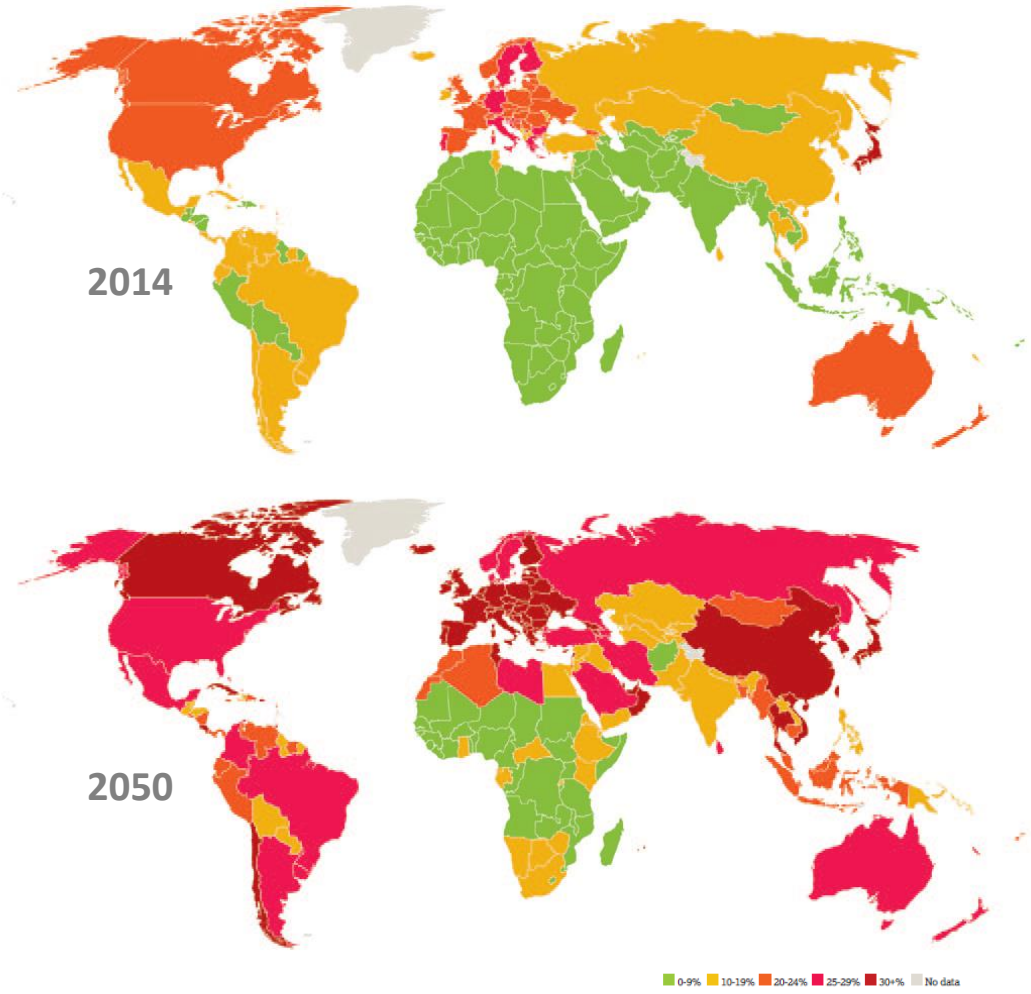

Figure 1.1 - Proportion of population aged 60-plus in 2014 and 2050. Data from UNDESA Population Division, *World population prospects.*

The increase in life expectancy can be seen as a result of public health policy and socioeconomic development. However, it is also challenge the society to adapt and promote better health and functional capacity of the elderly people, and to understand the biological basis of aging, as well as, the morphological and molecular aspects underlying various age-related diseases (Rezzani et al., 2012).

Aging is an age-dependent or age-progressive decline in intrinsic physiological function leading to an increase in age-specific mortality rate and a decrease in age-specific reproductive rate (Flatt, 2012). It is an extremely complex process subjected to scientific scrutiny based on the ever-expanding knowledge of the molecular and cellular bases of life and disease (López-Otín et al., 2013), once, aging as a progressive impairment of function, results in vulnerability increase to environmental challenge and a growing risk of disease including cardiovascular disease, cancer and neurodegenerative diseases (Kirkwood, 2005; Partridge et al., 2011).

Some theories have been proposed to explain aging, however the contribution of each theory to identify a primary cause of aging has been difficult to establish, and therefore, aging is conventionally regarded as a process of progressive decline of cellular homeostasis, cognitive impairment or dementia, regulated by intrinsic and extrinsic variables in relation to the individual (Bishop et al., 2010; Tripathi, 2012). Nevertheless, recently it was described the predominant cellular and molecular hallmarks of aging by López-Ótin and colleagues that represent common denominators of aging in different organisms, suggesting that aging begins at the cellular level and then spreads to the tissue. Of the nine hallmarks, genetic instability, telomere attrition, loss of proteostasis, and stem cell exhaustion are primarily genetically predetermined. Epigenetic alterations, deregulated nutrient sensing, cellular senescence, and altered intercellular communication have their primary source in life style and environment influences. The hallmarks of aging were defined taking into account that they should be manifested during normal aging, experimental aggravation should accelerate aging, and experimental amelioration should retard the normal aging process and increase healthy lifespan (López-Otín et al., 2013). While no mechanisms or drugs are available to increase lifespan and healthspan, understand the connections between hallmarks of aging and how they may be controlled, or even, prevented, is the challenge of reseachers of aging field.

1.1.1 Brain aging

Aging alters tissues throughout the body, albeit at different rates. In particular, the central nervous system (CNS) is especially vulnerable to the effects of aging, integrating sensory information and responding to changes from external environment. In addition, the interactions of CNS, mainly the hypothalamus, have been implicated in regulating organism lifespan (Zhang et al., 2013). Therefore, the brain represents as both a vulnerable site to aging effects of while also acting as a potential master regulator of systemic aging itself, showing a unique duality not reflected in other tissues (Bouchard and Villeda, 2015).

Consequently, aging in human brain results in stereotypical structural and neurophysiological changes and variable degrees of cognitive decline. Structural changes include shrinkage in total brain volume and morphological changes in specific regions. In addition, brain aging also involves alteration in levels of enzymes, hormones, genetic and epigenetic modulation, dysregulated metabolism, increased oxidative stress, altered protein processing and synaptic function, and these changes together lead to decline in physiological and cognitive functions (Thakur and Rattan, 2012).

With age, brains shrink in volume particularly in frontal cortex, temporal cortex, putamen, thalamus and nucleus accumbens. These reductions are due to neuronal shrinkage, reduction of synaptic spines, lower number of synapses and reduced length of myelinated axons (Dickstein et al., 2007). The cerebral ventricles enlarge with progression of age (Wilkie et al., 2012), as well as, the permeability of blood-brain barrier also increases with advancing age (Farrall and Wardlaw, 2009). Functional imaging studies have demonstrated that the coordination of brain activity between different brain regions becomes less robust in the aging brain, suggesting a loss of integrative function, which is also accompanied by neural activity decrease in some brain regions (Bishop et al., 2010). Contrary to the early investigations which pointed that of aged nervous tissue has a profound neuron loss in advanced age, most brain areas neuronal loss does not have a significant role in age-related cognitive decline (Burke and Barnes, 2006).

1.1.2 Molecular mechanisms underlying brain aging

Several mechanisms have been proposed to explain the brain aging process such as mitochondrial dysfunction, accumulation of damaged proteins and decline in stem-cell function.

The progressive degeneration of mitochondria function has often been associated with aging in general and in particular with CNS aging. Several studies of genes expression profiling suggest a decreased of mitochondrial function, which could contribute to the accelerated aging. Since mitochondrial oxidative phosphorylation is the key source of energy intensive in neurons, they are more susceptible to mitochondrial dysfunction. Moreover, irregularities in the electron transport chain in mitochondria during progressive aging cause an increased production of reactive oxygen species (ROS), which may cause damage to respiratory chain proteins. In normal course, the action of antioxidant defenses presented in cells counter the ROS, however, in aging process the action of the antioxidants is diminished, resulting in local oxidative damage to mitochondrial proteins and deoxyribonucleic acid (DNA) (Yankner et al.,

2008). In addition, the nuclear DNA lesions caused by oxidative damage in brain aging, accumulates in the promoters of a subset genes associated with synaptic function and protein transport. Thus, DNA damage may reduce the expression of these genes and also contributes to brain aging (Lu et al., 2004). Moreover, somatic mitochondrial DNA mutations occur in control regions where they impair the transcription and replication of mitochondrial DNA (Coskun et al., 2004) and reduce the activity of respiratory chain enzymes (Lin et al., 2002).

The accumulation of damaged molecules within the cells is one of the most widely documented alterations that occur in neurons during aging (Rubinsztein et al., 2011). A quality control mechanism in cells preserves the stability and functionality of the proteins, by stabilization of correctly folded proteins and degradation processes. Loss of protein homeostasis leads to the accumulation of oxidized, misfolded, cross-linked or aggregated macromolecules that contribute to the development of age-related pathologies (López-Otín et al., 2013). These aggregated macromolecules or simply damaged proteins are removed mainly by autophagy (Bergamini et al., 2003). However, with increasing age this process fails and consequently occurs neuronal dysfunction and cell death (Bi et al., 2000; Cuervo et al., 2005; Meijer and Codogno, 2006). Moreover, dysregulation of autophagy has also been implicated in multiple neurodegenerative diseases (Hara et al., 2006).

The decline in stem-cell function is also a hallmark of aging and is implicated in neural aging. The adult neural stem cells (NSCs) suffer from proliferative declines and cellular senescence with age (Kuhn et al., 1996). The decreased cell proliferation (Maslov et al., 2004; Luo et al., 2006), increased G0/G1 cell cycle arrest and reduced neuronal neurogenesis (Molofsky et al., 2006) gradually result in a loss of repair and regeneration capacity, contributing to neural aging and disorders.

Understanding the biological bases of aging and the molecular mechanisms that underlie age-related diseases is utmost important to future genetic and pharmacological interventions to increase lifespan with life quality.

1.1.3 Putative strategies to delay aging

A major goal of aging research is to extend healthspan by identifying approaches for delaying or preventing age-related diseases. Many are the preventive systemic strategies that have been shown to combat signs of aging in both CNS and the peripheral tissues. The systemic manipulations have been shown to ameliorate the cognitive impairments and neurodegenerative processes in the aged brain, as well as, the regenerative capacity of aged tissues (Bouchard and Villeda, 2015). Some studies suggest that some diets including omega 3

fatty acids, vitamin C, vitamin E, vitamin B12, vitamin B6, folic acid iron, calcium, zinc, docosahexanoic acid and breast milk proteins delay the effects of normal brain aging and cognitive decline (Tripathi, 2012).

The literature also suggests that physical exercise not only decreases vascular disease risk, but may also has benefit on cognitive function decline during aging (van Praag et al., 2005; Marlatt et al., 2012). By enhancing neurogenesis, synaptic plasticity and resistance of the brain to injury and disease, physical exercise may optimize brain function. Exercise can also prevent brain inflammation and promote releasing of systemic factors, such as vascular endothelial growth factor (VEGF) and insulin-like growth factor 1 (IGF-1), contributing to brain health (Trejo et al., 2001; Fabel et al., 2003; Pereira et al., 2007; Voss et al., 2013; Praag et al., 2014).

Heterochronic parabiosis, where the aged animal is exposed to blood of young animals, is also a systemic strategy with beneficial effect on cognition deficit related with aging (Katsimpardi et al., 2014; Villeda et al., 2014). The mechanisms underlying this benefitial effects of heterochronic parabiosis may include regenerative capacity of peripheral tissues and CNS, including stimulation of neurogenesis in hippocampus (Villeda et al., 2011; Katsimpardi et al., 2014).

1.1.3.1 Caloric Restriction (CR)

Caloric restriction (CR) is other systemic manipulation shown to counteract the brain aging and neurodegenerative disorders. It consists in a reduction of 20-60% of calories without reduction of essential nutrients (Tripathi, 2012). This nutritional and non-pharmacological intervention is the most robust anti-aging strategy, since it increases the average life expectancy of a wide variety of species, from yeast to mammals (Masoro, 2006; Roberts and Schoeller, 2007; Fontana and Partridge, 2015). Since the pioneer work of McCay in 1935, which showed that the restriction of calories by 40% extended two times the rats lifespan (McCay et al., 1935), several studies suggest that CR extends the longevity and/or have beneficial effects on aging delay and related diseases of various animals, including fruit flies, nematelmintes, rodents and rhesus monkeys (Table 1.1).

Table 1.1 - Benefitial effects of caloric restriction in different organisms.

Animals	*Beneficial effects*	*Refereneces*
Yeast	-Extends lifespan.	(Lin et al., 2000)
Nemathelminthes	-Extends lifespan.	(Braeckman et al., 2006)
Fruit flies	-Reduces mortality and extends lifespan.	(Burger et al., 2010; Metaxakis and Partridge, 2013)
Rodents	-Extends lifespan; -Improves motor learning and hippocampal-dependent memory; -Induces proliferation of neural progenitor cells in hippocampus; -Improves signs of neurodegeneration in disease models (Alzheimer's and Parkinson's diseases); -Delays the age-related hearing loss; -Reduces body core temperature, insulin secretion, blood glucose levels, and growth hormone and IGF-1 secretion; -Reduces cancer incidence.	(McCay et al., 1935; Weindruch et al., 1986; Pitsikas and Algeri, 1992; Lee et al., 2000, 2002; Seidman, 2000; Koubova and Guarente, 2003; Park et al., 2013; Ma et al., 2014; Talhati et al., 2014)
Rhesus monkeys	-Increases survival; -Improves metabolic health; -Maintains higher physical activity; -Delays the onset of sarcopenia, presbycusis, and brain atrophy; -Reduces the risk of developing and dying of type 2 diabetes, cancer, and cardiovascular disease.	(Fowler et al., 2002; Colman et al., 2009, 2014; Mattison et al., 2012; Yamada et al., 2013)

Overall, CR has been shown to improve longevity and resistance to stress in animal models; however in humans, the most corroborative data on the beneficial effects of CR is the weight loss in overweight and reducing the predisposition to cardiovascular incident. In humans is difficult to definitively answer whether or not CR prolongs life, because of the ethical and logistical limitations of research design. Table 1.2 summarized the human studies already performed and its effects on health.

Table 1.2 - Human Caloric Restriction studies and its impact on health.

Study	*Caloric restriction description*	*Beneficial effects*	*References*
Biosphere 2 experiment	30% CR for 2 years in subjects with 22-67 years	Reduction of blood pressure, fasting blood glucose, insulin, cholesterol, and triodothyronine (T3) and white blood cells.	(Walford et al., 2002)
CALERIE– Phase 1	25% CR , for 6-12 months, in healthy, overweight women and men aged 24-60 years	Decreased body weight, visceral and subcutaneous adipose tissue, core body temperature, fasting glucose and improve insulin sensitivity.	(Heilbronn et al., 2006; Weiss et al., 2006; Fontana et al., 2007; Lefevre et al., 2010)
CALERIE - Phase 2	25% CR in healthy, overweight women and men aged 21-50 years, for 2 years	Decreased resting metabolic rate, core body temperature, T3, TNF-α, CRP, triglycerides and total cholesterol, systolic and diastolic blood pressure.	(Ravussin et al., 2015)
Caloric restriction Society	CR in 18 society members for an average of 6 years	Decreased total body fat, total and LDL cholesterol, fasting glucose and levels of chronic inflammation.	(Fontana et al., 2004)
Human Studies Committee of Washington University School of Medicine	~ 30% CR for an average of 9.6 years (4–20 years)	Counteract the down-regulation of pathways associated with muscle contraction, electron transport and oxidative phosphorylation in skeletal muscle; Down-regulation of the activity of the insulin/IGF pathway in skeletal muscle.	(Mercken et al., 2013)

CR: Caloric Restriction; TNF-α: tumor necrosis factor alpha; CRP: C-reactive protein; LDL: low-density lipoprotein; CALERIE: Comprehensive Assessment of Long Term Effects of Reducing Caloric Intake).

1.1.3.1.1 Mechanisms underlying the caloric restriction's beneficial effects

Although the metabolic and physiological changes induced by CR have been investigated for over 70 years, the precise mechanism by which it is able to slow the progression of age-related degeneration remains a subject of much debate. The following table 1.3 summarizes the current understanding, including recent findings that implicate the involvement of specific enzymes and signaling pathways.

Table 1.3 - The possible mechanisms involved in the beneficial effects of caloric restriction effects.

Processes involved	*Mechanisms*	*References*
Protection against the age-associated oxidative stress	-Reduction of energy expenditure and the steady-state levels of ROS; -Elevation of mitochondrial membrane permeabilization. electron transport chain, and ATP production; -Induction of PGC-1α.	(Merry, 2004; López-Lluch et al., 2006; Michan, 2014)
Reduction of chronic systemic inflammation	-Increased anti-inflammatory genes (e.g. NF-κBiα); -Inhibition of the pro-inflammatory genes (e.g. TNFα, IL-6).	(Sung et al., 2004; Higami et al., 2006; Jung et al., 2009; Swindell, 2009)
Sirtuins involvement	-Increase of SIRT1 expression and promotion of mammalian cell survival; - CR did not extend lifespan in SIRT1 knockout mice.	(Cohen et al., 2004; Boily et al., 2008; Mercken et al., 2014)
Metabolic reprogramming	-Reduction of serum IGF-1, insulin, glucose, testosterone and estradiol levels; -Enhance of insulin sensitivity; -Decreased levels of hormones regulating thermogenesis and basal metabolism (T3); -Increased anti-inflammatory hormone levels (adiponectin and corticosterone).	(Shimokawa et al., 2003; Argentino et al., 2005; Fontana et al., 2006, 2010a; Fontana, 2009; Redman and Ravussin, 2009; Wang et al., 2009b)
Neurotrophic factors and neurogenesis promotion	-Increased BDNF levels in cortex and hippocampus; -Increase of the capacity of stem cells to self-renew, proliferate, differentiate, and replace cells in adult tissues; -Promotion of survival of glial cells.	(Bondolfi et al., 2004; Park et al., 2013; Mazzoccoli et al., 2014)
Autophagy	-Stimulation of autophagy (discussed in section 1.4.5).	(Donati, 2006; Bergamini et al., 2007; Hansen et al., 2008; Blagosklonny, 2010)

ATP: adenosine triphosphate; PGC-1α: proliferation-activated receptor coactivator 1 alpha; ROS: reactive oxygen species; NF-κBiα: nuclear factor kappa B inhibitor alpha; TNF-α: tumor necrosis factor alpha; IL-6: interleukin-6; SIRT1: Sirtuin 1; BDNF: brain-derived neurotrophic factor.

1.1.3.1.2 Neuroendocrine alterations induced by caloric restriction

CR also induces a neuroendocrine response which is reflected primarily by increased levels of neuropeptide Y (NPY) in the arcuate nucleus (ARC) of the hypothalamus (Brady et al., 1990; Bi et al., 2003; de Rijke et al., 2005; Minor et al., 2009). It will be discussed in section 1.2.3.3.

1.2 Neuropeptide Y

In 1982, Neuropeptide Y (NPY) was isolated from porcine brain and sequenced, based on its physicochemical properties, by Tatemoto et al. (1982), and, it is a member of NPY family or ‘‘PP-fold’’ family, together with peptide YY (PYY) and pancreatic peptide (PP). All these peptides have a common hairpin-like tertiary structure, consisting of an N-terminal poly-proline helix and a long alpha-helix connected by a beta turn (Glover et al., 1984; Allen et al., 1987a). With the highest degree of phylogenetic preservation, NPY is a linear polypeptide with 36 amino acid residues, with amidated C-terminal end and large number of tyrosine residues (which are abbreviated by the letter Y), included in both ends of the molecule (Michel et al., 1998) (Figure 1.2). In addition, the carboxyl-terminal of the peptide is responsible for its biological activity while the amino-terminal is involved in receptor affinity.

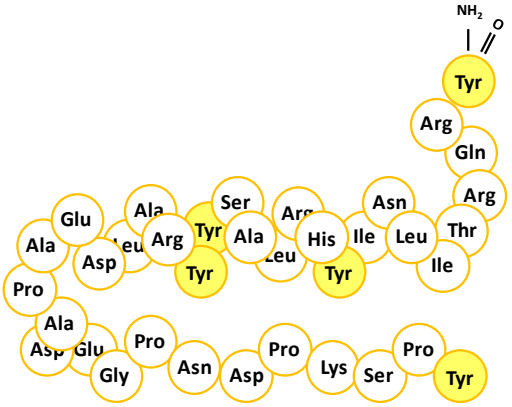

Figure 1.2 - Structure of human NPY. Human NPY has 36-amino acid peptides, with a carboxyl-terminal amidation. This α-amidated carboxyl terminus is essential during the purification of the NPY-related peptides. Adapted from (Schwartz et al., 1990).

1.2.1 Neuropeptide Y: synthesis, metabolization and localization

The human NPY gene is located in chromosome 7 at the locus 7p15.1, and is divided into 4 exons that are separated by 3 introns (Minth et al., 1984; Cerdá-Reverter and Larhammar, 2000). In mouse, NPY gene is placed in chromosome 6, locus 6 B3; 6 26.0 cM, while in rat is localized in chromosome 4, locus 4q24 (Pruitt et al., 2012).

NPY is first synthesized as a precursor peptide and only becomes active after processing, as many other peptides and proteins. NPY is produced by cleavage of a precursor peptide that consists in four post-translational enzymatic events (Figure 1.3). The messenger ribonucleic acid (mRNA) is translated in ribosomes, resulting the prepro-NPY, which is transported to the endoplasmic reticulum (ER) and thus to the Golgi complex. From prepro-NPY, with 97 amino

acid residues, a signal peptidase enzyme creats pro-NPY, removing a 28 amino acids peptide, in secretory granules. The pro-NPY usually travels to the Golgi complex and further to the trans-Golgi. This pro-NPY is a 69 amino acid peptide formed by $NPY_{1–39}$ where the carboxylic group is flanked by a group of 33 amino acids called the C- terminal flanking peptide of NPY (CPON). This pro-NPY is cleaved by prococonverting enzymes, prohormone convertase 1 (PC1) or 3 (PC3) and/or PC2, to originate NPY_{1-39}, by releasing CPON. NPY_{1-39} resultant is processed to NPY_{1-37} by a carboxypeptidase B (CPB), and by peptidylglycine alpha-amidating monooxygenase (PAM) to originate the biological active amidated $NPY_{1–36}$ (dos Santos Medeiros and Turner, 1996).

Although this peptide is already in its biologically active form, NPY can be further cleaved by two enzymes, namely dipeptidyl peptidase 4 (DPP-IV) and aminopeptidase P (AP-P). These enzymes give rise to different metabolites with varying selectivity to NPY receptors and, thus, different biological activities (dos Santos Medeiros and Turner, 1996; Pedrazzini et al., 2003).

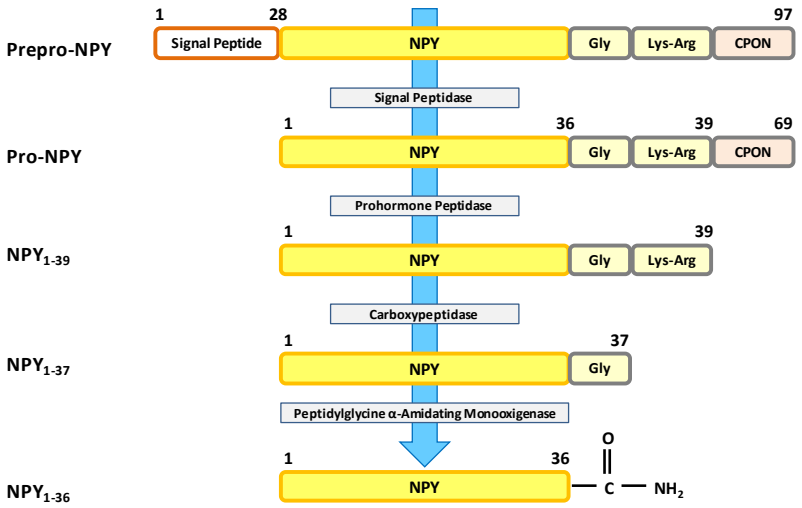

Figure 1.3 - Biosynthesis of NPY. The numbers are referred to base pairs; CPON: C-flanking peptide of NPY. Adapted from (Silva et al., 2002).

Active NPY form could be metabolized to generate truncated peptides with selective specificity to different receptors (Figure 1.4). DPP-IV is plasmatic membrane peptidase with a high affinity to NPY. This exopeptidase belongs to the family of propyl-oligopeptidases that cleaves proline amino acid in the penultimate position. Thus, DPP-IV acting on the NPY cleaves the bond Ser-Pro of the terminal amine, removing the N-terminal dipeptide Tyr-Pro to generate NPY_{3-36}, that loses affinity to NPY Y_1 receptor but retains its capacity to bind to the NPY Y_2 and Y_5 receptors (dos Santos Medeiros and Turner, 1996). There are also other aminopeptidases that are able to cleave NPY. Dipeptidyl-peptidase 8 (DPP-VIII) and dipeptidyl-peptidase 9 (DPP-IX) are

cytoplasmatic enzymes, and as DPP-IV, both cleave NPY in dipeptide Tyr-Pro (Abbott et al., 2000; Ajami et al., 2004).

The AP-P is another peptidase which hydrolyzes NPY. The AP-P has two isoforms: while AP-P1 is soluble in the cytosol, the AP-P2 is anchored to the plasma membrane. Both cleave peptide binds between first and second amino acid residues at the N-terminal side of protein, and in NPY, the hydrolyze of Try-Pro binding results in NPY_{2-36} peptide (Vanhoof et al., 1997; Venema et al., 1997). Peptides NPY_{3-36} and NPY_{2-36} can also be degraded by neutral endopeptidase-24-11 being the major cleavage sites the Tir^{20}-Tir^{21} and Leu^{30}-Ile^{31}, giving rise to biologically inactive peptides as NPY_{1-20} and NPY_{31-36} (dos Santos Medeiros and Turner, 1994, 1996).

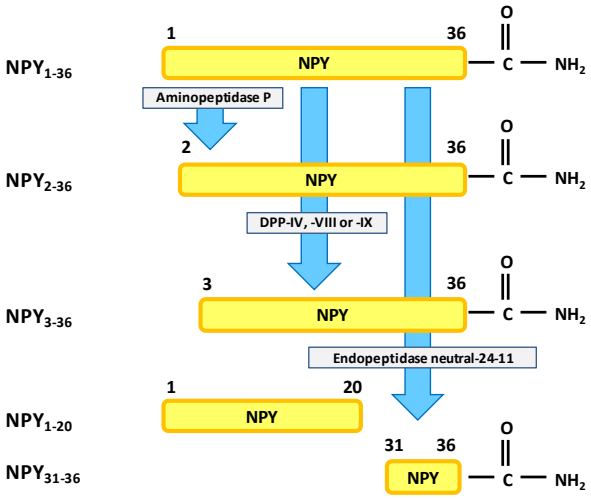

Figure 1.4 - Scheme of the processing of NPY. Active NPY could suffer post-secretory processing that can modify the receptor selectivity. Adapted from (Santos-Carvalho et al., 2015).

NPY is one of the most abundant peptides in human body, and is involved in numerous assorted physiological functions, showing both pre- and postsynaptic actions. NPY in the human (Adrian et al., 1983) and rodent (Allen et al., 1983) CNS is well described. NPY is widely expressed within mammalian brain, with a wide distribution in the central and peripheral nervous system and in other peripheral tissues (Everitt et al., 1984; Michel et al., 1998) (Table 1.4).

Table 1.4 - NPY distribution in tissues and organs (adaptated from Santos-Carvalho et al., 2015).

Localization of NPY	*Distribution*	*References*
Central Nervous System	Hypothalamus	(Adrian et al., 1983; Allen et al., 1983; Silva et al., 2005)
	Spinal cord, mesencephalon, metencephalon	(Allen et al., 1984; Halliday et al., 1988; Pammer et al., 1990)
	Córtex, basal ganglia, thalamus, septum, striatum, amygdala	(Allen et al., 1983; de Quidt and Emson, 1986a; Silva et al., 2005)
	Hippocampus (dentate gyrus)	(Caberlotto et al., 2000)
	Occipital lobe and temporal cortex	(Adrian et al., 1983; Beal et al., 1987; Delalle et al., 1997)
	Pituitary gland	(Grunditz et al., 1984; Jones et al., 1989; Silva et al., 2005)
	Retina	(Bruun et al., 1984; Tornqvist and Ehinger, 1988; Ferriero and Sagar, 1989)
Peripheral Nervous System	Sympathetic ganglion neurons	(Lundberg et al., 1982)
Immunologic System	Spleen	(Lundberg et al., 1989; Romano et al., 1991)
	Thymus	(Kranz et al., 1997)
Digestive system	Dental pulp	(Uddman et al., 1984)
	Colon, enteric neurons, pylorous	(Jackerott and Larsson, 1997a; Rettenbacher and Reubi, 2001; Cox and Tough, 2002; Lindeström and Ekblad, 2002; Anitha et al., 2006; Zalecki, 2012)
	Tongue	(Kusakabe et al., 1998)
	Liver	(Esteban et al., 2001)
Cardiovascular system	Heart and endothelial cells	(Ahmed et al., 1997; Jackerott and Larsson, 1997a; Zukowska-Grojec et al., 1998; Jacques et al., 2003; Silva et al., 2005)
Endocrine system	Thyroid gland	(Grunditz et al., 1984)
	Adrenal Glands	(de Quidt and Emson, 1986b; Pelto-huikko, 1989; Fernandez-Vivero et al., 1993; Cavadas et al., 2001)
	Pancreas, Langerhans islets	(Jackerott and Larsson, 1997b; Adeghate et al., 2000; Ponery and Adeghate, 2000; Lambert et al., 2002)
	Adipose tissue and adipocytes	(Valet et al., 1990; Rosmaninho-Salgado et al., 2012)
Reproductive system	Ovary and corpus luteum	(Keator et al., 2010)
	Corti organ	(Gomide et al., 2009)
	Testis	(Gong et al., 2009)
Skin and sensory systems	Sebaceous and lacrimal glands	(Ebara et al., 1992; Kirch et al., 1996; Seifert and Spitznas, 1996; Seifert et al., 1996)
	Nasal mucosa	(Zhao et al., 1998; Kniping et al., 2003)
	Skin nerve fibers	(Johansson, 1986)
Bone, Muscles	Skeletal muscle	(Jonhagen et al., 2006)
	Ligaments	(Jiang et al., 2008)
	Periostum Osteoblasts	(Togari et al., 1997)

1.2.2 Neuropeptide Y receptors

NPY exerts its functions through the class A G protein-coupled receptors (GPCR), with a heptahelical structure (Michel et al., 1998; Fredriksson et al., 2003). NPY family receptors are the same for all members of the NPY family (NPY, PYY, PP), and can be organized into three subfamilies: the Y_1 subfamily containing of subtypes Y_1, Y_4, y_6 and Y_8; the Y_2 subfamily including the subtypes Y_2 and Y_7; and the Y_5 subtype, alone in it subfamily (Larhammar and Salaneck, 2004). Each of these receptors could be responsible for particular NPY functions. NPY and PYY have similar Y-receptor binding profiles with greatest affinity for the Y_2 receptors, followed by Y_1, Y_5, and the least affinity for Y_4 receptors (Blomqvist and Herzog, 1997; Michel et al., 1998; Khor and Baldock, 2012). In contrast, PP has a high affinity to the Y_4 receptor with much lower affinity to all other Y receptors (Blomqvist and Herzog, 1997; Khor and Baldock, 2012). Humans comprise the receptor subtypes NPY Y_1, Y_2, Y_4, Y_5 and y_6.

The general distribution of NPY receptors in the brain and its functions, as well as, the molecular signaling pathways associated with NPY receptors activation are reported in the following tables (Table 1.5 and 1.6, respectively).

Table 1.5 - General overview of mammalian NPY receptor family in brain.

NPY receptor	*Agonists*	*Antagonists*	*Brain Localization*	*Examples of Functions in CNS*	*References*
NPY Y_1	[Leu^{31},Pro^{34}]NPY [Leu^{31},Pro^{34}]PYY [Pro^{34}]PYY [Pro^{34}]NPY	BIBP3226 BIBO3304	Hypothalamus, cerebral cortex, hippocampus, amygdala, thalamus.	Increases appetite; Anxiolytic and antidepressant effects; Proliferation; Alcohol consumption regulation; Neuroprotection.	(Zukowska-Grojec et al., 1998; Sajdyk et al., 1999; Corp et al., 2001; Redrobe et al., 2002; Thiele et al., 2002; Silva et al., 2003, 2005)
NPY Y_2	PYY-(3-36) NPY 3-36	BIIE0246 JNJ-5207787	Hippocampus, hypothalamus, thalamus, amygdale, brainstem and cortex.	Appetite regulation; Anxiolytic and Anti-epileptic action; Neuroprotection; Learning and memory; Circadian rhythms regulation.	(El Bahh et al., 2002; Sainsbury et al., 2002; Redrobe et al., 2003, 2004; Silva et al., 2005; Soscia and Harrington, 2005; Santos-Carvalho et al., 2013)
NPY Y_4	PP	NA	Hypothalamus, frontal brain, hippocampus. Thalamus and amygdala.	Food intake regulation; Luteinizing hormone release; Neuroprotection.	(Horvath et al., 2001; Campbell et al., 2003; Silva et al., 2005; Santos-Carvalho et al., 2013)
NPY Y_5	[Ala^{31},Aib^{32}]NPY	L-152,804	Hypothalamus, thalamus, amygdala, hippocampus and striatum.	Appetite regulation, Anxiolytic and anticonvulsant effects, Neuroprotection; Circadian rhythms regulation.	(Mashiko et al., 2003; Benmaamar et al., 2005; Silva et al., 2005; Morales-Medina et al., 2012; Santos-Carvalho et al., 2013)

Table 1.6 - Signal transduction induced by NPY receptors subtypes activation.

NPY receptor	*Signal transduction*	*References*
NPY Y_1	-Inhibition of adenylate cyclase by $G_{i/o}$ activation; -Intracellular Ca^{2+} mobilization; -Modulation of K^+ and Ca^{2+} channels; -MAPK pathway; -PI3K pathway; -PKA modulation; -PKC activation.	(Selbie et al., 1995; Mannon and Raymond, 1998; Nie and Selbie, 1998; Sun et al., 1998; Mullins et al., 2002; Holliday et al., 2004; Aveleira and Botelho, et al., 2015)
NPY Y_2	-Inhibition of adenylate cyclase by $G_{i/o}$ activation; -Intracellular Ca^{2+} mobilization; -Modulation of K^+ and Ca^{2+} channels; -MAPK pathway; -PKA modulation; -PI3K pathway; -PKC activation.	(Gerald et al., 1995; Nie and Selbie, 1998; Sun et al., 1998; Mullins et al., 2002; Holliday et al., 2004; Rosmaninho-Salgado et al., 2012)
NPY Y_4	-Inhibition of adenylate cyclase by $G_{i/o}$ activation; -Intracellular Ca^{2+} mobilization; -Modulation of K^+ and Ca^{2+} channels; -MAPK pathway; -PKC activation.	(Bard et al., 1995; Sun et al., 1998; Mullins et al., 2002; Holliday et al., 2004)
NPY Y_5	-Inhibition of adenylate cyclase by $G_{i/o}$ activation; -PI3K pathway; -MAPK pathway; -PKA modulation; -PKC activation.	(Pellieux et al., 2000; Mullins et al., 2002; Holliday et al., 2004; Igura et al., 2011; Rosmaninho-Salgado et al., 2012; Aveleira and Botelho, et al., 2015)

MAPK: Mitogen-activated protein kinase; PI3K: Phosphatidylinositol-3-Kinase; PKA: Protein kinase A; PKC: Protein kinase C.

1.2.3 NPY in the hypothalamus

NPY is produced in high levels in the hypothalamus where it has relevant functions namely on the regulation of food intake, energy expenditure, circadian rhythm, reproduction and sexual behavior (Kalra and Kalra, 2004; Silva et al., 2005). As a potent endogenous orexigenic factor, NPY levels increase in the arcuate nucleus (ARC) of the hypothalamus by caloric restriction (CR) (Brady et al., 1990; Bi et al., 2003; de Rijke et al., 2005; Minor et al., 2009).

Therefore, the hypothalamus is a brain region which regulates homeostasis by integrating external and internal sensory signals, processing them, then prosecuting regulatory autonomic signals and neuroendocrine releasing peptides to maintain homeostasis (Biran et al., 2015). In vertebrates, the hypothalamus is localized ventrally to the thalamus, dorsally to the anterior pituitary and is composed of several nuclei of interconnected cell populations (Figure 1.5). Several neuronal types are present in each nucleus, and they work in an organized manner within and between nuclei to regulate several physiological functions. Metabolism regulation,

water balance, satiety, reproductive physiology, circadian rhythm, thermoregulation, emotional responses and aging are physiological mechanisms regulated by the hypothalamus (Zhang et al., 2013; Biran et al., 2015), in those nuclei, namely the arcuate nucleus (ARC), paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), dorsomedial hypothalamus (DMH), lateral hypothalamic area (LH) and suprachiasmatic nucleus (SCN), known as the internal biological clock (Kalra et al., 1999).

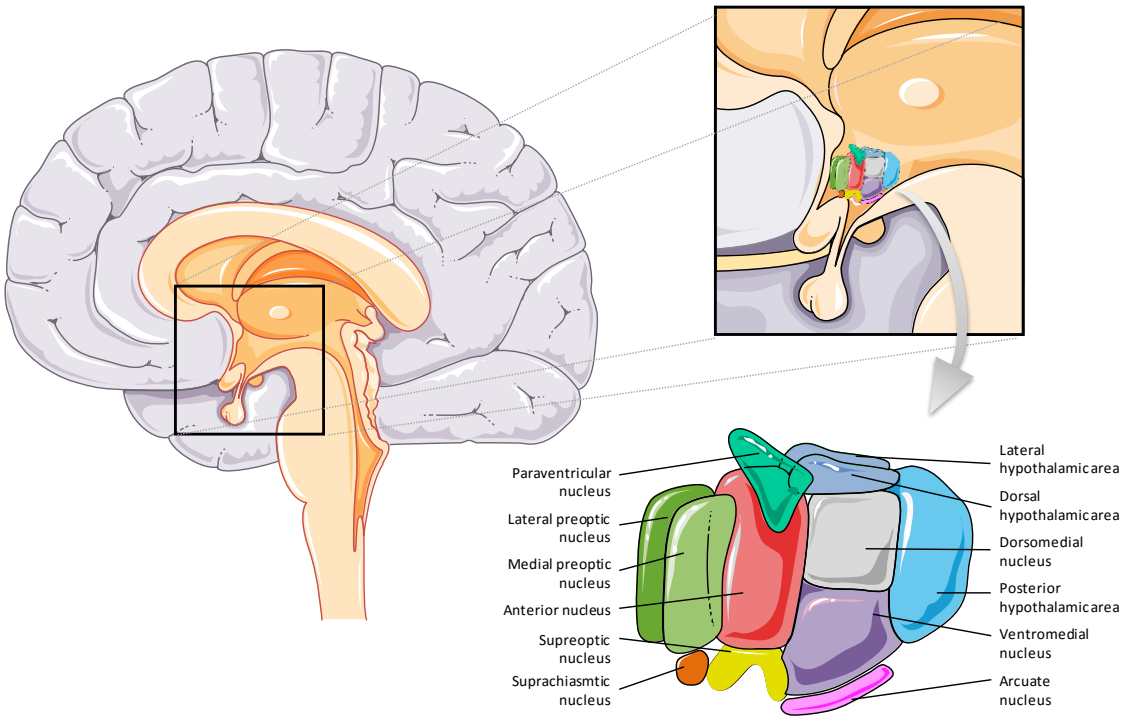

Figure 1.5 - Anatomical localization of the hypothalamus and hypothalamic nuclei in human brain. Sagittal section near the midline of the human brain (left panel) shows the hypothalamus localization in the botton of brain, above the brainstem (black box). High-magnifications (right panels) represent the three-dimenensional organization of hypothalamic nuclei. Adapted from Selvier Medical Art.

1.2.3.1 Hypothalamus and regulation of energy balance

The hypothalamus is a critical center for regulation of energy balance, a crucial mechanism for the maintenance of life. Regulation of energy homeostasis involves a crosstalk between the appetite-regulating network, the energy expenditure network and peripheral metabolic organs. The principal hypothalamic area containing these circuits is the ARC, but within the hypothalamus the PVN and DMH also have a role in regulation of energy balance (Kim et al., 2014). The hypothalamic ARC is the primary feeding control area. It is a region close to the third ventricle and the median eminence (Kalra et al., 1999).

Hormonal signals, such as leptin, insulin and ghrelin, from peripheral metabolic organs reach the ARC, due to the absence of an intact blood brain barrier in this region (Norsted et al., 2008). Neurons of the ARC receive and integrate these metabolic hormonal signals through specific receptors (Unger et al., 1989; Schwartz et al., 1996b; Harrold et al., 2008) and synthesize appetite-related neuropeptides (Schwartz et al., 2000; Stanley et al., 2005). The circulating factors modify principally the activity of two functionally opposing neuron populations within ARC: the agouti-related peptide (AgRP)/ NPY-expressing neurons and pro-opiomelanocortin (POMC) and cocaine-amphetamine-regulated transcript (CART)-expressing neurons (Schwartz et al., 2000). Moreover, a subset of ARC NPY neurons are also a γ-aminobutyric acid (GABA) co-producing neurons (Horvath et al., 1997) (Figure 1.6).

In detail, leptin is produced by fat cells and is expressed according to the size of fat stores (Friedman, 2002). Together with pancreas derives hormone, insulin, they regulate the signaling brain of energy status, triggering a negative energy balance. As a majority of both POMC/CART and NPY/AgRP neurons co-express leptin receptors (Elmquist et al., 1999; Schwartz et al., 2000), leptin is able to activate POMC/CART neurons and inhibit NPY/AgRP neurons (Elias et al., 1998a, 1999; Cowley et al., 2001). The same inhibitory effect on NPY/AgRP can be seen under insulin surge that also activates the POMC/CART neurons, since the ARC exhibit a high expression of insulin receptors (Baskin et al., 1988; Sipols et al., 1995), decreasing the food intake. Beyond leptin and insulin signals, satiaty information generated during the course of meals has a major effect in decreasing meal size. Satiety signals, such as amylin, glucagon-like peptide 1 and cholecystokinin, can be secreted in response to either mechanical or chemical stimulation of the stomach or small intestine during ingestion of food. Many of the signals provide information to the brain directly by penetrating the blood-brain-barrier and interacting with specific neuronal receptors, or indirectly by stimulating neurons that in turn forward signals to the brain, influencing energy homeostasis.

In addition, ghrelin, the hunger hormone predominantly secreted by specialized endocrine cells of the stomach when it is empty, has been reported to have a central role on feeding regulation. The peripherally-secreted ghrelin exerts its orexigenic effects through the growth hormone secretagogue receptor (GHSR), where in the hypothalamus is mainly express in the AgRP/NPY neurons (Willesen et al., 1999; Mondal et al., 2005). Therefore, ghrelin stimulates AgRP/NPY neurons and inhibits POMC neuron activity, an effect that could be mediated by the activation of AgRP/NPY neurons (Cowley et al., 2003), thereby stimulating food intake in an attempt to restore energy homeostasis.

Afterwards, neuronal projections from the NPY/AGRP and POMC/CART neurons converge to hypothalamic nuclei including the PVN, LH, DMH and VMH (Eskay et al., 1979; Sim and Joseph, 1991; Elias et al., 1998b; Bagnol et al., 1999; Broberger, 1999; Haskell-Luevano et al., 1999), where neuropeptides are released and act on secondary neurons (Figure 1.6). NPY can also be produced by hypothalamic DMH, through only transiently during certain periods of negative energy balance (Grove et al., 2001, 2003), and these neuronal NPY population project mainly to the brainstem and sympathetic nervous system (SNS) (Mercer et al., 2011). Among these, the PVN is a crucial site of action and integration of feeding signals (Cowley et al., 2001) once most of the axons of ARC neurons project to PVN (Baker and Herkenham, 1995; Elmquist et al., 1998; Bagnol et al., 1999; Parker and Herzog, 1999). Second-order neurons in the PVN express neuropeptides such as Thyrotropin-releasing hormone (TRH), Corticotrophin-releasing hormone (CRH) and Oxytocin, making the connection to the neuroendocrine system (Schwartz et al., 2000) (Figure 1.6). While the neuronal population in the LH expresses Orexins (Hypocretins) and Melanin-concentrating hormone (MCH), and project to the central cortex, suggesting an interaction to conscious behavior (Schwartz et al., 2000; Broberger and Hökfelt, 2001).

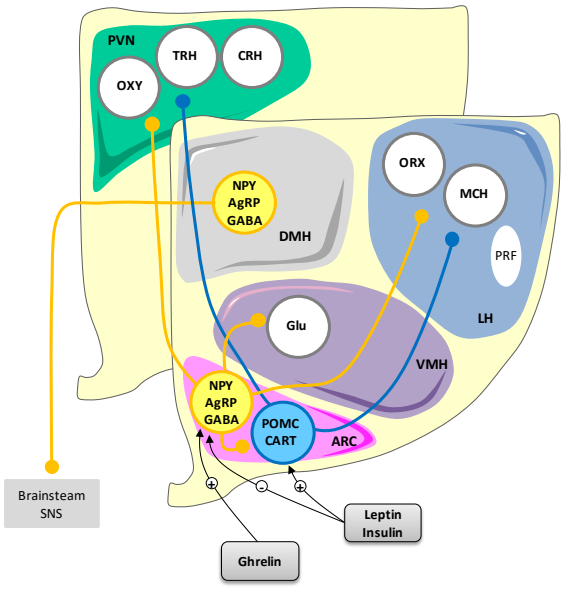

Figure 1.6 - NPY/AGRP and POMC/CART neurons in the arcuate nucleus (ARC). AGRP and NPY stimulate food intake and decrease energy expenditure, whereas α-melanocyte stimulating hormone (a post-translational derivative of POMC) and CART inhibit food intake and increase energy expenditure. Leptin and insulin activate NPY/AgRP neurons and inhibit POMC/CART neurons; ghrelin can activate NPY /AgRP neurons, thereby stimulating food intake. First-order ARC NPY/AgRP and POMC/CART neurons send projections to second-order neurons in the paraventricular nucleus (PVN) and lateral hypothalamus (LH), which express feeding-related peptides and make the connection to the neuroendocrine system, brain stem and higher centers in the brain. The density of NPY fibers also reaches the glutamatergic neurons in ventromedial hypothalamus (VMH). The main efferent projections from NPY neurons in the dorsomedial nucleus (DMH) are within the brainstem and sympathetic nervous system (SNS). TRH, Thyrotropin-releasing hormone; CRH, Corticotrophin-releasing hormone; OXY, Oxytocin; ORX, Orexins; MCH, Melanin-concentrating hormone; Glu, Glutamate; PRF, Perifornical Region. Adapted from (Mercer et al., 2011).

1.2.3.2 Neuropeptide Y receptors in the hypothalamus

The distribution of the hypothalamic NPY receptors shows individual patterns in hypothalamic neurons, which are important to understand NPY-mediated effects.

Within the ARC, NPY Y_1 and Y_5 receptors are located at the postsynaptic membrane, whereas the Y_2 receptors are found both postsynaptically and at the presynaptic terminal (Broberger et al., 1997; Fetissov et al., 2004b; Ghamari-Langroudi et al., 2005; Chee and Colmers, 2008) (Figure 1.7).

Outside the ARC, the expression of NPY Y_1 receptors has been reported on hypothalamic nuclei that receive NPY projections like the PVN, VMH, LH and SCN (Larsen et al., 1993; Broberger, 1999; Wolak et al., 2003). The distribution of NPY Y_5 receptor in the hypothalamus is similar to the distribution of NPY Y_1 and includes the PVN, LH and supraoptic nucleus (Gerald et al., 1996; Nichol et al., 1999; Wolak et al., 2003; Morin and Gehlert, 2006). NPY Y_2 receptor is also present in PVN, DMH and in medial preoptic nucleus (Dumont et al., 1998; Fetissov et al., 2004a; Stanic et al., 2006). NPY Y_4 receptors are present in LH, specially in orexin-neurons (Campbell et al., 2003) which also receive NPY projections from the ARC (Horvath et al., 1999).

In the hypothalamus, NPY has an inhibitory tonus on target neurons. In fact, the firing activity of NPY-sensitive neurons is inhibited upon postsynaptic activation of Y_1 or Y_2 receptors in the VMH and ARC, or Y_4 receptors in the LH (Cowley et al., 1999; Campbell et al., 2003; Fu et al., 2004; Chee et al., 2010). However, NPY does not act directly in PVN neurons (Cowley et al., 1999), but rather modulates PVN neuronal activity by inhibition of GABA interneurons, through pre-synaptic activation of NPY receptors Y_1, Y_2 and Y_5 (Cowley et al., 1999; Pronchuk et al., 2002; Pronchuk and Colmers, 2004). This effect produces a decreased in GABA release and disinhibition of the neurons receiving NPY-sensitive GABA inputs, increasing their output (Cowley et al., 1999; Pronchuk and Colmers, 2004; Chee and Colmers, 2008). In summary, NPY actions on feeding circuits in the hypothalamus include both direct inhibition of target neurons and indirect disinhibition of second-order neurons (Chee and Colmers, 2008). These apparent contradictory actions probably depend on the orexigenic/anorexigenic phenotype of target cells; however more detailed studies are needed to understand the nature of NPY target neurons.

1.2.3.3 Regulation of neuropeptide Y levels in the hypothalamus

The hypothalamic NPY expression is regulated by multiple neural and peripheral signals including the food deprivation, hormones and circadian clock.

During food deprivation and food restriction in rodents, the levels of NPY increases in the ARC (Beck et al., 1990; Brady et al., 1990; Swart et al., 2002; Bi et al., 2003; de Rijke et al., 2005) with the consequent increase of NPY content in the PVN (Sahu et al., 1988; Beck et al., 1990; Kalra et al., 1991). Moreover, the NPY levels return to initial values within 6 to 24 hours after re-feeding (Beck et al., 1990; Kalra et al., 1991; Sánchez et al., 2008). Therefore, NPY release is enhanced immediately prior to the onset of feedind and, thereafter, levels decreases significantly during the course of eating (Kalra et al., 1991).

In addition, NPY levels in ARC NPY neurons is regulated by peripheral hormones, namely by hunger signals, such as ghrelin, and by satiety-signaling hormones, like insulin and leptin (Näslund and Hellström, 2007). Ghrelin is an orexigenic peptide mainly synthesized by the stomach (Kojima et al., 1999; Ariyasu et al., 2001) and regulated by ingestion of nutrients. Peripheral ghrelin levels rise before a meal and rapidly decrease after food intake (Ariyasu et al., 2001; Cummings et al., 2001; Tschöp et al., 2001). The activation of ghrelin receptors on NPY neurons in the ARC (Willesen et al., 1999; Mondal et al., 2005) stimulates NPY production and increases food intake (Kamegai et al., 2001; Guan et al., 2010). Insulin and leptin are anorexigenic signals produced by the pancreas and white adipose tissue, respectively. Fasting suppresses the release of insulin and leptin into circulation (Malabu et al., 1992; Schwartz et al., 1992; Frederich et al., 1995; Saladin et al., 1995). Insulin and leptin receptors localized in NPY neurons in the ARC (Baskin et al., 1988, 1999; Hakansson et al., 1996; Mercer et al., 1996; Schwartz et al., 1996b) allow these hormones act to decrease NPY levels and decrease food intake (Schwartz et al., 1992, 1996a; Sipols et al., 1995).

Additionally, NPY neurons located in the ARC are activated by low glucose concentrations, *in vivo* and *in vitro* (Akabayashi et al., 1993; Muroya et al., 1999; Sergeyev et al., 2000; Fioramonti et al., 2007). Fatty acids can also signal nutrient availability in the hypothalamus, once central administration of long-chain fatty acids, namely oleic acid and conjugated linoleic acid, inhibits food intake and decreases the hypothalamic expression of NPY (Obici et al., 2002; Cao et al., 2007). It was also reported that high fat diet with higher levels of saturated fatty acids decreases NPY expression in the ARC compared to a high fat diet with higher levels of polyunsaturared acids (Wang et al., 2002; Barson et al., 2012).

1.2.3.4 Other neuropeptides in arcuate nucleus

Pro-opiomelanocortin (POMC) is a melanocortin precursor molecule expressed in ARC neurons, acting to decrease food intake (Giraudo et al., 1998). Most of the POMC-expressing neurons are located in the ARC of the hypothalamus, but they are also present in the nucleus

of the solitary tract (NTS) of the brainstem and pituitary gland (Dubé et al., 1978; Eskay et al., 1979; Yamazoe et al., 1984).

Different melanocortins result from the cleavage of POMC precursor depending on the tissue-specific proteases and exert biological actions by melanocortin receptors. The different POMC-derived peptides include adrenocorticotropin hormone (ACTH) and α-, β- and γ-melanocyte–stimulating hormones (MSH) (Cone, 2005), being α-MSH a key melanocortin system component implicated in energy homeostasis (Wardlaw, 2011). POMC cleavage product α-MSH acts as an agonist at melanocortin 3 (MC3R) and melanocortin 4 receptors (MC4R) in the hypothalamus. The MC4R is widely distributed throughout the brain and highly present within the hypothalamus, specifically in neurons of the PVN, VMH, supraoptic nucleus (SON) and perifornical hypothalamus (PFR) (Mountjoy et al., 1994; Kishi et al., 2003; Liu et al., 2003; Siljee et al., 2013). MC3R is localized in the ARC (Roselli-Rehfuss et al., 1993). Activation of these receptors leads to a reduction in food intake and an increase in energy utilization.

Additionally, POMC neurons in the ARC also express non-melanocortin molecules including the anorexigenic peptide CART (Vrang et al., 1999), which is less studied due to the lack of understanding of the receptors for this peptide.

Agouti-related protein (AgRP) is a 132-amino acid orexigenic neuropeptide synthesized only in the ARC of the hypothalamus (Shutter et al., 1997), where is co-localized with NPY in the NPY/AgRP neurons and released to the PVN (Broberger et al., 1998; Cowley et al., 1999). Like NPY, AgRP mRNA increases during fasting (Hahn et al., 1998; Mizuno and Mobbs, 1999), and its levels is also regulated by satiety hormones leptin and insulin. In fact, leptin decreases AgRP levels (Mizuno and Mobbs, 1999) while leptin-deficiency mice show elevated AgRP content compared to wild-type (Shutter et al., 1997).

Despite of the similarities between AgRP and NPY on feeding regulation, they act through different mechanisms to increase food intake. AgRP acts as endogenous antagonist of the MC3R and MC4R, to counterwork the anorectic effect of α-MSH (Ollmann et al., 1997; Haskell-Luevano et al., 2001). Therefore, release of endogenous MC3/MC4 receptor antagonist AgRP in the PVN represents an additional mechanism to increase food intake after activation of NPY/AgRP neurons.

Additionally, the modulatory feeding role of AgRP may be more important during conditions of high energy requirements, such as pregnancy and lactation (Sorensen et al., 2002).

1.2.3.5 Interplay between NPY/AGRP neurons and POMC/CART neurons

The integrated model of feeding regulation includes functional connections between NPY/AgRP neurons and POMC/CART neurons, within the hypothalamic ARC (Figure 1.7).

Within ARC, NPY/AgRP and POMC/CART neurons establish a reciprocal innervations mainly formed by dense NPY/AgRP fibers projecting to POMC cell bodies (Cowley et al., 2001). NPY directly activates NPY Y_1 and Y_2 receptors expressed by POMC neurons, leading to membrane hyperpolarization and an inhibition of firing activity (Cowley et al., 2001; Roseberry et al., 2004; Acuna-Goycolea and van den Pol, 2005; Ghamari-Langroudi et al., 2005). Additional involvement of the NPY Y_5 receptor has been implicated in the regulation of spontaneous release of α-MSH from POMC neurons (Galas et al., 2002), further supporting the NPY-mediated inhibition of the anorexigenic melanocortin system. Moreover, presynaptic NPY Y_2 receptors are also present in ARC NPY neurons implying a local inhibitory feedback on the orexigenic signal (Broberger et al., 1997; Acuna-Goycolea and van den Pol, 2005). In addition, AgRP itself acts as an endogenous antagonist of both MC3R and MC4R (Fong et al., 1997), blocking the actions of α-MSH in downstream hypothalamic nuclei, and inhibiting POMC neurons directly through the MC3Rs that they express (Jégou et al., 2000). In summary, activation of NPY/AgRP neurons by orexigenic peptides like ghrelin acts to increase food intake by complementary mechanisms: a) stimulation of feeding by direct activation of NPY receptors in the PVN; b) antagonism of the MC3R and MC4R activation by AgRP in the ARC and PVN; c) inhibition of the anorexigenic POMC neurons in the ARC via NPY Y_1 and Y_2 receptors and also by GABA release.

On the other hand, satiety signals like leptin also operate trough complementary mechanisms to decrease food intake: a) inhibition of feeding directly by activation of POMC neurons with release of α-MSH in the PVN; b) inhibition of MC4R antagonist AgRP release in the PVN; c) activation of MC3R and MC4R inhibits ARC NPY/AgRP neurons; d) decrease release from NPY/GABA terminals, wherein diminished GABA release desinhibits the POMC neurons.

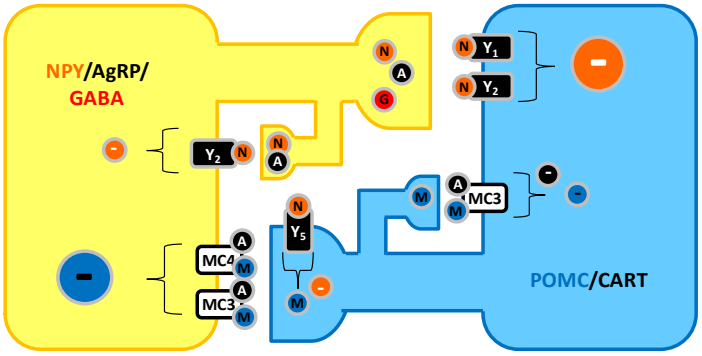

Figure 1.7 - Reciprocal innervation between NPY/AgRP and POMC/CART neurons within the arcuate nucleus (ARC) of the hypothalamus. Nerve terminals from NPY/AGRP neurons densely innervate POMC/CART neurons. Release of NPY (N, orange) from ARC NPY/AgRP neurons activates postsynaptic NPY Y_1 and Y_2 receptors to directly inhibit (-) anorexigenic ARC POMC/CART neurons and activates NPY Y_5 receptors to inhibit α-MSH (M, blue) release. GABA release (G, red) also inhibits POMC/CART neurons. Activation of MC3R or MC4R inhibits ARC NPY/AgRP neurons; this action may be inhibited by the release of AgRP (A, black), the endogenous antagonist of MC3R and MC4R. Taken together, these actions provide a robust, multiplex mechanism by which NPY/AgRP neurons mediate orexigenic activity in the hypothalamus. Abbreviations: AgRP, Agouti-related peptide; GABA, γ-amminobutiric acid; NPY, Neuropeptide Y; POMC, Proopiomelanocortin; MC3/MC4R, melanocortin-receptor type 3 and 4; $Y_1/Y_2/Y_5$, Neuropeptide Y receptor type 1, 2 and 5. Adapted from (Mercer et al., 2011).

1.2.4 Neuropeptide Y: an anti-aging player?

Several studies suggest that the NPY system is linked to the aging process. Long-lived female centenarians have higher NPY plasma levels compared to younger women (Baranowska et al., 2006), and transgenic rats overexpressing NPY live longer (Michalkiewicz et al., 2003), while NPY Y_2-receptor knockout mice show learning deficits and memory deterioration, a decline in cognitive function also observed in aging (Hedden and Gabrieli, 2004; Redrobe et al., 2004). In contrast, NPY plasma levels in humans decline with increasing age (Chiodera et al., 2000). In animal models of aged rodents, and in brain samples that have suffered neurodegenerative diseases, levels of NPY and NPY receptors also decrease in several brain areas (Table 1.7). In addition, reduced NPY is associated with the development of "anorexia of aging", characterized by reduced food intake and body weight (Matsumoto et al., 2000; Morley, 2001).

The major neuroendocrine effect of caloric restriction (CR) is the increase of NPY in the arcuate nucleus of the hypothalamus, due to its hyperphagic effect and to recover the low energy availability induced by CR, the most robust anti-aging strategy described in section 1.1.3.1 (de Rijke et al., 2005; Bi, 2007; Minor et al., 2009). Although it is not yet well established whether NPY is essential to CR effects, the increase in NPY can lead to several physiological modifications similar to those induced by CR, namely the reduction of core body temperature, the blood glucose levels and the fertility, and the increase of glucocorticoid secretion (Minor et al., 2009). Thereby, is NPY a mediator of the beneficial effects of CR? Increasing evidence show

the involvement of NPY in the beneficial effects of CR: 1) NPY is implicated in tumorigenesis repression induced by CR (Minor et al., 2011; Chiba et al., 2014); 2) CR does not increase lifespan in NPY knockout mice (Chiba et al., 2014); and 3) recently, it was suggested that the thermoregulatory role of NPY could represent a potential mechanism underlying the anti-inflammatory effects of CR in hypothalamus (Radler et al., 2015). However, the mechanisms underlying the role of NPY on CR-beneficial effects are not completely known. Moreover the contribution of NPY on lifespan extension remains to be explored.

Moreover, NPY is able to interfere in six of the nine cellular hallmarks of aging: loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication, as discussed below. There are no reports on the other three hallmarks of aging (genomic instability, epigenetic alterations and telomere attrition) that are related to NPY.

Table 1.7 - Alterations of NPY levels and its receptors in aged brain and in neurodegenerative diseases.

Brain Region	*Relative NPY levels in brain areas of*				*Relative NPY receptors levels of*			*References*
	rat aged brain ( )*	*Alzheimer's disease patients (**)*	*Parkinson's disease patients (**)*	*Huntington 's disease patients (**)*	*Y_1 in rat aged brain (*)*	*Y_2 in rat aged brain (*)*	*Y_5 in rat aged brain (*)*	
Cortex	↓ NPY-IR ↓ NPY-IR neurons	↓ NPY-IR ↓ NPY-IR neurons	No alteration ↓ NPY-IR	No alteration ↑ NPY-IR	↓ IR in cingulate cortex	N.D.	↓ IR in superficial layers	(Allen et al., 1985, 1987a; Dawbarn et al., 1985; Beal et al., 1988; Davies et al., 1990; Kowalski et al., 1992; Cha et al., 1997; Huh et al., 1997; Mazurek et al., 1997; Veyrat-Durebex et al., 2013)
Hippocampus	↓ NPY-IR ↓ NPY-IR neurons	↓ NPY-IR ↓ NPY-IR neurons	≈	≈	↓ IR in CA1	↓ IR in CA1	↓ IR in CA2	(Allen et al., 1985, 1987b; Dawbarn et al., 1985; Chan-Palay et al., 1986; Higuchi et al., 1988; Kowalski et al., 1992; Cha et al., 1997; Cadiacio et al., 2003; Veyrat-Durebex et al., 2013)
Hypothalamus	↓NPY mRNA ↓ NPY-IR	ND	ND	≈	↓ IR ARC, LH ↑ IR VMH ↓ mRNA PVN	↓ IR anterior hypothalamus	↓ IR ARC ↓ NPY mRNA PVN	(Dawbarn et al., 1985; Kowalski et al., 1992; Gruenewald et al., 1994; Sohn et al., 2002; Coppola et al., 2004; Veyrat-Durebex et al., 2013)
Olfactory nucleus	↓ NPY-IR neurons	ND	ND	ND	ND	ND	ND	(Hwang et al., 2001)
Striatum	↓ NPY-IR ↓ NPY-IR neurons	ND	↑ NPY mRNA	↑ NPY-IR neurons ↑ NPY-IR	ND	≈	ND	(Dawbarn et al., 1985; Allen et al., 1987a; Beal et al., 1988; Higuchi et al., 1988; Kowalski et al., 1992; Zoli et al., 1993; Huh et al., 1997; Mazurek et al., 1997; Cannizzaro et al., 2003; Veyrat-Durebex et al., 2013)
Medulla	↓ NPY-IR	ND	ND	ND	ND	ND	ND	(Higuchi et al., 1988)
Thalamus	ND	ND	ND	ND	↓ IR	≈	↓ IR	(Veyrat-Durebex et al., 2013)
Spinal cord	↓ NPY-IR	ND	ND	ND	ND	ND	ND	(Higuchi et al., 1988)

↓: lower levels; ↑: higher levels; ≈: similar levels (*) compared to young adults; (**) compared to controls (age-matched brain); ND: not determined; IR: immunoreactivity; VMH : Ventromedial nucleus; PVN: Paraventricular nucleus; LH : Lateral hypothalamus; ARC: Arcuate nucleus.

Does NPY change loss of proteostasis related to aging?

Molecular chaperones are modulators of protein homeostasis, mediating protein folding and stability. Pharmacological induction of chaperones contributes to stable cellular conditions, as has been shown with the heat-shock protein 72 (HSP72), which preserves muscle function and delays the progress of dystrophic pathology (Gehrig et al., 2012). In the human neuroglia cell line, NPY increased the expression and release of HSP72 (Panossian et al., 2012). Both the effectiveness of this NPY-induced HSP72 effect on muscular dystrophy and its involvement with chaperone activity should be explored more fully. In addition to this chaperone activity, the autophagy-lysosomal system is also a proteolytic system implicated in protein quality control. The NPY effect on autophagy will be discussed further in chapther 3 of this dissertation.

Does NPY change nutrient-sensing deregulation observed in aging?

Growth hormone (GH) and sirtuins (Sirt) are examples of critical nutrient signaling pathways involved in aging and aging-related diseases (Barzilai et al., 2012). Sirt1 activation can improve health and survival, while reduction of GH, and signaling by its downstream effector insulin-like growth factor 1 (IGF-1) extends longevity (Barzilai et al., 2012). The involvement of the transcription factor forkhead box O (FOXO) on longevity in worms and flies is well established. Sirt1 overexpression, or a reduction on the insulin/IGF-1 signaling flux, activate the FOXO orthologue, and, in this way, promote longevity (Berdichevsky et al., 2006; Kenyon, 2010).

Since the beneficial effects of caloric restriction could be mediated, at least in part, by the molecular pathways mentioned above, and NPY is critical for these effects, we predict that NPY could interfere with signaling pathways involving FOXO and/or GH. However, few studies have addressed these issues. In one report that did examine this issue, in mammalian cells and in *Drosophila melanogaster* orthologue, it was shown that NPY upregulates the dual specificity tyrosine-phosphorylation-regulated kinase 1a (Dyrk1a) expression that activates Sirt1 deacetylase, which in turn deacetylates and activates FOXO (Hong et al., 2012).

Results on the impact of NPY on GH levels are controversial, and are most probably species-dependent. In rats, intracerebroventricular NPY injection reduces circulatory GH levels, with this effect mediated by both the Y_1- or Y_2-receptors (Härfstrand et al., 1987; Suzuki et al., 1996). However, in pituitary cells from pigs or goldfish, NPY stimulates GH secretion (Peng et al., 1993; Barb and Barrett, 2005), and the intracerebroventricular injection of NPY in pigs did not change circulating GH levels (Cho et al., 2010). Since the reduced functions of the GH/IGF-1

axis have been linked to greater longevity, the effect of NPY on GH release should be investigated further.

The hypothalamus is implicated in central-metabolic regulation, and the NPY neurons, located in the hypothalamic arcuate nucleus and lateral hypothalamus, are metabolic sensing neurons (Maejima et al., 2011; Marston et al., 2011), as mentioned above. Thus, the orexigenic effect of NPY may have beneficial effects in "anorexia of aging", characterized by food intake decrease, possible malnutrition and increased disease risk (Morley, 2001). Since reduced NPY is associated with the development of "anorexia of aging", restore NPY levels could ameliorate the phenotype. However, in elderly subjects that become overweight and with glucose intolerance/leptin resistance, leading to obesity, diabetes and other metabolic disorders, the NPY beneficial effects could be questioned. Some studies show that NPY system is differentially changed on obese rodents. For example, in the arcuate nucleus of genetic obesity rodent models, namely in Zucker rat or in *ob/ob* mouse, the NPY levels and release is increased and receptor density is reduced (Mckibbin et al., 1991; Beck, 2006). On the other hand, in diet-induced obesity rodents and obese humans the hypothalamic NPY levels are lower compared to controls with normal body mass index (Zahorska-Markiewicz et al., 2001; Goldstone et al., 2002; Hansen et al., 2004; Beck, 2006). Therefore, taking into account the complexity of NPY system and the discrepancies found between obesity models, the net NPY effect on overweight elderly subjects should be further investigated. Finally, hypothalamic NPY cells have been implicated in the nutrient-sensing pathways regulated by caloric restriction. In fact, in NPY-knockout mice, caloric restriction was not able to induce certain beneficial effects, such as the increase of adiponectin levels in blood (Minor et al., 2011). Although these studies are not conclusive, they suggest that NPY is a mediator of nutrient-sensing pathways in the hypothalamus and, consequently, may mediate the slowing of aging effects.

Does NPY change mitochondrial dysfunction associated with aging?

The loss of mitochondrial integrity and function has a deep impact on aging, although it is still a challenge for aging research to identify the mechanisms underlying this process. In particular, the mitochondrial dysfunction associated with decreased oxidative capacity or increased oxidative damage, and mitochondrial DNA mutations, are still controversial topics, making understanding the role of mitochondria in aging a complex undertaking (López-Otín et al., 2013). Some studies have suggested that NPY may have a relevant role in mitochondrial function.

The fact that NPY polymorphism could engage mitochondrial function is one piece of evidence for its involvement in properties of mitochondria. NPY mRNA has two initiate translation sequences which allow two proteins to be formed with different intracellular locations: the NPY_{1-36} peptide, directed to secretory vesicles, and NPY_{17-36} form with a mitochondrial targeting signal. In addition, the functional Leucine[7] to Proline [7] polymorphism in the signal sequence of preproNPY affects the processing of proNPY. The L7P polymorphism increases both the levels of mature NPY release in humans after sympathetic stimulation (Kallio et al., 2001), and the mobility of the secretory vesicles (Kaipio and Pesonen, 2009). Furthermore, it has been suggested that the putative mitochondrial fragment NPY_{17-36} is attached to the outer membrane of the mitochondria, where it may affect the energy balance of the membranes of the mitochondrion in the cell (Kaipio and Pesonen, 2009). NPY also contributes to oxidant-antioxidant balance, and, in an epilepsy mouse model, has been shown to protect against oxidative stress and mitochondrial dysfunction (Kir et al., 2013). This study showed that intraperitoneal NPY administration decreased malondialdehyde levels and increased glutathione levels in the hippocampus of rats with pentylenetetrazole-induced epileptic seizures (Kir et al., 2013). Finally, the NPY system has been linked to mitochondrial bioenergetics. The lack of an NPY Y_1 receptor is associated with an increased capacity for mitochondrial fatty acid transport, β-oxidation, and, also, increases the protein levels of subunits of the respiratory chain complex and PGC-1α in muscle and liver. These effects were suggested to improve mitochondrial oxidative capacity in an NPY Y_1 deficient mouse (Zhang et al., 2010). However, more studies are needed to explore the potential involvement of the NPY system in mitochondria protection as a relevant mechanism to delay aging.

Does NPY delay cellular senescence?

Cellular senescence is characterized by an irreversible arrest of cell proliferation that occurs when a cell experiences potential stress. It is a potent suppressor mechanism, and is shared by the degeneration which occurs in cells and tissues upon aging and is characterized by gradual loss of function, and by hyperplasia pathologies, where gain-of-function changes allow cells to proliferate inappropriately (Campisi, 2013). Therefore, cellular senescence could be a beneficial compensatory response to prevent propagation of damaged, and potentially oncogenic, cells, and trigger their removal by the immune system (López-Otín et al., 2013).

Regarding the blocking proliferation induced by cellular senescence, some studies indicated that NPY changes cell proliferation. NPY increases proliferation of endothelial cells, inducing angiogenesis (Zukowska-Grojec et al., 1998); while important in skin wound repair and in

vascular ischemia, this may also contribute to tumour progression (Kitlinska et al., 2005). NPY receptor activation in tumour cells may change cell proliferation, and could also be useful to target and deliver anti-cancer drugs to tumours (Lu et al., 2010; Sheriff et al., 2010; Li et al., 2015).

In old organisms, senescent cells accumulate in tissues; this could be linked to a failure of the immune system to remove these cells (López-Otín et al., 2013; Burton and Krizhanovsky, 2014). Therefore, strategies to improve immune surveillance may prevent the accumulation of senescent cells and rescue aging phenotype. NPY has been reported to have a beneficial role in the immune system. In rodents, NPY increases lymphocyte proliferation, adherence and chemotaxis (Medina et al., 1999, 2000; Puerto et al., 2005). Although some results are controversial, NPY may increase natural killer cells activity (De la Fuente et al., 2001b; Puerto et al., 2005). It also stimulates adherence capacity, motility, ingestion and superoxide production of rodent macrophages (De la Fuente et al., 2000, 2001a) and modulates functions of neutrophils (Bedoui et al., 2008).

In conclusion, further investigation of the role of NPY in delaying or dealing with the effects of cellular senescence seems crucial. Specifically, because the elimination of senescent cells could be a conceptual intervention to delay aging, the ability of NPY to change cell proliferation and/or to stimulate the immune system to eliminate those cells should be the subject of intense study.

Does NPY delay stem cell exhaustion?

Stem cell decline, as the result of multiple types of damage, is a characteristic of aging, and decreases the regenerative potential of tissues. Stem cell rejuvenation may reverse the aging phenotype, and some studies have shown an active role of NPY in this process. NPY, through activation of Y_1 or Y_5 receptors, has the potential for maintaining self-renewal and pluripotency of human embryonic stem cells (Son et al., 2011). In addition, NPY Y_5 receptor activation increases the proliferation of rat bone marrow cells, which have declined with aging (Igura et al., 2011), and inhibits differentiation of marrow progenitor cells (Lee et al., 2010). Additionally, it was recently reported that NPY prevents bone marrow impairments in nerve-induced injury by regulating the hematopoietic stem cell microenvironment (Park et al., 2015).

It is well established that NPY has a direct effect on progenitor cell proliferation in different neurogenic niches, such as the olfactory epithelium (Doyle et al., 2008), retina (Alvaro et al., 2008a), and the subventricular (Agasse et al., 2008; Stanic et al., 2008; Thiriet et al., 2011),

subcallosal (Howell et al., 2007), and subgranular zones (Howell et al., 2005). The NPY Y_1 receptor is the main component responsible for the proliferative effect of NPY in both subgranular and subventricular zones (Howell et al., 2005; Agasse et al., 2008; Thiriet et al., 2011). In retinal progenitor cells, in addition to the Y_1 receptor, activation of NPY Y_2 or Y_5 also increases proliferation (Alvaro et al., 2008a). In addition to these effects of the proliferative factor, the intracerebroventricular injection of NPY also stimulates migration of the neuronal precursor from the subventricular zone to the olfactory bulb and to the striatum (Decressac et al., 2009; Thiriet et al., 2011), where they can differentiate (Agasse et al., 2008; Decressac et al., 2009); NPY also promotes neuronal differentiation in dentate gyrus from adult mice (Decressac et al., 2011).

The hypothalamus, where the highest amounts of NPY are produced, is also a neurogenic niche (Kokoeva et al., 2007); however the role of NPY in hypothalamic neurogenesis is not known. Since NPY hypothalamic levels (Higuchi et al., 1988; Gruenewald et al., 1994) and hypothalamic neurogenesis are reduced in aged mice (Zhang et al., 2013), it will be of interest to examine the role of NPY in the maintenance of the hypothalamic neurogenic niche.

Overall, the ability of NPY to stimulate stem cell self-renewal and the proliferation, migration and differentiation of neural precursor cells could counteract tissue aging-associated damages triggered by stem cell exhaustion. This NPY effect could be used as a strategy in cell replacement therapy in neurodegenerative disorders.

Does NPY promote intercellular communication and reduce inflammation?

Both the reduction of intercellular communication and overall inflammation are important hallmarks of aging that NPY may have the ability either to inhibit or to delay.

NPY is able to enhance gap junctional intercellular communication between bone cells in vitro, inducing the proliferation and activity of osteoblasts, and, consequently, improve age-related bone impairments (Ma et al., 2013).

Recently, Zhang and colleagues showed that the hypothalamic inflammatory changes mediated by IKKβ/NF-κB underlie the whole-body aging process. In turn, IKKβ/NF-κB activation inhibits GnRH (Gonadotropin releasing hormone) release to mediate aging-related hypothalamic GnRH decline, while GnRH therapy prevents aging-impaired neurogenesis and decelerates aging development (Zhang et al., 2013). NPY is able to inhibit GnRH neurons' excitability through Y_1 receptors and to stimulate the same neurons through Y_4 receptor activation (Roa and Herbison, 2012). Although GnRH neurons are known to respond differently

according to the type of NPY receptor activated, more studies should be performed to clarify the role of NPY in aging-delay via GnRH modulation.

Some studies have shown that NPY has anti-inflammatory properties in murine macrophages and microglia; acting to decrease levels of pro-inflammatory cytokines, such as TNF-α and IL-1β (De la Fuente et al., 2001a; Puerto et al., 2005; Ferreira et al., 2010; Li et al., 2014). NPY can also inhibit lipopolysaccharide-induced microglia phagocytosis and motility (Ferreira et al., 2011, 2012). It has also been reported that NPY increases TGF-β1 (transforming growth facto beta 1) production in murine macrophage-like cell line (Zhou et al., 2008). Finally, NPY-stimulated dendritic cells produce lower levels of IL-12 and promote a Th2 polarization of naïve T cells, increasing production of cytokines IL-6 and IL-10 (Buttari et al., 2014).

Although some studies have reported a relationship between NPY and atherosclerosis, this is still controversial. Systemic administration of an NPY Y_1 receptor antagonist increases atherosclerotic lesion area in a hypercholesterolemic mouse model (Jääskeläinen et al., 2013). In addition, several single nucleotide polymorphisms in the NPY gene and a gain-of-function polymorphism have been associated with increased atherosclerosis in human (Karvonen et al., 2001). Furthermore, another study suggested that NPY increases proinflammatory cytokine release from isolated rodent mast cells and may contribute to lesion progression and vascular inflammation in atherosclerosis (Lagraauw et al., 2014).

These studies suggest, within the context of intercellular communication, that NPY acts as a fine regulator in inflammatory response. Modulation of NPY levels could, thus, preserve cell-cell contacts and counteract the age-associated inflammation.

1.3 Autophagy

Normal cell growth and development requires a well-controlled balance between biosynthesis and catabolic processes. Protein synthesis and organelle biogenesis versus protein degradation and organelle turnover are antagonistic processes that regulate cellular homeostasis (Klionsky and Emr, 2000; Wang and Klionsky, 2003; He and Klionsky, 2009). Therefore, depending on the availability of nutrients and environmental conditions, cells have specific mechanisms to regulate either growth and biosynthesis, or constituents' turnover and nutrient recycling. In eukaryotic cells, there are two major pathways for large-scale cellular constituents degradation: the ubiquitin-proteasome system (UPS), which is mostly responsible for the turnover of short-lived proteins (Ciechanover and Brundin, 2003; Inobe and Matouschek, 2014) and the lysosomal system, the only known mechanism that cells possess to dispose long-lived proteins, protein aggregates and intracellular organelles. At least four different delivery pathways to the lysosome of substrate proteins and cytosolic constituents for degradation are known (Mizushima, 2004). One of the four pathways is endocytosis/phagocytosis, which mediates the degradation of extracellular materials and plasma membrane proteins. The other three pathways are characterized by the sequestration of cytosolic components and subsequent delivery to the lysosome. They are intended for the turnover of intracellular constituents and are considered the three different types of autophagy (Figure 1.8).

The term autophagy, coined from the Greek words of αυτς, meaning 'autos' (self) and φαγειν ('phagein'), meaning 'eating', was first used by Christian de Duve at the CIBA Foundation Symposium on Lysosomes in 1963, to distinguish the lysosomal degradation of a part of the cell's self from the breakdown of extracellular material, establishing a nomenclature for different cellular pathways and compartments in the endosomal-lysosomal pathway (Klionsky, 2008; Jaeger and Wyss-Coray, 2009). Moreover, early work done in autophagy research was published in 1967 and autophgay was characterized as a physiological response to starvation in order to degrade and recycle non-essential intracellular macromolecules in rat liver cells (Deter and De Duve, 1967; Deter et al., 1967; Schworer and Mortimore, 1979).

1.3.1 Types and mechanisms of autophagy

Autophagy is a genetically-regulated pathway that governs the intracellular turnover of subcellular proteins, organelles, and foreign pathogens through the mobilization of lysosome-dependent degradation processes; and has an important role in regulating cell fate in response to stress (Shintani and Klionsky, 2004; Levine, 2005; Yang and Klionsky, 2010a). It is an

evolutionarily-conserved process that occurs in all species and cell types studied thus far, and provides a fundamental mechanism to maintain cellular homeostasis under normal and pathophysiological conditions (Yang and Klionsky, 2010a). Recently, increasing attention has been focused on the role of autophagy on metabolism of misfolded proteins and neuronal cell death, in neurodegeneration (Martinez-Vicente and Cuervo, 2007; Cherra and Chu, 2008; Tooze and Schiavo, 2008; Radad et al., 2015). The three main types of mammalian autophagy identified are: chaperone-mediated autophagy (CMA), microautophagy and macroautophagy, the most common subtype (Mizushima, 2004) (Figure 1.8). In CMA, target proteins containing a specific consensus peptide sequence (KFERQ) are recognized by a cytosolic chaperone (Dice, 1990), the heat shock cognate protein of 70 kDa (Hsc70) that along with its modulatory co-chaperones deliver the substrate to the lysosome (Chiang et al., 1989) across the membrane, through a specific receptor, the lysosome-associated membrane protein type 2A (LAMP-2A) (Cuervo and Dice, 1996). The substrate protein is unfolded before the internalization, a step not required in the other types of autophagy (Salvador et al., 2000). Microautophagy refers to a process by which the lysosomal membrane directly invaginates or exvaginates, in order to sequester the cytosolic component and buds into the lysosomal lumen for degradation (Ahlberg and Glaumann, 1985; Kunzt et al., 2004). Finally, macroautophagy, the most well studied type of autophagy, is characterized by the formation of a double membrane vesicle named autophagosome, which engulfs long-lived proteins and/or organelles and, subsequently fuses with a lysosome, degrades them. Autophagic cargoes, delivered to the lysosome, are digested by lysosomal hydrolases to their basic components (e.g., amino acids, fatty acids), which are released by membrane permeases, to be reutilized for biosynthetic pathways (Ravikumar et al., 2010; Mizushima and Komatsu, 2011). In addition, both micro- and macroautophagy can be selective or nonselective and these processes have been best characterized in yeast (Shintani and Klionsky, 2004).

Other specialized forms of autophagy exist, such as mitophagy, direct targeting of mitochondria to lysosomes (Tolkovsky, 2009); pexophagy, selective degradation of peroxisomes (Manjithaya et al., 2010); ribophagy, degradation of ribosomes (Beau et al., 2008); xenophagy, degradation of intracellular bacteria and viruses (Levine, 2005; Huang and Klionsky, 2007); crinophagy, lysosomal fusion with re-directed exosomes (Sandberg and Borg, 2007); and piecemeal microautophagy of the nucleus, partial sequestration and degradation of the nucleus (Kvam and Goldfarb, 2007), but most of them have only been observed in yeast or under special conditions. Therefore, the different types of autophagy mentioned differ in

mechanism whereby substrates are delivered to lysosomes, their regulation and their selectivity.

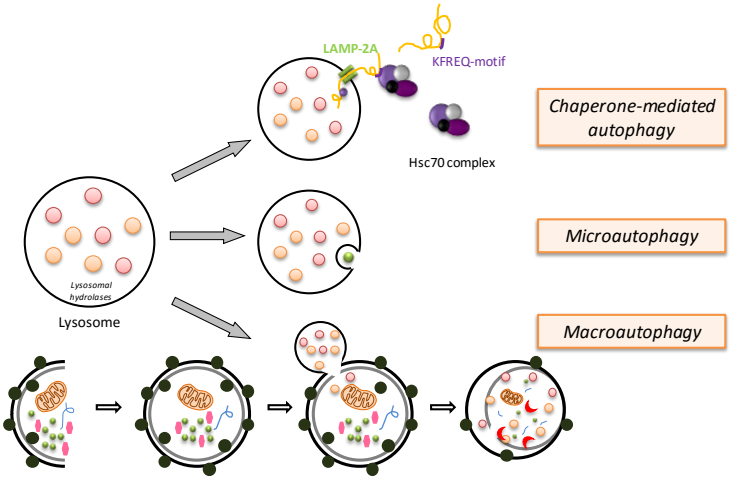

Figure 1.8 - The three different types of autophagy in mammals. During chaperone-mediated autophagy, proteins carrying the pentapeptide KFERQ-like motif are recognized by the cytosolic heat shock cognate 70 (Hsc70) complex, which then associates with the integral lysosome membrane protein LAMP-2A, and proteins are translocated into the lysosome. During microautophagy the lysosomal membrane invaginates to engulf portions of the cytoplasm, which are consequently broken down once entirely enclosed. During macroautophagy specialized double-membrane vesicles, autophagosomes, are formed for cargo transportation. Complete autophagosomes fuse with lysosome and expose their content to to the hydrolytic enzymes for degradation. Adapted from (Boya et al., 2013).

1.3.2 The autophagic machinery

Macroautophagy (hereafter referred to as autophagy) was morphologically identified in the late 1950s in mammalian cells, almost in parallel to the discovery of the lysosome through electron microscopy (Clark, 1957; Novikoff, 1959; Deter and De Duve, 1967). Although the concept is more than half a century old, the main advances in understanding the machinery behind autophagy were achieved in slight more than a decade ago (Klionsky, 2007), through the identification of autophagy-related genes (ATG). Autophagy is not only simply a degradative process, but it has an integral role in human physiology and pathophysiology, and the discovery of genes, initially in yeast, was a major milestone in understanding of autophagy mechanisms, allowing the identification of key proteins and interactions (Tsukada, 1993). Furthermore, many of the autophagy factors found in yeast have already known homologs in higher eukaryotes (Yang and Klionsky, 2009).

Thus, autophagy is a complex pathway conserved through evolution, where the coordinated actions of autophagic and non-autophagic proteins lead to the induction of autophagosome formation (I), autophagosome nucleation (II), autophagosome elongation (III), cargo recognition and selectivity (IV) and autophagosome maturation and cargo degradation (V). In

this dissertation the main focus will be on mammalian autophagy. Each of these steps involves different machinery and protein interactions that will be further elaborated below (Figure 1.9).

Induction of autophagosome formation

Autophagy induction is tightly controlled by complex regulatory mechanisms that could involve diverse input signals, including nutrients, growth factors, hormones, intracellular Ca^{2+}-concentrations, ATP levels, hypoxia, accumulation of misfolded proteins and many more. Sharing a convergent level, many signals could induce autophagy through the inhibition of the mechanistic target of rapamycin (MTOR) or through independent pathways (Wirawan et al., 2012). MTOR, an evolutionarily conserved serine/threonine protein kinase, is a key regulator of the balance between cell growth and autophagy, in response to cellular physiologic or extracellular conditions, controlling transcription, translation and metabolism (Jung et al., 2010). MTOR is known to bind several proteins, forming two distinct complexes: the mTOR complex 1 (mTORC1) and the mTOR complex 2 (mTORC2).

The mTORC1 integrates upstream nutrient and growth factor-derived signals, from several pathways, such as insulin, class I phosphatidylinositol-3-kinase (PI3KC1) and protein kinase B (PKB) (also known as AKT) (Laplante and Sabatini, 2009; Sengupta et al., 2010), and together with its downstream effector ribosomal protein S6 kinase (S6K1) have emerged as critical signaling components in the development and growth of the organism (Nobukuni et al., 2007). Upon nutrient rich conditions, this kinase is active and therefore autophagy is downregulated. An alternative pathway for direct induction of autophagy, in an MTOR independent way, can occur by different stimuli of the class III phosphatidylinositol-3-kinase (PI3KC3) or Atg6-Beclin-1, crucial for the nucleation step.

Under nutrient-rich conditions, active mTORC1 binds and phosphorylates uncoordinated-51 (unc-51)-like kinase 1 or 2 (ULK1 or ULK2) and Atg13 within the ULK complex, to repress the kinase activity of ULK1, thereby inhibiting autophagosome formation. Under starvation conditions, inhibition of mTORC1 causes mTORC1 to dissociate from the ULK1−Atg13−FIP200 complex, leading to dephosphorylation of Atg13 and ULK1 by mTORC1 and leading to autophagy induction, whereas ULK1 still phosphorylates Atg13, FIP200 and itself, resulting in the initiation of autophagy (Ganley et al., 2009; Hosokawa et al., 2009; Wong et al., 2013) (Figure 1.9A).

It is still unclear how these phosphorylation events may regulate the autophagosome formation and how the ULK1−Atg13−FIP200 complex localization at the autophagosome initiation site is coordinated. However, recently it was demonstrated that the ULK complex is involved in the proper localization of PIK3C3. In nutrient-rich conditions, the PIK3C3 complex

connects to the cytoskeleton, an interaction mediated by the activating molecule in Bcl-2-interacting protein-regulated autophagy 1 (Ambra1), which binds both the PIK3C3 complex and the microtubule-associated dynein motor complex (Di Bartolomeo et al., 2010). During starvation, ULK1 phosphorylates Ambra1, which releases Ambra1 and the PIK3C3 complex from the microtubules. The PIK3C3 complex is allocated to the ER, which is pointed as the major organelle contributing to autophagosome formation (Di Bartolomeo et al., 2010).

Autophagosome nucleation

The nucleation step overall begins with the formation of the phagophore (also called isolation membrane), a small portion of membrane which elongates to engulf the cargo and culminates with the formation of a whole vesicle, the autophagosome (Beau et al., 2011). The origin of these lipid bilayers has been under debate for many years and is still uncertain. There are several hypotheses: the ER, the Golgi apparatus, the ER-Golgi intermediate compartment, the mitochondria, the plasma membrane and recycling endosomes (Shibutani and Yoshimori, 2014). Although the autophagic membrane may originate from several locations, it is not known whether all these membrane sources are active in all types of autophagy, or each source represents a type of autophagosome with different contents (Weidberg et al., 2011).
Overall, the nucleation step starts with the interaction between the ULK complex (ULKs-Atg13-FIP200) and the class III PI3K (PI3KC3) complex located at the isolation membrane (IM) (Figure 1.9B). The PI3KC3 complex contains PI3KC3 subunits, namely PI3KC3 and p150, Ambra-1, mammalian Atg14L, and Bcl-2-interacting protein (Beclin-1), working as interacting partner (Volinia et al., 1995; Kihara et al., 2001; Wirawan et al., 2012). Within this complex, Beclin-1 constitutes a platform for the binding of several interactors regulating the kinase activity of PI3KC3 (Funderburk et al., 2010). In turn, PI3KC3 generates phosphatidyl-inositol-3-phosphate (PI3P), which recruits Atg proteins to the site of autophagosome formation.

Autophagosome elongation

The initial nucleation of the phagophore is followed by expansion and elongation of the membrane. Autophagosome membrane expansion is contingent on the coordinated actions of several core autophagy machinery proteins involved in two ubiquitin-like conjugation systems: the Atg12-Atg5 and the microtubule associated protein-1 light chain-3B (LC3B)-phosphatidylethanolamine (PE) system (Xie et al., 2008) (Figure 1.9C). These systems have been proposed to act during the elongation of the phagophore membrane and autophagosome closure (Yang and Klionsky, 2010a; Beau et al., 2011).

In the Atg5-Atg12 system, Atg12 is activated by Atg7, an ubiquitin-activating enzyme (E1)-like protein, transferred to Atg10, an ubiquitin-carrier enzyme (E2)-like protein, and then covalently linked to Atg5. Lastly, Atg12-Atg5 forms a multimer complex with Atg16L (forming Atg12-Atg5-Atg16L) (Kuma et al., 2002; Mizushima et al., 2003), which is localized to the outer portion of the autophagossomal membrane (Hanada et al., 2007), and has been suggested to determine its curvature. Though crucial for pre-autophagosomal elongation, once a fully functional autophagosome forms, the Atg12-Atg5-Atg16L complex dissociates (Ravikumar et al., 2010).

The second ubiquitin-like conjugation pathway involves LC3B lipidation. LC3B is the most studied member of the LC3 family, and is initially synthesized as a precursor (Pro-LC3B). Pro-LC3B has an additional arginine residue at the C-terminus that is immediately cleaved by a cisteine protease, Atg4, to become LC3B-I, exposing a glycine at the C-terminal end (Tanida et al., 2004). LC3B-I is then conjugated with Atg7 by a thioester bond, and finally with Atg3, another E2 ubiquitin conjugating enzyme, to form an amide bond with PE (Kirisako et al., 2000; Tanida et al., 2004), an important phospholipid found in biological membranes. This lipidation, more specifically the site where it occurs, appears to be dependent on the Atg16L complex (Yang and Klionsky, 2010a). Whereas the unconjugated form of LC3B, known as LC3B-I resides in the cytosol, the LC3 lipidated conjugated form, called LC3B-II, is membrane-bound to the phagophore, both to the inner and the outer membrane and so it is suited to serving as an autophagy-specific marker (Shibata et al., 2010).

Cargo recognition and selectivity

Autophagy in higher eukaryotes was regarded as non-selective process for bulk assimilation, so that any cytosolic components may be randomly sequestered for degradation and recycling (Ryter et al., 2013). This has an important role under starvation condition, supplying nutrients to ensure the cell viability. However, increasing evidence suggests that autophagy has a more selective role in delivery of a specif cytoplasmic cargo to the lysosome for degradation, removing through a selective way potentially damaging proteins and/or unwanted organelles. For instance, autophagy could play an important function in the clearance of cytosolic ubiquitinated substrates and aggregate-prone proteins (He and Klionsky, 2009).

Recent studies suggest that the autophagy selectivity related to ubiquitinated substrates may be mediated by Sequestosome 1 (SQSTM1, also called p62) (Bjørkøy et al., 2005; Pankiv et al., 2007). SQSTM1 is a signaling-adaptor protein, playing critical roles in several cellular functions at the organism level, such as bone remodeling, obesity and cancer (Manley et al., 2013). The multidomain structure of SQSTM1 allows it to interact with a myriad of other proteins, namely

it was reported that SQSTM1 interacts with ubiquitinated proteins for UPS degradation (Seibenhener and Babu, 2004; Babu et al., 2005) and co-localizes with ubiquitinated protein aggregates, LC3B and lysosomes. Also, the formation of autophagosomes proved to be dependent of SQSTM1 even upon starvation (Bjørkøy et al., 2005). SQSTM1 interacts directly with ubiquitinated proteins through an ubiquitin-associated (UBA) domain (Ichimura and Komatsu, 2010). Futhermore, SQSTM1 can interact with LC3B through its LC3-interacting region, which in turn, integrates the autophagosome membrane and is ultimately degraded upon the autophagy late stages, leading to the concomitant degradation of SQSTM1 and proteins connected to it (Weidberg et al., 2011). Also, apparently, SQSTM1 acts as a "garbage disposer" in the cytoplasm, building up aggregates of poly-ubiquitinated proteins, to enhance their degradation by autophagy. But at the same time, excess SQSTM1 delays the proteasomal degradation of poly-ubiquitinated aggregates (Korolchuk et al., 2009; Moscat and Diaz-Meco, 2009). This mechanism implies that autophagy may not only be a complementary process to UPS, but also a major alternative process, by enhancing the clearance of ubiquitinated substrates.

In addition, there is also selective autophagic degradation of certain organelles, such as mitochondria (mitophagy), peroxisomes (pexophagy), ribosomes (ribophagy), and microbial invaders (xenophagy), as mentioned above. Nevertheless, autophagy selectivity is still a matter of debate, while new data uncovering autophagic cargo specificity arise (Weidberg et al., 2011).

Autophagosome maturation and cargo degradation

The final step of autophagy involves the sealing of the membrane in order to form complete autophagosomes, which then fuse to endosomes and/or to lysosomes, to form autolysosomes, where the cargo is degraded (Tong et al., 2010) (Figure 1.9D). The fusogenic properties of LC3B-II seem to be responsible for the closure membrane (Nakatogawa et al., 2007; Fujita et al., 2008). In addition, along with LC3B lipidation machinery the Atg16L complex appears to be important for complete closure of autophagosome (Sou et al., 2008; Longatti and Tooze, 2009). Then, the fusion between autophagosomes and lysosomes is a dynamic process mediated by the cytoskeleton, namely, the microtubules network, to which the autophagosomes are associated. They move bidirectionally along it and accumulate at the microtubule-organizing centre, where lysosomes are usually clustered (Jahreiss et al., 2008). In mammalian cells, the fusion event requires the lysosomal membrane protein LAMP-2 and the small guanosine triphosphatase Rab7 (Jäger et al., 2004). After the autolysosome formation, with the fusion between the lysosome and the outer membrane of the autophagosome, the

inner membrane and the cargo, including bound LC3B-II, are released in the lysosomal lumen and degraded by several proteases, namely cathepsins B, D and L (Tanida et al., 2005). The degradation products are then released to the cytosol, potentially leading to the activation of MTOR and the disassembly of the autolysosome (Tong et al., 2010).

In mammalian cells, most of the Atg proteins are found in isolation membranes (e.g., ULK1/2, Atg13, FIP200, Atg101, Beclin-1, Atg14, LC3B, Atg12, Atg5, Atg16L) but not on complete autophagosomes (Longatti and Tooze, 2009). To date, only the autophagic protein LC3B was systematically found on autophagosomes being therefore the most widely monitored autophagy-related protein (Klionsky et al., 2012). Importantly, an increased number of autophagosomes may not necessarily correspond to an increased autophagic activity, but instead a defect in autophagosome turnover due to a block in fusion with lysosome or disruption of lysosome function (Klionsky and Al., 2012).

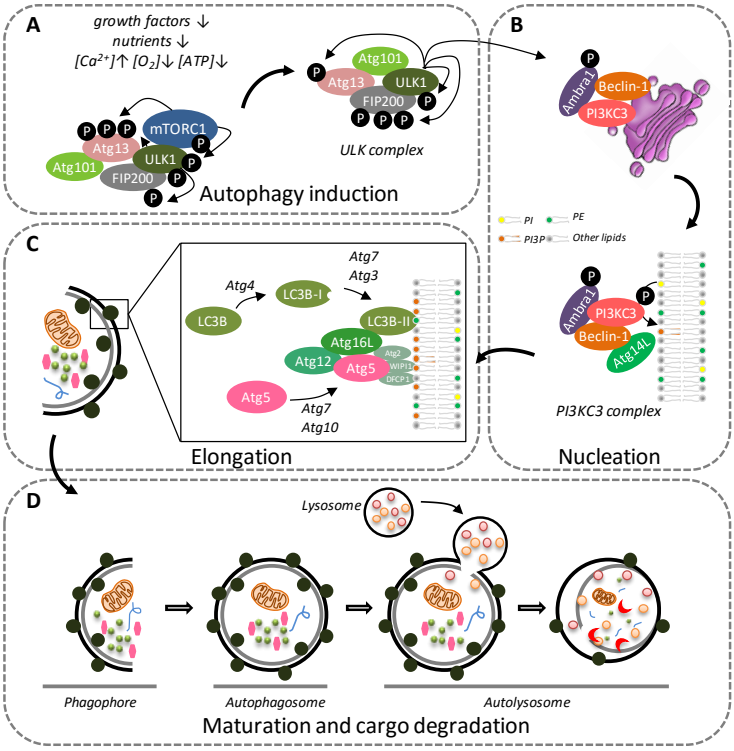

Figure 1.9 - The core molecular machinery of autophagy in mammalian cells. A, Autophagy induction: during cellular and metabolic stress the mTORC1 is inactivated, which allows ULK complex activation. B, Nucleation: ULK1 phosphorylates Ambra1, interacting with the class III PI3K (PIK3C3) complex. Then, the PIK3C3 complex generates phosphatidyl-inositol-3-phosphate (PI3P), which recruits Atg proteins to the site of autophagosome formation. C, Elongation: two ubiquitin-like processes are carried out. Atg7 and Atg10 mediate Atg5-Atg12 complex formation. This Atg5-Atg12 complex subsequently binds to Atg16L1, generating the Atg16L1 complex. LC3 cleaved by Atg4 generates LC3-I, which is then conjugated to phosphatidylethanolamine (PE), to generate LC3-II, a process mediated by Atg7, Atg10 and the Atg16L1 complex. D, Maturation and cargo degradation: after the completion of autophagosome formation, the autophagic cargo is released into the lysosomal lumen by the fusion of the outer autophagosomal membrane with the lysosome, and the content is degradaded. Adapted from (Wirawan et al., 2012).

1.3.3 Signaling pathways regulating autophagy

Autophagy controls the degradation of organelles and cytosolic proteins under different environmental stress and even under normal growth conditions. Therefore, tight regulation mechanisms exist to control its induction. The main pathway, considered the canonical one, culminates in the inhibition of MTOR, but there are other pathways, which induce autophagy downstream of MTOR, at known, or yet unknown points. Overall, there is still much to uncover regarding the pathways and factors regulating the induction of autophagy in mammalian cells, as well as some discrepancies, which may be related to cell type specificity.

The class I PI3K/AKT/mTORC1 pathway

As previously stated, MTOR acts as a core regulator of the balance between cell growth and autophagy (Jung et al., 2010), thus, it is understandable that many cell signaling pathways interact with MTOR, in response to different intra- and extracellular conditions. The canonical pathway leading to MTOR activation, and negatively controlling autophagy, is the phosphatidylinositol-3-kinase (PI3K)−AKT−mTORC1. Upon growth factors, such as insulin or IGF-1, binding to the insulin receptor, autophosphorylation of the insulin receptor on tyrosine residues results in recruitment and phosphorylation of insulin receptor substrates 1 and 2 (IRS1/2), forming a docking scaffold for PI3KC1 (He and Klionsky, 2009). This class of enzymes preferentially reacts with phosphatidylinositides (4,5)-bisphosphate ($PI(4,5)P_2$), phosphorylating them and leading to the production of phosphatidylinositides (3,4,5)-trisphosphate ($PI(3,4,5)P_3$) (Kong et al., 2010). This step is regulated by phosphatase and tensin homologue (PTEN), which reverts the action of PI3K (Arico et al., 2001). Generation of $PI(3,4,5)P_3$ recruits AKT to the plasma membrane where it becomes activated by direct phosphorylation by phosphoinositide-dependent protein kinase 1 (PDK1). mTORC2 also plays a role at this point, contributing to the activation of AKT, although its upstream regulating mechanisms remain unknown (Dunlop et al., 2009). After activation, AKT phosphorylates protein 2 from the tuberous sclerosis complex (TSC2), preventing it from complexing with TSC1. When these two proteins are complexed, forming the TSC1/2 complex, they function as a GTPase activating protein (GAP) for Ras homologue enriched in brain (Rheb), leading to the conversion of bound guanosine triphosphate (GTP) into guanosine diphosphate (GDP) (Rheb-GTP to Rheb-GDP) and, therefore, preventing it from activating mTORC1 (Garami et al., 2003). Hence, with an active AKT, TSC1/2 is inhibited, which allows GTP-bound Rheb to activate MTOR and prevent autophagy (Figure 1.10) (He and Klionsky, 2009; Sengupta et al., 2010).

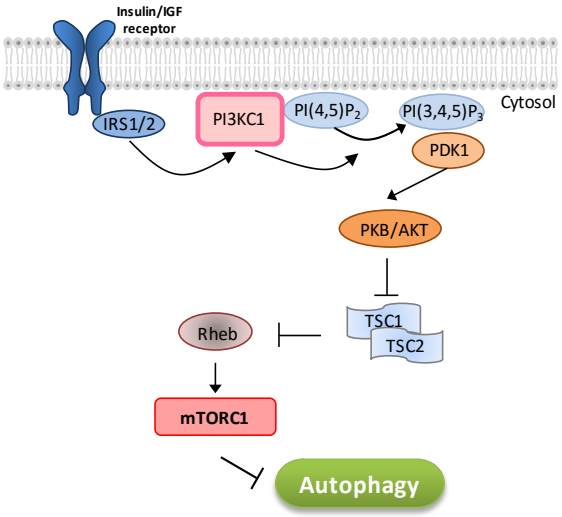

Figure 1.10 - The class I PI3K–AKT–MTOR pathway for inhibition of autophagy. Schematic representation of the canonical pathway regulating autophagy in mammals. Upon insulin receptor activation, IRS1/2 form a docking scaffold for class I PI3K (PI3KC1), which in turn, phosphorylates $PI(4,5)P_2$ in the cellular membrane, to $PI(3,4,5)P_3$. This recruits PDK1, which activates AKT. AKT inhibits the conjugation of TSC1/TSC2, therefore inducing Rheb effect on the activation of mTORC1 and the inhibition of autophagy. Adapted from (Yang and Klionsky, 2010a).

The AMPK pathway

The canonical pathway has several branching points that connect it to other response mechanisms. One of these mechanisms may be generically called the energy-sensing mechanism and acts primarily through adenosine monophosphate (AMP)-dependent protein kinase (AMPK) (Yang and Klionsky, 2010a).

Low-glucose intracellular availability leads to decreased ATP production by mitochondria, and therefore, an increase of the AMP/ATP ratio (Hardie et al., 2003). This reduced cellular energy level activates AMPK, a heteromeric kinase composed of a catalytic (α) and two regulatory subunits (β and γ) (Kahn et al., 2005). AMP binds AMPK and puts the trimer in a conformation that allows the phosphorylation by the upstream activating liver kinase B-1 (LKB-1, also known as serine/threonine kinase 11) (Sanders et al., 2007). Active AMPK induces the formation of TSC1/2 complex and the consequent inhibition of mTORC1 through Rheb (Inoki et al., 2003; Corradetti et al., 2004). In addition, the LKB-1-AMPK pathway stabilizes the cell-cycle inhibitor p27kip1, which also induces autophagy (Liang et al., 2007). It was shown that AMPK also inhibits mTORC1 in a TSC1/2-independent manner, through phosphorylation of raptor, on two serine residues that are conserved throughout evolution. This phosphorylation allows the binding of 14-3-3 proteins to raptor, thus inhibiting its assembly with MTOR and recruitment of the other mTORC1 components (Gwinn et al., 2008). Furthermore, a recent study has shown that AMPK directly phosphorylates and activates ULK1, inducing autophagy, upon glucose starvation (Kim et al., 2011).

AMPK has also been implicated in pathways responsive to cellular stress, namely the increase of the intracellular Ca^{2+} and hypoxia. The calcium response is dependent on the activation of calcium-activated calmodulin-dependent kinase kinase-β (CaMKKβ), which directly activates AMPK (Høyer-Hansen et al., 2007), while hypoxia response relates mainly to the decrease in AMP/ATP ratio in hypoxic cells (Liu et al., 2006). Hypoxia, as well as other cellular stresses, also induces autophagy in an AMPK-independent manner, through hypoxia-inducible factor (HIF) activation of regulated in development and DNA damage 1 (REDD1) and REDD2, which induce the TSC1/2 complex (Dunlop et al., 2009).

Amino acid-sensing mechanisms

Being autophagy a process intended to respond to low-nutrient conditions, it would be expected that the levels of amino acids would have a close interaction with the autophagy-regulating mechanisms. Indeed, several factors have been shown to respond to amino acid levels, either inducing or inhibiting autophagy.

mTORC1 could be positively regulated by amino acids which represent a strong nutrient signal. It is proposed that mTORC1 senses and is phosphorylated in response to nutrient signals (Long et al., 2005). In addition, other studies show that amino acids activate PI3KC3 (Byfield et al., 2005; Nobukuni et al., 2005), which in turn leads to MTOR activation and autophagy inhibition. The way through which PI3KC3 activates MTOR is not fully established yet, but it may presumably be dependent on the recruitment of FYVE and PX domain-containing proteins, by PI3P (Nobukuni et al., 2007). This responsive effect is the opposite of that in which PI3KC3 has in the autophagic machinery induction downstream of ULK−Atg13−FIP200 complex. The contradiction signals may be related to the existence of different PI3K protein sub-populations or complexes in the cell, carrying out different functions, under different signals (He and Klionsky, 2009).

Nevertheless, the link between amino acids signaling and PI3KC3 has been attributed to calcium increase, which binds calmodulin and, in turn, activates PI3KC3 (Gulati et al., 2008). Another contradiction rises, this time regarding calcium effects on autophagy induction: while calcium increase induces AMPK to activate ULK1, the TSC1/2 complex and inhibit mTORC1, leading to autophagy, it also induces PI3KC3 to activate mTORC1, inhibiting autophagy. Byfield et al. have previously shown that the PI3KC3 activating effect on mTORC1 is abolished under glucose starvation conditions and, consistently, by AMPK activation (Byfield et al., 2005). Thus, it is assumable that the increased Ca^{2+}-dependent inhibitory effect on autophagy, being either AMPK- dependent (Høyer-Hansen et al., 2007) and/or –independent (Grotemeier et al., 2010), may overcome the PI3KC3 activating effect on mTORC1, upon starvation.

Another factor linking amino acid availability to mTORC1 is the mitogen-activated protein kinase kinase kinase kinase-2 (MAP4K2), a MAPK family protein. MAP4K2 overexpression was found to increase the phosphorylation of ribosomal S6 protein kinase β 1 (S6K1), a substrate of mTORC1, while its knockdown decreased S6K1 phosphorylation, after amino acid stimulation (Findlay et al., 2007). Though, no direct link has yet been established between MAP4K2 and autophagy induction.

In addition, it has been suggested that Ras-related small GTPases (Rag) active TORC1 in response to amino acids (Kim and Guan, 2009), through Rheb in a specific subcellular compartment (Sancak et al., 2008). But rather than modulating its kinase activity, when induced by amino acids, the Rag proteins mediate translocation of TORC1 to a spefic subcellular compartment that also contains its activator Rheb (Sancak et al., 2008). Yet, it is not known how this translocation increases mTORC1 activity (Kim and Guan, 2009) and also, no direct connection has been made with autophagy induction.

The MAPK/ERK pathway

A study revealed that the Ras pathway also contributes to the canonical pathway regulating autophagy, since, upon insulin receptor stimulation, the small GTPase Ras is activated, leading to both the induction of PI3KC1 activity and Raf-1/MAP (mitogen-acitvated protein) kinases cascade activation (Marshall, 1996; Furuta et al., 2004). While PI3KC1 signals through AKT, as previously described, Raf-1 (MAP3K) phosphorylates MEK1/2 (MAP2K), which in turn phosphorylates ERK1/2 (extracellular signal-regulated kinases 1/2; MAPK). ERK1/2 then phosphorylates TSC2, inhibiting its assembly with TSC1, thus allowing Rheb to activate mTORC1 (Ma et al., 2005). A substrate of ERK, ribosomal s6 protein kinase α 1 (RSK1, or p90) has also been found to phosphorilate TSC2, contributing to the activation of mTORC1 (Roux et al., 2004; Kwiatkowski and Manning, 2005). Plus, both ERK1/2 and RSK1 phosphorilate S6K1 (Steelman et al., 2011), further indicating their pro-proliferative role in this pathway (Figure 1.11).

Conversely, a study has found that ERK1/2 stimulates autophagy in the human colon cancer cell line HT-29. They have shown that ERK1/2 phosphorylate G-protein subunit α (Gα)-interacting protein (GAIP), thus leading to accelerated GTPase activity of inhibitory Gα-3 (Gαi3). When in heterotrimeric GDP-bound form, rather than GTP-bound, Gαi3 induces the formation of autophagosomes, so, as GAIP induces the accumulation of the GDP-bound form, it induces autophagy (Ogier-Denis et al., 2000). It was also shown that this ERK1/2 effect was dependent on amino acid starvation, since amino acids lead to the inhibition of Raf-1 (Pattingre et al., 2003). This discrepancy may be due to cell type specificity.

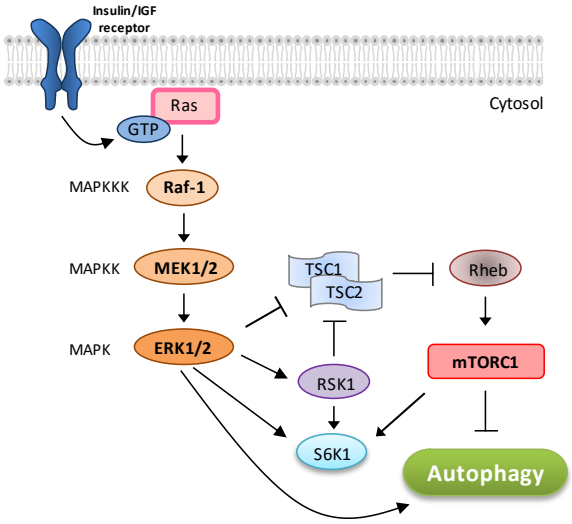

Figure 1.11 - The MAPK pathway in the regulation of autophagy. Schematic representation of the Ras–ERK1/2 MAPK cascade and its action on autophagy. Upon insulin receptor activation, Ras induces the MAPK cascade Raf-1–MEK1/2–ERK1/2. This pathway seems to have contradictory, possibly cell type-dependent, effects on autophagy. ERK1/2 and its substrate RSK1 inhibit TSC1/2, leading to mTOR activation, while also phosphorylating mTORC1 substrate, S6K1, which regulates proliferation-related ribosomal function. However, autophagy-inducing effects have also been reported with ERK1/2 inducing autophagosome formation, through an mTORC1-independent mechanism and dependent on amino acids depletion. Adapted from (Yang and Klionsky, 2010a).

The cAMP-Epac-PLC-ε-IP3 and Ca^{2+}-calpain-Gαs pathways

An independent mTORC1 signaling pathway has been suggested in autophagy regulation, namely through inositol triphosphates (IP_3) (Williams et al., 2008). Firstly, it was observed that lithium-induced autophagy is mediated through inositol monophosphatase (IMPase) inhibition and thus, on low IP_3 levels, which had no effect on MTOR or S6K1 activation (Sarkar et al., 2005). Consequently, they proposed a cyclical MTOR-independent pathway negatively regulating mammalian autophagy, comprising cyclic adenosine monophosphate (cAMP)–exchange protein activated (Epac) by cAMP –phospholipase C-ε (PLCε) –IP_3 and Ca^{2+}–calpains–stimulatory protein G subunit α (Gαs) (Williams et al., 2008; Sarkar et al., 2009). Basically, an increase in cAMP caused by Gαs-induced adenylate cyclase (AC) will activate Epac, which in turn activates a small GTPase Ras-related protein Rap2B, which will further induce PLCε and increase the production of IP_3. As IP_3 induces Ca^{2+} release from the ER, the intracytosolic concentration of Ca^{2+} increases, leading to the activation of a family of Ca^{2+}-dependent cystein proteases called calpains. Active calpains cleave and activate Gαs, which creates a positive feedback loop effect. Thus, inhibiting this pathway would lead to autophagy induction downstream mTORC1 (Sarkar et al., 2009). The fact that this model implies intracytosolic Ca^{2+} decrease in autophagy induction conflicts with what has been described,

regarding Ca^{2+} increase inducing autophagy in AMPK-dependent and -independent manner (Grotemeier et al., 2010).

Ras/cAMP-dependent PKA pathway

Besides Ras signaling pathways mentioned before, the Ras/cAMP-dependent protein kinase A (PKA) also regulates autophagy (Kopperud et al., 2003; Budovskaya et al., 2004). This pathway plays a key role in cell proliferation, stress response, and longevity (Thevelein et al., 2000). In yeast, PKA contains a heterotetramer that is composed by a regulatory subunit Bcy1 and three apparently redundant catalytic subunits, Tpk1, Tpk2, and Tpk3. Under nutrient rich conditions, two redundant Ras GTPases, Ras1 and Ras2, are activated and subsequently stimulate AC to produce cAMP. Then, elevated cAMP is able to bind to the regulatory PKA subunit, Bcy1, allowing its dissociation from the PKA catalytic subunits (Portela and Moreno, 2006), and consequently the activation of PKA. When this activation occurs, the autophagy induced by MTOR inhibition is suppressed in yeast, suggesting that Ras-PKA pathway is a potent negative regulator of autophagy (Budovskaya et al., 2005; Yorimitsu et al., 2007). In addition, it was demonstrated that Atg family proteins are PKA substrates. Apparently, in the presence of nutrients, PKA phosphorylation promotes the presence of Atg1, homolog of mammalian ULK1 and ULK2, and dissociated from the phagophore assembly site (PAS), whereas during nutrients depletion, Atg1 is dephosphorylated and it is localized to the PAS. However, it is still unknown if the phosphorylation of these proteins by the PKA is linked to autophagy regulation (Budovskaya et al., 2005). Mammalian PKA negatively regulates autophagy either by directly phosphorylating LC3B (Cherra et al., 2010) or by activating TORC1, which inhibits autophagy (Mavrakis et al., 2006). In yeast, the abrogation of the conserved MTOR, Ras/cAMP-dependent PKA, that integrate the network of nutrient sensing pathways, is known to promote longevity (Gomes et al., 2007; Chen and Klionsky, 2011). These signaling pathways are negative regulators of autophagy, reinforcing that autophagy and aging are coordinately regulated by a complex network of different signaling pathways, with partial overlapping branches and yet undisclosed hierarchic connections.

1.3.4 Physiological role of autophagy

As a basic cellular mechanism for recycling of nutrients, autophagy is involved in many physiological processes in higher eukarytes. It has been well established that autophagy regulates important biological functions, such as cell survival, cell death, cell metabolism, development, aging, infection and immunity (Rami, 2009). Since it has been shown to be a

highly conserved cellular process in evolution, it is thus possible to assign an important role in the organism's survival and adaptation to environmental changes (Yang and Klionsky, 2010b).

The principle homeostatic functions of autophagy include the selective clearance of aggregated protein to preserve proteostasis, and the selective removal of dysfunctional mitochondria (mitophagy). Autophagy has also a vital role in mammals, during embryonic development, in response to low-nutrient levels and after birth, when the nutrient supply from the progenitor is suddenly interrupted (Kuma et al., 2004). There is also evidence for the role of autophagy tissue-specific processes, as in the formation of multilamellar bodies, which are responsible for the production of surfactant in type II pneumocytes (Hariri et al., 2000) and in the mitochondria removal from maturing erythrocytes (Mortensen et al., 2010). Additionally, autophagy plays a central role in innate and adaptive immunity, with diverse functions such as regulation of inflammatory responses, antigen presentation, and pathogen clearance. Autophagy can preserve cellular function in a wide variety of tissue injury and disease states, however, maladaptive or pro-pathogenic outcomes have also been described. Among the many diseases where autophagy may play a role include proteinopathies which involve aberrant accumulation of proteins (e.g., neurodegenerative disorders), infectious diseases, cancer and metabolic disorders such as diabetes and metabolic syndrome. Targeting the autophagy pathway and its regulatory components may eventually lead to the development of therapeutics (Ravikumar et al., 2010; Ryter et al., 2013).

There are numerous evidences showing that autophagy is constitutively present in all eukaryotic cells, but their activity may vary between different tissues or cell types and it is especially important in terminally differentiated cells (Rami 2009). Under basal levels, autophagy is mainly responsible for the elimination of damaged organelles and potentially toxic proteins (Beau et al., 2011). Though, the activation of autophagy beyond a certain threshold may lead to cell death (called autophagic cell death), cause atrophy and collapsed cellular functions (Rami, 2009).

The role of autophagy in neurons and in CNS is now a largely object of study. The definitive proof of the importance of autophagy in the CNS was obtained from engineered mice lacking either the Atg7 or Atg5 autophagy genes, demonstrating that the loss of autophagy causes neurodegeneration even in the absence of any disease-associated mutant proteins, and it is also implicated in the pathogenesis of neurodegenerative disorders (Hara et al., 2006; Komatsu et al., 2006). Moreover, the specific deletion of Atg genes in the hypothalamus showed that the selective loss of autophagy in anorexigenic proopiomelanocortin (POMC) neurons in mice decreases α-MSH levels, increases food intake and body weight, promotes adiposity and alters glucose homeostasis (Kaushik et al., 2012; Quan et al., 2012). Moreover,

mice with POMC neuron-specific deletion of Atg7 display abnormal development of POMC neuronal projections (Coupé et al., 2012). The AgRP autophagy-null mice resulted in decreased body weight, reduced total body fat content, and decreased food intake in response to fasting (Kaushik et al., 2011), demonstrating the role on autophagy in hypothalamic metabolic regulation.

1.3.5 Autophagy and aging

In addition to playing critical roles in development and pathophysiological conditions, autophagy has also been implicated in aging, at least in model organisms. Aged cells are characterized by the accumulation of altered or damaged DNA, protein, lipids, and cellular organelles (Vellai et al., 2009), and this hints that aging may be able to attenuate by enhancing their clearance. This accumulation of damaged macromolecules during aging occurs due to defective clearance and a general decline in the housekeeping mechanisms. It was recently reported autophagy impairment as an hallmark of aging since the basal autophagic activity and lysosomal proteolytic activities decrease with age, contributing to the accumulation of altered macromolecules a characteristic feature of aging (Cuervo et al., 2005; Meijer and Codogno, 2006; Mariño and López-otín, 2008; López-Otín et al., 2013). Autophagy deficiency contributs to different aspects of aging phenotype and to the aggravation of detrimental age-related diseases (Cuervo, 2008; Rubinsztein et al., 2011). Moreover, evidence show that aged-associated autophagy impairment is also observed in the hypothalamus (Kaushik et al., 2012), brain area critical for the development of whole-body aging and with impact on lifespan (Zhang et al., 2013).

Regarding the contribution of autophagy impairment to aggravation of neurodegenerative diseases (Cuervo, 2008), accumulation of autophagic vacuoles (AV) was found in brains of animal models and patients with Alzheimer's disease (AD) (Haung Yu et al., 2005; Nixon et al., 2005; Yang et al., 2008; Ułamek-Kozioł et al., 2013), Parkinson's disease (PD) (Anglade et al., 1997; Spencer et al., 2009) and Huntington's disease (HD) (Sapp et al., 1997; Kegel et al., 2000; Mizushima et al., 2008). Moreover, in HD, the ability of autophagic vacuoles (AV) to recognize cytosolic cargo is largely defective in HD cells, even with a normal or increased autphagosome-lysosome pathway (Vicente et al., 2010). In addition, the accumulated mutant huntingtin protein can recruit cytosolic beclin-1, and impairs beclin-1 complex-mediated autophagy, leading to the accumulation of mutant protein and neuronal toxicity (Shibata et al., 2006). In PD, overexpression of the wild-type α-synuclein is associated with autophagy inhibition (Winslow et al., 2010). However, the mechanisms by which autophagy are regulated in PD

remains unknown, since overexpression of mutant α-synuclein induces autophagy (Cuervo, 2004; Xilouri et al., 2009). In AD, beclin-1 downregulation is observed in early AD (Pickford et al., 2008). In addition, in Machado-Joseph Disease (MJD), abnormal expression of endogenous autophagic markers, decrease levels of beclin-1 and accumulation of autophagosomes were reported (Nascimento-Ferreira et al., 2011). This accumulation of AV in late stages of disease was initially incorrectly associated with autophagic cell death (Bursch et al., 2000; Bursch, 2001), believing now that autophagy has a protective role in neurodegenerative diseases and that the term "autophagic cell death" was misnamed, occurring in some cases cell death with autophagy but not cell death executed by autophagy (Kroemer and Levine, 2008; Kroemer et al., 2009; Levine and Kroemer, 2009). Accumulated evidence revealed that neuronal autophagy is essential for the healthy aging neurons. Moreover, deregulated excessive autophagy can also cause neurodegeneration (Cherra and Chu, 2008; Cheung and Ip, 2009).

Overall, environmental conditions or use of agents that promote the induction of autophagy have been shown to improve cellular fitness and survival. The systemic administration of autophagy-inducing drugs has proven to increase lifespan in the nematode *Caenorhabditis elegans*, in the fruit fly *Drosophila melanogaster* and in mice (Eisenberg et al., 2009). Furthermore, the beneficial effects of calorie restriction (CR), the most robust anti-aging intervention known so far (Masoro, 2006; Bergamini et al., 2007), appear to be largely mediated by autophagy (Donati, 2006; Hansen et al., 2008; Blagosklonny, 2010). Also, it has been found that autophagy response decreases with aging, but that decrease is countered by CR (Bergamini et al., 2007). Knowing that autophagy is a major mechanism in response to nutrient starvation, and CR induces it, beneficial effects of CR and autophagy are directly related.

1.4 Synaptic Plasticity

1.4.1 Types of Synapses

Synaptic plasticity is known to play a central role in a range of brain-related behaviors, such as learning, memory and addiction (Pang and Han, 2012). Chemical synapses enable cell-to-cell communication via the secretion of neurotransmitters; these chemical agents released by the presynaptic neuron produce secondary current flow in postsynaptic neuron by activating specific receptor molecules. The secretion of neurotransmitters is triggered by the influx of Ca^{2+} through voltage gated channels, which give rises to a transient increase in Ca^{2+} concentration within the presynaptic terminal. The rise in intracellular Ca^{2+} concentration causes synaptic vesicles to fuse with the presynaptic plasma membrane and release their contents into the space between the pre- and postsynaptic cell (synaptic cleft). The neurotransmitter concentration in the synaptic cleft remains high for only a very brief period. Neurons communicate with each other primarily through fast chemical synapses. The neurotransmitter binding to postsynaptic receptors produces a postsynaptic conductance change as ion channels are opened (or sometimes closed). This conductance change typically generates an electrical current, the postsynaptic current (PSC), which in turn changes the postsynaptic membrane potential to produce a postsynaptic potential (PSP). According with the effect of presynaptic stimulation on the PSP, chemical synapses can be classified as excitatory or inhibitory. Excitatory synapses occur mainly on very small protrusions from the dentritic shaft called dentritic spines, and they are characterized by a morphological and a function specialization of the postsynaptic membrane called the postsynaptic density (PSD) (Sheng and Kim, 2011). The PSD was first observed in electron micrographs as a fuzzy electron-dense thickening of the postsynaptic membrane that is opposed to the presynaptic active zone (Gray, 1959; Siekevitz, 1985), and therefore also called “asymmetric synapses”. The PSD contains the glutamate receptors that are activated by the glutamate neurotransmitter from the presynaptic terminal, as well as associated-signaling molecules and structural molecules. In contrast, inhibitory synapses are formed on the shaft of dendrites, or on cell bodies and axon initial segments, and by electron microcopy (EM), they show only a slight electron-dense thickening associated with the postsynaptic membrane and hence were described as “symmetric synapses” (Gray, 1959) (Figure 1.12). The postsynaptic side of excitatory synapses differs from inhibitory synapses not only in their content of neurotransmitters receptors but also in their morphology and molecular composition and organization (Sheng and Kim, 2011).

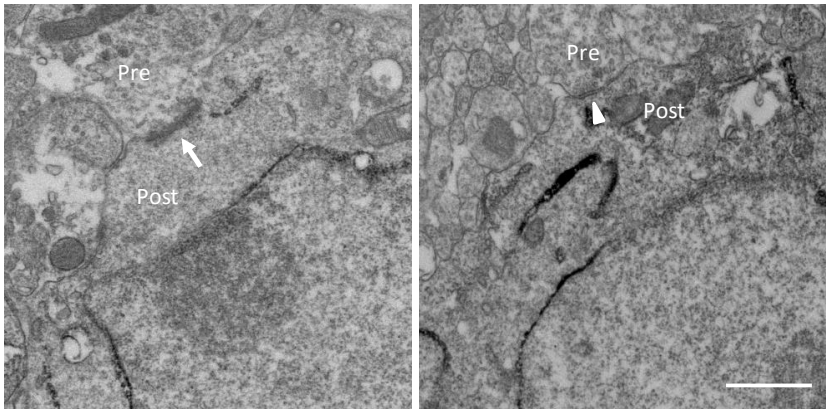

Figure 1.12 - Electron micrographs from cross sections of mouse neurons. Representative electron micrographs showing presynaptic terminals with vesicles and asymmetrical, putative stimulatory (arrow, left), and symmetrical, putative inhibitory (arrowhead, right) synapses. Scale bar, 500 nm.

1.4.2 Excitatory neurotransmission

The vast majority of excitatory neurotransmission in CNS is mediated by glutamate. In neurons, glutamate is packed within synaptic vesicles by dedicated vesicular transporters (vGluTs) and following presynaptic activity it is released in the synaptic cleft. It then diffuses rapidly across the synaptic cleft and activates glutamate receptors localized primarily on the postsynaptic membrane. There are two major classes of glutamate receptors: the ionotropic receptors (iGluRs), ion channels, and the metabotropic receptors (mGluRs), G protein-coupled receptors (GPCRs) (Watkins and Jane, 2006).

mGluRs are broadly distributed throughout the CNS and are specifically localized at discrete synaptic and extrasynaptic sites in both neurons and glia in virtually every major brain region. Activation of mGluRs results in diverse actions on neuronal excitability and synaptic transmission by modulation of a variety of ion channels and other regulatory and signaling proteins (Niswender and Conn, 2010).

Mammalian iGluRs (Smart and Paoletti, 2012) are encoded by a total of 18 genes that assemble into four major families: AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), kainate, NMDA (N-methyl-D-aspartate), and delta receptors, named after their most selective agonist (AMPARs, kainateRs, NMDARs, and deltaRs, respectively) (Watkins et al., 1981; Hollmann et al., 1989; Seeburg, 1993; Nakanishi and Masu, 1994; Dingledine et al., 1999). There are four AMPAR genes (GluA1-4, also known as GluR1-4); five kainateR genes (GluK1-5, also known as GluR5-7, KA1-2); seven NMDAR genes (GluN1;GluN2A-D; GluN3A-B, also known as NR1; NR2A-D; NR3A-B), and two d-subunits (GluD1-2, also known as d1 and d2).

KainateRs are found both on presynaptic and postsynaptic compartments and have a more modulatory rather than direct role at synapses (Lerma, 2006; Contractor et al., 2011). DeltaRs

are the least understood iGluRs largely because, in contrast to other iGluRs, their apparent incapacity to gate an ion channel following ligand binding makes them electrically "silent" (Kohda et al., 2000; Schmid et al., 2009). Thus, the excitatory postsynaptic current (EPSC) is typically mediated by members of the AMPAR and NMDAR families.

iGluRs form tetramers and each subunit is composed by a large extracellular amino-terminal domain; an agonist binding domain binding glutamate and glycine; a transmembrane domain composed of three membrane-spanning segments plus a short reentrant loop that forms the ion channel; and a cytoplasmic carboxyterminal domain involved in receptor trafficking, subcellular localization and coupling to intracellular signaling complexes (Wo and Oswald, 1995; Traynelis et al., 2010).

Even in a milliseconds range response of iGluRs, activation of AMPA receptors by glutamate mediates a component that has rapid onset and decay, whereas the component mediated by NMDA receptor activation has a slower rise time and a decay lasting up to several hundred milliseconds.

AMPA and NMDA receptors contain ion channels that conduct fluxes of Na^+, Ca^{2+} and K^+. Activation of AMPA receptors leads to a high Na^+ influx and a small K^+ flux and the net effect is depolarization of the postsynaptic neuron. Hence, the influx of current through GluA2-containing AMPA receptors (most AMPARs contain at least one subunit of GluA2) is normally largely carried by the movement of Na^+ from the extracellular face to the intracellular compartment; these receptors have very low Ca^{2+} permeability. On the other hand, GluA2-lacking AMPA receptors (e.g., GluA1 homomeric channels or GluA1/3 heteromeric channels) have a glutamine in the pore region instead of the arginine residue present in edited GluA2 subunits. Such channels have a high conductance for Na^+ and are permeable to Ca^{2+} (Liu and Zukin, 2007).

1.4.2.1 Regulation AMPA receptors in cell surface

AMPARs, the principal transducer of fast excitatory neurotransmission in the mammalian brain, are tetrameric assemblies of highly homologous GluA1-4 subunits. The trafficking of AMPARs into and out of synapses is highly dynamic and is regulated by subunit specific AMPAR-interacting proteins as well as by various post-translational modifications that occur on their cytoplasmic carboxyl terminal (C-terminal) domains (Anggono and Huganir, 2012). Receptors with subunits containing short cytoplasmic termini (GluA2/3) continuously cycle in and out of the synapse, while those with long cytoplasmic tails (GluA1/2 and GluA2/4) are

driven into synapses by synaptic activity (Hayashi et al., 2000; Zhu et al., 2000; Passafaro et al., 2001; Shi et al., 2001).

The number of AMPARs at the synapse is dependent on relative rates of exocytosis and endocytosis at the postsynaptic membrane. Enhanced receptor exocytosis and recycling occurs during synaptic potentiation, while increased rate of endocytosis results in synaptic depression (Shepherd and Huganir, 2007; Kessels and Malinow, 2009). Some observations support the idea that AMPARs do not directly exchange between the PSD and cytosolic compartments. Instead, exocytosis and endocytosis appear to occur at extrasynaptic membrane sites from which AMPARs laterally diffuse into and out of the synaptic PSD. Therefore, AMPARs are inserted into the plasma membrane in the soma or dendrites at extrasynaptic sites and travel to dendritic spines via lateral diffusion (Adesnik et al., 2005; Yudowski et al., 2007; Lin et al., 2009). The synaptic targeting of AMPARs is largely regulated by both AMPARs interaction partners and phosphorylation (Henley et al., 2011; Anggono and Huganir, 2012) (Figure 1.13). In the absence of neuronal activity, AMPARs undergo constitutive recycling between synapses and the cytosol, where they are sorted for either degradation or reinsertion at synapses (Ehlers, 2000; Passafaro et al., 2001). Upon synaptic potentiation they are more actively recycled through an endosomal pathway to enhance exocytosis (Park et al., 2004). For incorporation of GluA1-containing AMPARs into sinapses requires phosphorylation of GluA1, mobilizing receptors to extrasynaptic sites, and calcium/calmodulin (CaM)-dependent protein kinase II (CaMKII) activity upon NMDAR activation, which promote Ca^{2+} influx enhancing stabilization of the receptor in the synapse. CaMKII could act by promotion the exocytosis of AMPARs-containing vesicles (Maletic-Savatic et al., 1998) and/or the trapping at the PSD of laterally diffusing AMPARs (Opazo et al., 2012).

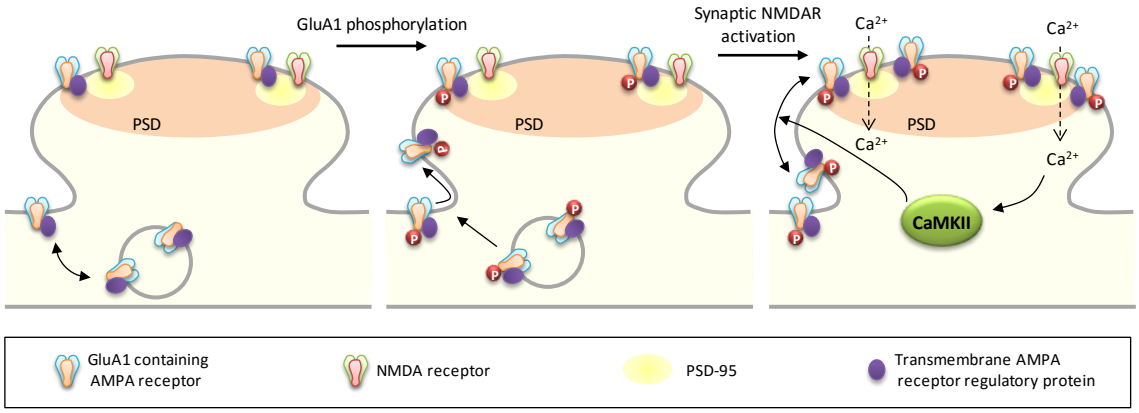

Figure 1.13 - Multistep trafficking of AMPARs to synapses. Left: under basal conditions, AMPA receptors constitutively cycle to and from the synaptic membrane. Middle: phosphorylation of GluA1 is associated with the insertion of GluA1-containing AMPARs into extrasynaptic sites. Right: potentiation requires trafficking of these extrasynaptic AMPARs to synapses, triggered by Ca^{2+} influx through NMDA receptors activation and presumably Ca^{2+}-dependent signaling, including CaMKII. PSD-95, postsynaptic density protein 95. Adapted from (Derkach et al., 2007).

1.4.2.2 Regulation NMDA receptors in cell surface

NMDA receptors are highly permeable tetramers to Ca^{2+}, composed of multiple NR1 subunits, essential for channel function. The receptors have with at least one NR2 subtype, which has a long C-terminal tails serving as anchoring points for signal transduction enzymes, and influencing receptor assembly, receptor trafficking and synaptic targeting (Monyer et al., 1992; Seeburg, 1993; Kennedy and Manzerra, 2001). Neurons can control the level of NMDA receptor expression at the synapse in response to different levels of activity, through rapid translation of specific NR1A splice variants, once NR1 gene encoded motifs that could prevent surface expression of the splice variant (Standley et al., 2000; Mu et al., 2003). During receptor assembly, binding NR2 subunit masks the NR1 retention signal, and promotes forward trafficking of the heterodimeric receptors through the secretory pathway to the cell surface (Lau and Zukin, 2007). NMDARs are transported to different subcellular sites, some receptors are localized to synaptic sites (pre- and postsynaptic) while others are localized extrasynaptically, which is determined by subunit composition (Stocca and Vicini, 1998; Tovar and Westbrook, 1999; Li et al., 2002). Exocytosis of NMDAR occurs at or near synapse, or can reach the synapse trough mobility in surface membrane (Tovar and Westbrook, 2002; Triller and Choquet, 2005) (Figure 1.14). NMDAR in mature synapses are associated with the scaffolding proteins, namely PSD-95 and synapse-associated protein (SAP)-102, important to link NMDAR to a number of signaling proteins, such as PKA and PKC. Activation of PKC and PKA regulates NMDAR trafficking by phosphorylation NR1 and promoting endoplasmic reticulum (ER) export and slow delivery of NMDAR to the plasma membrane (Scott et al., 2001, 2003). Furthermore, NMDARs are cycled to and from synaptic via both constitutive and regulated

pathway, involving direct effects of agonist binding activity (Vissel et al., 2001; Nong et al., 2003) and activation of metabotropic glutamate receptors (Snyder et al., 2001). Therefore, the number of NMDAR stabilized at the cell surface results from the balance between insertion, facilitated by SAP-102, and clathrin-mediated internalization at specialized endocytic zones located tangential to the PSD, for where NMDAR laterally diffuse (Blanpied et al., 2002; Rácz et al., 2004). Following endocytosis, NR2A-containing NMDAR are preferentially targeted for degradation, whereas NR2B receptors are targeted for recycling (Figure 1.14).

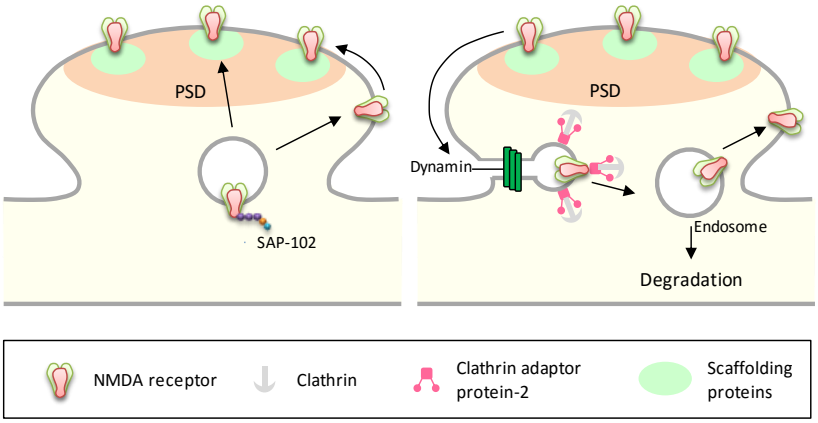

Figure 1.14 - Trafficking of NMDA receptor to synapses. The number of NMDARs stabilized at cell surface represents a balance between clathrin-mediated internalization and insertion, which is facilitated by scaffolding proteins. Left: packets of nascent receptors in the synaptic sited deliver NMDA receptors to the plasma membrane at the synapse through the involvement of synapse-associated protein (SAP)-102, or the exocytosis occurs near to synapse Right: NMDARs internalization is thought to occur by lateral diffusion of receptors away from to synapse to endocytic zones, through clathrin-coated pits. Following endocytosis, NR2B-containing NMDA receptors are targeted for recycling, whereas NR2A-containing NMDA receptors are preferentially targeted for degradation. Adapted from (Lau and Zukin, 2007).

1.4.3 Excitatory neurotransmission in the hypothalamus

In the hypothalamus, glutamate is present in some neurons and its receptors are distributed throughout the brain (van den Pol and Trombley, 1993). GluA1 and GluA2 are among the most widely subunits expressed of non-NMDA ionotropic receptor in the hypothalamus. GluA1 is expressed in the arcuate and periventricular region, and throughout anterior and medial hypothalamus (Martin et al., 1993; van den Pol et al., 1994). Similar to GluA1, strong GluA2 label was found in the arcuate and dorsomedial nuclei, and over both dorsomedial and ventrolateral parts of the ventromedial nucleus (van den Pol et al., 1994). Neurons that express NR1 mRNA were found in the lateral, ventromedial and anterior hypothalamus. Cells in arcuate and paraventricular nuclei also express NMDA receptors (van den Pol et al., 1994).

The iGluRs activation is central to physiological processes associated with learning and memory. The involvement of glutamatergic system on feeding behavior it has only recently

been studied. The intracerebroventricular and the lateral hypothalamic injection of glutamate, or its excitatory amino acid agonists, kainic acid, AMPA, and NMDA, rapidly elicit an intense food intake in rats (Stricker-Krongrad et al., 1992; Stanley et al., 1993). The glutamate involvement on food control was also studied by Guyenet et al., and they observed that feeding rapidly increases extracellular glutamante concentration in mediobasal hypothalamus and that this response is greater during high-fat diet than chow feeding (Guyenet et al., 2013).
The role of glutamatergic input to NPY/AgRP and POMC neurons on food intake regulation was studied by Liu and colleagues. They reported that body weight, fat stores and food intake were markedly reduced in mice with specific deletion of NMDARs in AgRP neurons. Interestingly, the deletion of NMDARs in POMC neurons had no effect on energy homeostasis. Furthermore, they showed that fasting activates AgRP neurons and increases the synaptic strength due to increased AMPA mediated synaptic transmission, and this effect was abolished when NMDARs were eliminated from postsynaptic neurons (Liu et al., 2012).

1.4.4 Inhibitory neurotransmission

Inhibitory neurotransmission in CNS is mediated by γ-aminobutyric acid (GABA) and glycine, whereas GABA predominates as the major inhibitory transmitter throughout the CNS, whereas glycine is of greater importance in the spinal cord and brainstem. The regulation of GABA itself is achieved by several specialized molecular mechanisms mediating transport, sequestration, synthesis and GABA degradation.
GABA exerts its inhibitory control by acting on two classes of receptors with distinct electrophysiological and pharmacological properties: GABA type A receptors ($GABA_AR$), ionotropic fast-acting receptor that belongs to a superfamily of pentameric ligand-gated ion channels, while GABA type B receptors ($GABA_BR$) belong to the metabotropic G protein-coupled receptor superfamily and produce slow prolonged inhibitory responses (Ong and Ib Kerr, 2000; Olsen and Sieghart, 2008).
Under normal physiological conditions $GABA_ARs$ respond to the binding of GABA by opening an integral chloride channel and allowing chloride to enter the neuron. The result is the increase in the anion conductance, leading to hyperpolarization of a depolarized membrane and neuronal inhibition. Deficits in $GABA_AR$ function have been associated with both physiatric diasases and neurobiological disorders (Olsen and Sieghart, 2008).
The $GABA_A$ receptors are the major inhibitory neurotransmitter receptors in mammalian brain. Each isoform consists of pentameric heterologous assembly from at least three different proteins selected from 19 different subunits (Olsen and Sieghart, 2008). In humans, these

include α1-6, β1-3, γ1-3, ε, θ, δ, π and ρ1-3 (Whiting, 2003; Olsen and Sieghart, 2008, 2009). Mature subunits are composed of approximately 450 amino acids residues in length, what about half of the subunit is a hydrophilic extracellular N-terminal domain containing the Cys loop, followed by four transmembrane sequences (M1-M4) (Chen and Olsen, 2007; Jacob et al., 2008).

Even assuming that a functioning $GABA_AR$ requires a combination of at least one α, one β and one γ subunit, the number of different subunits renders the possibility of the constitution of a large number of pentameric $GABA_AR$ combinations (Simon et al., 2004), determining the receptor's regional and development expression pattern, as well, as its physiological and pharmacological properties. The major adult isoform is generally accepted to be composed of α1, β2 and γ2 subunits (Whiting et al., 1995). Receptors composed of a α1, α2 or α3 subunits together with β and γ subunits are benzodiazepine-sensitive, and largely synaptically located, mediating most phasic inhibition in the brain (Rudolph and Möhler, 2004). Instead, $GABA_AR$ composed of a α4 or α6 subunits, together with β and δ subunits, are predominantly extrasynpatic, mediate tonic inhibition and are insensitive to benzodiazepine modulation (Brünig et al., 2002). $GABA_ARs$ are also present at presynaptic sites (Draguhn et al., 2008).

1.4.4.1 Regulation of $GABA_A$ receptors in cell surface

$GABA_AR$ can be delivered to the cell surface either as newly assembled channel complexes, via the novo secretory pathway, or reinserted following internalization. $GABA_AR$ are assembled from their component subunits in the endoplasmic reticulum and trafficked to the cell surface. The entry of $GABA_AR$ in the secretory pathway is regulated by interaction of α and β subunits with the protein that links integrin-associated protein with cytoskeleton-1 (PLIC-1) (Bedford et al., 2001). This protein promotes the surface expression of the receptor, inhibiting ubiquitination and proteosomal degradation of α and β subunits.

$GABA_ARs$ can access inhibitory postsynaptic specializations or extrasynaptic sites, depending on their subunits composition. Once on the neuronal surface $GABA_ARs$ are not static but are in a continue cycle between the plasma membrane and intracellular compartments (Thomas et al., 2005; Bogdanov et al., 2006) (Figure 1.15). The most important protein for stabilization of $GABA_ARs$ at synapses is gephyrin, considered the principal subsynaptic scaffold protein of both GABAergic and glycinergic synapses (Fritschy et al., 2008). On the other hand, the clustering of $GABA_ARs$ at the extrasynaptic site is mediated by radixin, which is an α5 $GABA_AR$ subunit-interacting protein linking the transmembrane proteins to the actin cytoskeleton (Loebrich et al., 2006). Moreover, the lateral diffusion of receptors from and into the synaptic regions also

plays an important role in the regulation of $GABA_A$Rs density at the synapse (Dahan et al., 2003; Tardin et al., 2003; Groc et al., 2004). The exchange between extrasynaptic and synaptic population is modulated by the activity of protein phosphatase 2B (PP2B). This calcium dependent mechanism activated via NMDA receptors leads to an increase in the lateral mobility of $GABA_A$Rs and reduces the size of inhibitory synapses, a process that favors neuronal depolarization (Bannai et al., 2009).

The regulation of receptor exo- and endocytosis is important in the control of the postsynaptic pool size and the strength of synaptic inhibition (Thomas et al., 2005; Bogdanov et al., 2006). The process of $GABA_A$R endocytosis occurs mainly via clathrin- and dynamin-dependent mechanisms upon interaction of the $GABA_A$R subunits with the clathrin-adaptor protein AP2-complex (Kittler et al., 2000, 2005, 2008) (Figure 1.15). The AP2, as a central component in the formation of these vesicles, forges a link between membrane proteins and clathrin, which forms the outer layer of the coat. $GABA_A$Rs are intimately associated with AP2 in the brain through a direct binding of the β1–3 and γ2 $GABA_A$R subunits (Kittler et al., 2000). The interaction of the AP2 with $GABA_A$R is negatively regulated by phosphorylation of $GABA_A$R β subunits.

Following internalization, $GABA_A$Rs are rapidly recycled back to the neuronal plasma membrane or target for lysosomal degradation. The density of internalized receptors is determinant for surface receptor levels. The decision regarding the sorting of endocytosed $GABA_A$Rs depends on the interaction of $GABA_A$R β1-3 subunits with huntingtin-associated protein 1 (HAP1) (Kittler et al., 2004). HAP1 is a $GABA_A$R-associated protein that binds the intracellular loop of β subunits (Kittler et al., 2004), however it is not known yet if HAP1 promotes recycling of $GABA_A$Rs or prevents their lysosomal degradation. Nevertheless, the selective suppression of hypothalamic HAP1 by siRNA induced a decrease in feeding behavior in mice that was attributed to reduced surface expression and activity of $GABA_A$Rs, demonstrating the importance of HAP1-dependent regulation of $GABA_A$R trafficking (Sheng et al., 2006).

Endocytosed $GABA_A$Rs that fail to be recycled are targeted for lysosomal degradation (Kittler et al., 2004). This process is regulated by ubiquitination of a series of lysine residues within intracellular domain of the γ2 subunit. Accordingly, an increase of $GABA_A$Rs accumulation at the synapses is observed when lysosomal activity is blocked or trafficking of ubiquitinated cargo to lysosomes is disrupted (Arancibia-Cárcamo and Kittler, 2009).

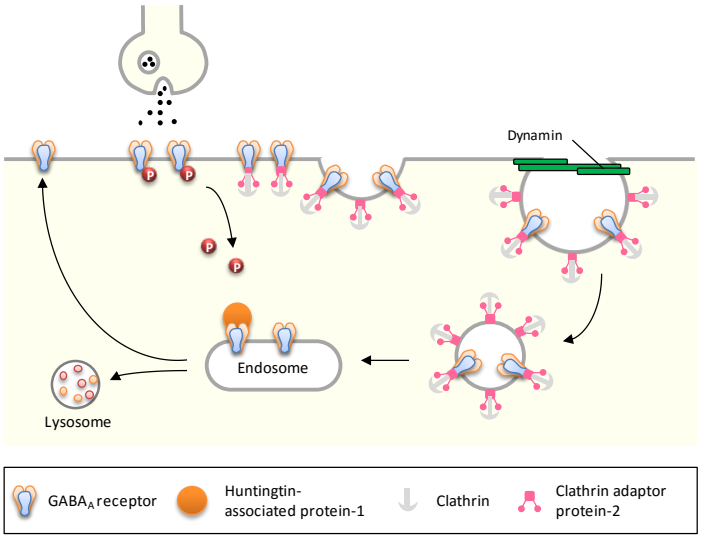

Figure 1.15 - GABA$_A$ receptor clathrin-mediated endocytosis. The receptors cluster in specializesd sites at the plasma membrane known as clathrin-coated pits. These structures invaginate and pinch off to form clathrin-coated vesicles, a process that is dependent on dynamin. The clathrin adaptor protein (AP)-2 forges a link between membrane proteins and clathrin that forms the puter layer of the coat, being a central component in the formation of these vesicles. The vesicles, after lose their coat, fuse together to form an early endosome. Internalized receptors are then either subjected to rapid recycling or targeted for lysosomal degradation, an endocytic sorting decision that is regulated by the huntingtin-associated protein (HAP)-1. Adapted from (Vithlani et al., 2011).

1.4.5 Inhibitory neurotransmission in the hypothalamus

There is a dense network of GABAergic terminals within the entire hypothalamus, and GABA is synthesized in neurones of several hypothalamic nuclei, which are known to be involved in control of feeding behaviour. GABA has been described as an orexigenic neurotransmitter (Kalra et al., 1999), acting through both GABA$_A$R and GABA$_B$R (Grandison and Guidotti, 1977; Kelly et al., 1979; Ebenezer, 1995).

Among α GABA$_A$R subunits, the α1 subunit is the most widely distributed and is practically present in all brain regions. In the hypothalamus, the GABA$_A$R α1, α2, α3 and α5 subunits mRNAs are found, being GABA$_A$R α2 subunit the predominant variant (Wisden et al., 1992; Fritschy and Mohler, 1995). NPY/AGRP-containing neurons are only positive for the GABA$_A$R α3 subunit. POMC/CART-containing neurones are positive for GABA$_A$R α1-3 subunits immunoreactivity. Regarding that POMC/CART neurones appear not to be GABAergic and that GABA and GABA$_A$ receptor agonist muscimol inhibit α-MSH release and POMC gene expression, the GABA$_A$R complex located on POMC/CART neurons does not represent somatodendritic autoreceptors (Bäckberg et al., 2004; Delgado, 2013).

The studies exploring the role of GABA as an orexigenic neurotransmitter show that the intracerebroventricular administration of the GABA$_A$ receptor agonist, muscimol, stimulates feeding in satiated pigs, a response blockable by the specific GABA$_A$ receptor antagonist,

bicuculline (Baldwin et al., 1990). Also, systemic and intracerebroventricular administration of $GABA_B$ receptor agonist, baclofen, causes an increase in food intake in satiated pigs (Ebenezer and Baldwin, 1990). Kelly et al. also injected muscimol into the ventromedial hypothalamus (VMH) and observed an increase of food intake dose-dependently in lean rats and a blocked effect by local pretreatment with the $GABA_AR$ antagonist picrotoxin (Kelly et al., 1979). In contrast to the feeding elicit effect of muscimol in VMH, when it is injected into the lateral hypothalamus (LH) attenuates feeding stimulated by orexin/hypocretin neurons, suggesting that $GABA_AR$ play divergent or even opposite roles in different hypothalamic areas (Turenius et al., 2009).

Moreover, the synaptic release of GABA by AgRP-expressing neurons in the hypothalamic arcuate nucleus is required for normal regulation of energy balance. In fact, acute ablation of AgRP neurons in adult mice has a severe anoretic effect, that can be prevented by chronic infusion of bretazenil, a partial $GABA_A$ receptor agonist, into the parabrachial nucleus, restoring feeding and body weight (Wu et al., 2009). In addition, the $GABA_B$ agonist baclofen significantly inhibits NPY expression stimulated by dexamethasone, and the inhibitory action of insulin is completely abolished in the presence of either the $GABA_A$ antagonist, bicuculline, or the $GABA_B$ antagonist, CGP35348 (*p*-3-aminopropyl-*p*-diethoxymethyl phosphoric acid), in hypothalamic organotypic cultures (Sato et al., 2005). Recently, deletion of $GABA_B$ receptor in POMC neurons in mice on high-fat diet results in a decrease of POMC expression and in obesity (Ito et al., 2013). These observations corroborate the role of GABA and its receptors in the feeding system.

1.4.6 Synaptic regulation in hypothalamic arcuate nucleus

Synaptic plasticity has not been considered previously as a critical regulator of energy homeostasis. Recent studies have revealed that synaptic vesicle release and continual plasticity in the feeding circuits may be a key component in energy balance control (Dietrich and Horvath, 2009).

The two neuronal populations – POMC/CART and NPY/AgRP neurons – are responsible for energy homeostasis in hypothalamic arcuate nucleus, as described in section 1.2.3.1. Activation of POMC neurons triggers the release of α-MSH from POMC axon terminals, leading to the activation of MC3R and MC4Rs, to suppress the food intake and increase energy expenditure. On the other hand, activation of the ARC NPY/AgRP neurons leads to release of AgRP, which antagonizes α-MSH effect on MC3R and MC4Rs. NPY/AgRP neurons also directly inhibits POMC perikarya through NPY and GABA release from NPY/AgRP terminals on basket-like synaptic

innervations of POMC neurons (Dietrich and Horvath, 2013a). Moreover, these neuronal populations receive abundant excitatory and inhibitory synaptic inputs, and the two neurotransmitters that account for most of the synaptic activity in the hypothalamus are the amino acids glutamate and GABA (Delgado, 2013), as mentioned above. The synaptic organization of hypothalamic neurons is able to rapidly rewiring the afferents neurons in response to metabolic hormones, even before any changes in feeding behavior and body weight (Sternson et al., 2005).

In the ARC, leptin differentially regulates catabolic/anorexigenic and anabolic/orexigenic neurons, as mentiones before. Leptin in ARC mainly contributes to satiety. Leptin enhances firing of POMC neurons via both presynaptic mode, depolarizating through a nonspecifc cation channel; and postsynaptic way reducing inhibition by local NPY/GABA neurons (Cowley et al., 2001). Moreover, recent evidence show that 'first-order' effectors of leptin signaling are mainly inhibitory-neurons, meaning that leptin directly acts on presynaptic GABAergic neurons and reduces inhibitory tone to postsynaptic POMC neurons, and thus prevents animals from over-feeding (Vong et al., 2011). Leptin-deficient *ob/ob* mice presents more excitatory synapses accompanied by fewer inhibitory synapses on NPY neurons, and more inhibitory synapses are formed on POMC neurons, contributing for the increased food intake in the *ob/ob* mice (Pinto et al., 2004). However, the balance of synaptic inputs is restored 6h after leptin administration in this mouse model (Pinto et al., 2004).

Insulin acts through insulin receptors present in hypothalamic nuclei, but unlike leptin, insulin directly inhibits the firing of a subpopulation of POMC neurons (Hill et al., 2010), by mechanisms still unknown. High-fat feeding in mice activates insulin receptor and PI3K signaling pathway to inhibit steroidogenic factor 1 expressing VMH neurons (Klockener et al., 2012), reducing the excitatory strength from VMH to ARC (Sternson et al., 2005), and thus contributes to obesity development. In hippocampus, the studies show that insulin facilitates the internalization of AMPAR (Man et al., 2000; Wang and Linden, 2000; Huang et al., 2003, 2004), and also recruits $GABA_AR$ (Wan et al., 1997). Moreover, insulin-induced hyperphagia could be blocked by $GABA_AR$ antagonist applied in VMH region (Kamatchi et al., 1984), suggesting $GABA_AR$ trafficking could be induced by insulin and contributes to food intake regulation.

Ghrelin is also involved in feeding regulation and energy homeostasis via activation of growth hormone secretagogue receptor in the hypothalamus. Ghrelin regulates NPY/AgRP-expressing

neurons in an opposite manner from leptin, attenuating the anorexic effect of leptin (Cowley et al., 2001, 2003; Horvath et al., 2001; Kamegai et al., 2001). Ghrelin directly stimulates the depolarization of NPY/AgRP neurons, and hyperpolarizes POMC neurons (Cowley et al., 2003). The decreased firing rate of POMC neurons results from presynaptic activation of GABAergic NPY/AgRP neurons (Tong et al., 2008), since the inhibitory effects of ghrelin on POMC neurons could be blocked by NPY and $GABA_A$ receptor blockers (Cowley et al., 2003). In addition, ghrelin increases the frequency of excitatory postsynaptic currents in the NPY/AgRP neurons through a mechanism dependent on calcium/calmodulin-dependent protein kinase kinase (CaMKK) activation of AMPK to promote synaptic plasticity (Yang et al., 2011). This enhanced synaptic transmission is switched off by leptin, once POMC-derived opiods (probably beta-endorphin) reverse the AMPK-mediated upregulation of excitatory inputs to NPY/AgRP neurons (Yang et al., 2011). Other mechanisms were already reported to be linked to synaptic plasticity, namely the presence of uncoupling protein 2 (UCP2) which seems to be essential for cellular changes that occur in the NPY/AgRP neurons in response to food deprivation and/or ghrelin (Coppola et al., 2007; Andrews et al., 2008); the inhibition of SIRT1 in the NPY/AgRP also affects cellular activity and synaptic plasticity (Dietrich et al., 2010). Moreover, the induction of spine formation in NPY/AgRP neuros in response to fasting could be mediated by ghrelin, which is completely abrogated in AgRP-NMDAR-knockout mice (Liu et al., 2012).

Generally, the synaptic rearrangement observed in hypothalamic area that controls the feeding behavior is a continuous phenomenon, dependent of daily metabolic environment. Therefore, the proposition assumes that the perikaryal inputs of orexigenic NPY/AgRP neurons are dominated by inhibitory connections when circulating leptin levels are high and ghrelin levels are low. When leptin levels diminish and ghrelin levels increase, stimulatory synapses dominate over inhibitory inputs. On POMC perikarya, the changes occur in the opposite direction described for the NPY/AgRP inputs. Some of the inhibitory inputs on POMC cells are likely to originate from NPY/AgRP neurons, and some of the stimulatory inputs on both cell types originate in the lateral hypocretin neurons (Horvath, 2006) (Figure 1.16).

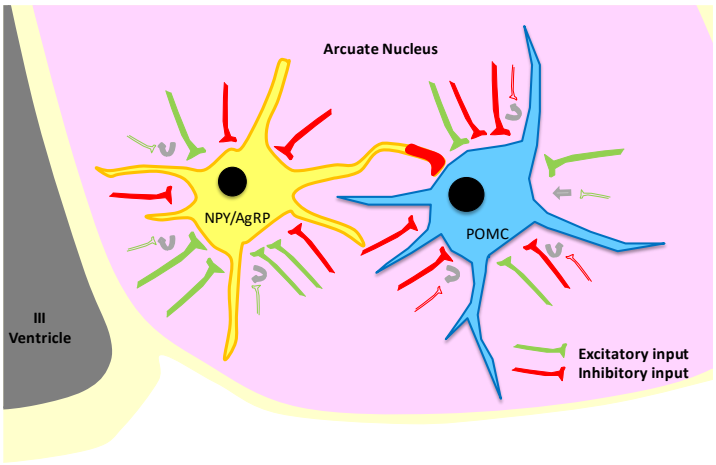

Figure 1.16 - Synaptic plasticity in the melanocortin system in the hypothalamic arcuate nucleus in response to different hormonal flutuations. When levels of ghrelin, an orexigenic hormone produced by the gut, are high there is an increase in the activity of the NPY/AgRP. The orexigenic NPY/AgRP neurons (yellow neuron) have an increased excitatory (green)/inhibitory (red) input ratio. In contrast, neighboring anorexigenic POMC neurnons (blue cells) have an elevated inhibitory/excitatory input ratio during negative energy balance compared to satiety. Arrows indicate the direction of synaptic movement after leptin levels increase and ghrelin levels diminished in satiety. When the levels of the anorexigenic hormone leptin are high there is an increase in the activity of POMC cells and a decrease in the activity of NPY/AgRP neurons. Positive energy balance leads to decrease in the number of excitatory inputs to NPY/AgRP neurons and inhibitory inputs to POMC neurons. Adapted from (Abizaid et al., 2006; Dietrich and Horvath, 2013a).

1.4.7 Synaptic plasticity and aging

Age-associated changes in neurotransmission can contribute to a decline in cognitive function or lead to neuronal degeneration (Shi et al., 2007). In the hypothalamus, there were already reported alterations in synaptic transmission. In ovary-intact rats, synaptic density was significantly less in middle aged 240 day animals than in 90-, 120-, or 180-day animals with greatest decrease occurring between 180- and 240-day animals (Moore and Leuschen, 1998). The reduction of synaptic network, namely GABAergic terminals, in the aging in the hypothalamic suprachiasmatic nucleus could also underlie the aging-related hypothalamic dysfunction (Palomba et al., 2008).

In addition, the down-regulation of many genes involved in synaptic transmission is also observed with aging in the hypothalamus. The expression levels of synaptotagmin I in the hypothalamus, a synaptic vesicle-associated protein that is involved in the regulation of neurotransmitter release, decreased 17-fold in aged animals. N-ethylmaleimide-sensitive factor-attachment receptor, a protein involved in synaptic vesicle exocytosis, was down-regulated 3.4-fold in hypothalamus of aged mice. Expression of PSD-95, which plays a role in NMDA-dependent signaling, decreased 16-fold, and GluR1 subunit decrease 2.7-fold in the aged hypothalamus. Expression of the $GABA_AR$ α-1 subunit, which plays an essential role in the signal transduction of neurotransmitter GABA, decreased 7-fold in the aged cortex but not in

the hypothalamus (Jiang et al., 2001). These results suggest that the age-related molecular alterations lead to synaptic modifications and a compromised interneuronal communication in different brain regions, even in the hypothalamus, during the aging process.

CHAPTER 2

Objectives

2. Main objectives

The hypothalamic arcuate nucleus, through its two neuronal populations – CART/POMC expressing neurons and AgRP/NPY expressing neurons – receives and integrates peripheral hormonal and nutritional signals and regulates appetite, food intake and energy expenditure. The neuronal synaptic input organization in this brain region is able to adapt and rearrange rapidly in response to metabolic hormones, contributing to energy homeostasis and metabolic phenotype.

The hypothalamus was recently pointed as important for the development of whole-body aging (Zhang et al., 2013). In spite of human life expectancy has increased, the world population aging is a current concern, with an increased prevalence of cognitive impairment and dementia. Thus aging research is focused in finding strategies that increase both lifespan and healthspan. Caloric restriction (CR) delays the aging and has a myriad of beneficial effects in several animal models. CR improves resistance to disease in yeast to primates and prevents the age-associated cognitive impairment and neurodegeneration, in mammals. Although, at the organism level, CR leads to other complex changes, whose effects and implications have not yet been fully elucidated. One of the major changes is the strong release of NPY in the hypothalamus, a peptide known to induce feeding behavior, additionally having neuroprotective effects. Moreover, it has been shown that CR extends lifespan, at least in part by autophagy induction. Autophagy, as a high-regulated intracellular process, is fundamental for cellular homeostasis, is required for lifespan extension in some animal models, and recently it has been reported that autophagy is a mechanism which plays a role in the control of energy homeostasis in the hypothalamus (Kaushik et al., 2011; Coupé et al., 2012).

Since NPY produces vast physiological effects, mostly consistent with the ones observed after CR (Minor, et al., 2009), and hypothalamic autophagy has an impact in energy homeostasis, the fact that the role of NPY in autophagy in the hypothalamus and the relationship between the autophagy and the synaptic plasticity in the hypothalamic circuits has never been studied has motivated this work.

Taking these evidences into account, we focused our work in two main objectives:

1 – To investigate the role of NPY receptors activation on autophagy in rodent hypothalamic neurons;

2 – To evaluate the impact of autophagy inhibition on synaptic plasticity and neuronal activation in rodent POMC neurons.

CHAPTER 3

Neuropeptide Y induces autophagy in hypothalamic neurons through a MTOR-independent pathway

3.1 Abstract

Growing evidence indicate that basal autophagic activity decreases with age, thus contributing to the aging phenotype and to the aggravation of age-related diseases. Moreover, it was described that hypothalamus is a critical brain area for whole-body aging development and has impact on lifespan. On the other hand, caloric restriction (CR) is a robust anti-aging intervention, increasing lifespan and decreasing the incidence of age-related diseases. CR increases autophagy and increases neuropeptide Y (NPY) levels in the hypothalamus. NPY has neuroprotective effects and increases resistance to stress and mean lifespan. However its role in autophagy has never been addressed.

Therefore, the aim of this study was to investigate the role of NPY on autophagy regulation in the hypothalamus. Using both hypothalamic neuronal *in vitro* models and mice overexpressing NPY in the hypothalamus, the present study demonstrates, for the first time, that NPY increases autophagy in the hypothalamic neurons through NPY Y_1 or Y_5 receptors activation. Moreover, this effect is tightly associated with the concerted activation of PI3K, MEK/ERK and PKA signaling pathways, independent of MTOR inhibition.

Since both hypothalamic autophagy and NPY levels decrease with age, modulation of NPY may act as a protective mechanism against impaired hypothalamic dysfunction associated with age. In addition, a better understanding of hypothalamic neuronal autophagy regulation by NPY system will provide new putative therapeutic strategies to ameliorate age-related deteriorations and extend longevity.

3.2 Introduction

Aging and longevity are determined by multifactorial and complex processes whose molecular basis remain incompletely understood (Kirkwood, 2005). Aging is associated with accumulation of specific cellular proteins within neurons, a pathologic hallmark of many neurodegenerative diseases. Moreover, aging also induces neuroendocrine control dysfunctions, altering the intimate relationship between the nervous and endocrine systems influencing the progression of age-related diseases (Smith et al., 2005). Since average human life expectancy has increased, but also the prevalence of cognitive decline and dementia, aging research is now focused in finding strategies that increase both lifespan and healthspan.

Autophagy is a highly regulated intracellular process involved in the turnover of most cellular constituents and in the maintenance of cellular homeostasis (Hansen et al., 2008; Blagosklonny, 2010). Long-lived proteins and organelles are first engulfed by specialized double membrane vesicles, termed autophagosomes, and delivered to the lysosomes for subsequent degradation. The constant flow of autophagosomes to lysosomes is tightly regulated by a number of proteins. It is well established that the basal autophagic activity of living cells decreases with age, thus contributing to the accumulation of altered macromolecules during aging (Mariño and López-otín, 2008). In addition, autophagy impairment contributes to different aspects of aging phenotype and to aggravation of age-related diseases (Cuervo, 2008). On the other hand, the activity of this catabolic pathway is required for lifespan extension in animal models such as *Caenorhabditis elegans* and *Drosophila melanogaster* (Hansen et al., 2008; Simonsen et al., 2008; Eisenberg et al., 2009).

Caloric restriction (CR), the reduced intake of food without malnutrition, is one of the few environmental manipulations that has been reported to consistently extend lifespan of many organisms, from yeast to mammals, and delays the progression of age-related diseases, at least in part, by stimulating autophagy (Donati, 2006; Masoro, 2006; Hansen et al., 2008; Blagosklonny, 2010). One major neuroendocrine effect of CR is the increase of neuropeptide Y (NPY) in the hypothalamus (Brady et al., 1990; Bi et al., 2003; de Rijke et al., 2005; Minor et al., 2009). The hypothalamus has a key role in the control of body homeostasis, neuroendocrine outputs and feeding behavior. Recently, it was described that this brain area is critical for the development of whole-body aging and has impact on lifespan (Satoh et al., 2013; Zhang et al., 2013).

NPY regulates food intake (Wieland et al., 2000), and it has also been implicated in several other physiological functions, such as blood pressure, body temperature, hormone and neurotransmitters release, and modulation of pain, sexual behavior, circadian rhythms, memory processing and cognition (Wettstein et al., 1995; Nguyen et al., 2011; Wiater et al., 2011; Decressac and Barker, 2012; Beck and Pourié, 2013). Moreover, NPY has a neuroprotective role against excitotoxicity in rat and mouse hippocampus and also in the retina (Silva et al., 2005; Alvaro et al., 2008b; Santos-Carvalho et al., 2013). These diverse actions of NPY are mediated by G-protein-coupled receptor subtypes named NPY Y_1, Y_2, Y_4, and/or Y_5 (Michel et al., 1998; Silva et al., 2005), all of which have been reported to be present in the hypothalamus (Acuna-Goycolea and van den Pol, 2005).

Others showed that CR does not increase lifespan in NPY knockout mice (Chiba et al., 2014) and aging decreases NPY levels in rodent hypothalamus (Higuchi et al., 1988; Gruenewald et al., 1994; Vela et al., 2003). On the other hand, transgenic rats overexpressing hypothalamic NPY have improved stress resistance and increased mean lifespan (Michalkiewicz et al., 2003). Although these observations suggest that NPY may play a relevant role in aging and lifespan, the effect of NPY on autophagy remains unknown.

Given the key role of hypothalamus in the regulation of body homeostasis and being autophagy a central process in CR-induced extension of lifespan, the aim of this study was to investigate the role of NPY and the involvement of its receptors on autophagy regulation in the hypothalamus and the mechanisms underlying this process. A better understanding of the role of NPY in the regulation of autophagy will provide a novel potential therapeutic strategy to extend longevity and ameliorate age-related deteriorations.

3.3 Materials and Methods

3.3.1 Animals

Female Wistar rats and male C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Mice were housed two per cage under a 12 h light/dark cycle in a temperature/humidity controlled room with *ad libitum* access to water and a standard chow diet. All experimental procedures were performed in accordance with the European Union Directive 86/609/EEC for the care and use of laboratory animals. Moreover, all the people working with animals have received appropriate education (FELASA course) as required by the Portuguese authorities. In addition, animals are housed in our licensed animal facility (International Animal Welfare Assurance number 520.000.000.2006). The present study is included in a project approved and financed by the Portuguese Science Foundation that approved the animal experimentation described. CNC animal experimentation board approved the utilization of animals for this project (reference PTDC/SAU-FCF/099082/2008).

3.3.1.1 Neuropeptide Y overexpression in the mouse hypothalamic arcuate nucleus

Recombinant AAV particles were generated as described before (Sousa-Ferreira et al., 2011b). Rat NPY cDNA from p46F06444D-NPY (RZPD) was cloned into adeno-associated viral vectors (AAV) back-bone. For the validation of NPY vector, HEK 293 cells (American Type Culture Collection, ATCC CRL-11268, Manassas, USA, ATTC) were co-transfected with pAAV-hSyn-NPY in a 1:1 ratio, using standard calcium phosphate method. Forty-eight hours after transfection, cell lysates were obtained and thirty micrograms of protein were used for 16% polyacrylamide gel Tricine-sodium dodecylsulphate (SDS) electrophoresis. Protein samples were transferred to a 0.22 µm nitrocellulose membrane (Applichem, Darmstadt, Germnay). The membrane was incubated with anti-NPY antibody (1:3000; Sigma-Aldrich, St. Louis, MO, USA) overnight at 4°C followed by incubation with alkaline phosphatase-linked antibody and detection by enhanced chemifluorescence (ECF) substrate. NPY and tubulin expression levels were calculated by densitometry analysis (Bio-Rad Fluor MaxS software, Hercules, CA, USA).

The recombinant plasmids with NPY cDNA under a neuronal specific promoter, the human synapsin promoter, were packaged into an AAV-1/2 chimerical capsid using AAV-1 and AAV-2 packaging plasmids in a 50:50 ratio, and injected in mice hypothalamic arcuate nucleus

(ARC), in order to induce constitutive NPY overexpression. The human synapsin promoter in the viral vector guarantees that only mature neurons will express the transgene. The proximal region of the synapsin promoter is sufficient for directing neuron-specific gene expression. This proximal region is highly conserved between mouse and human (Schoch et al., 1996). Recombinant plasmids with EGFP (enhanced green fluorescent protein) cDNA were used as a control for the procedure.

Thirty male C57BL/6 mice (eight weeks old) weighing 20-24g were randomly divided into two groups (n=15 per group) and housed two per cage, under 12 hrs light/dark cycles. Mice were anesthetized with an intraperitoneal injection of ketamine/xylazine (100 mg/kg and 10 mg/kg, respectively) and placed on a stereotaxic frame. The ARC was defined using The Paxino's Mouse Brain Atlas. Injection was performed bilaterally into the ARC: 0,5 mm lateral to the middle line, 1,65 mm posterior to the bregma and -5,8 mm ventral to the brain surface. The control group (AAV-GFP) received $1x10^9$ v.g./side of AAV-hSyn-EGFP-miR-ctr (v.g., viral genomes), in a final volume of 1.5 µl/side. The NPY overexpression group (AAV-NPY) received $3.6x10^9$ v.g./side of AAV-hSyn-NPY, in a final volume of 1.5 µl/side. Injection was performed at a rate of 0.5 µl/min with a 10 ml-Hamilton syringe attached to an automatic Pump Controller (WPI). Needle was kept in place for 5 minutes to minimize backflow. Mice were allowed to recover for 2 days.

3.3.1.2 Pair feeding and body weight gain analysis

Mice were housed in pairs and monitored for one month after AAV injections. Since overexpression of ARC NPY increases food intake and body weight, leading to an obese phenotype (Sousa-Ferreira et al., 2011b), which can potentially affect autophagy regulation in the hypothalamus (Ost et al., 2010; Las et al., 2011), AAV-NPY mice were pair-fed to control AAV-GFP mice (given the same amount of food that AAV-GFP mice ate, daily – approximately 4-5 g/day). Each mouse was weighted every other day, for weight control.

3.3.1.3 Serum analysis

Glucose levels were measured using Accu-Check Blood Glucose Sensor (Roche Diagnostics, Basel, Switzerland). Cholesterol and triglycerides were measured on an automated Synchron® Clinical System (Beckman Coulter).

3.3.1.4 Behavioral assessment

For the assessment of mice locomotor horizontal activity, open field tests were performed 29 days after AAV injections. Mice were acclimated into test room for one hour. Mice were placed in a 50×50 cm arena with 50 cm high walls and their movement activity was recorded for 40 min using the Acti-Track System (Panlab, Barcelona, Spain). Mean values for each measure were calculated. Data was analyzed using Student's unpaired *t* test with two-tailed *p* value, using Prism 5.0 (GraphPad Software, San Diego, CA, USA). A value of $p<0.05$ was considered significant. No alterations were found in the locomotor activity of AAV-NPY mice, 29 days post-injection. The distance travelled (8592 ± 2060.7 cm) and mean velocity (3.5 ± 0.7 cm/s) of AAV-NPY mice was similar to AAV-GFP mice (9050 ± 458.4 cm and 3.8 ± 0.2 cm/s, respectively).

3.3.1.5 Tissue and blood collection

AAV-injected animals were euthanized one month after the stereotaxic injections, by an intraperitoneal administration lethal dose of sodium thiopental (B. Braun, Hesse, Germany). Animals from both groups were randomly selected either for collection of blood and hypothalamic tissue for protein extraction, or for whole brain removal for immunohistochemistry experiments. For tissue lysates and blood analysis, first, the blood was collected and serum was separated by centrifugation (2,000 g for 15 min, at 4°C). Serum samples were kept at -20°C until use. Then, after decapitation, hypothalami were individually collected and stored at -80°C until use.

For immunohistochemistry, animals were intracardially perfused with 4% (w/v) paraformaldehyde/ 0.1 M phosphate buffered saline (PBS; 137 mM NaCl; 2.7 mM KCl; 10 mM Na_2PO_4; 1.8 mM KH_2PO_4; pH 7.4) fixative solution and, after decapitation, the brains were removed and cryoprotected in 25% sucrose/0.1 M PBS solution for 48 h at 4°C. Brains were then stored at -80°C until use. For immunohistochemistry purposes, brains were cut at a cryostat-microtome (Leica CM3050S, Leica Microsystems Nussloch GmbH, Nußloch, Germany) in 25 µm coronal sections. Slices were collected and stored in 48-well trays, free-floating in 0.1 M PBS supplemented with 0.12 µmol/L sodium azide. The plates were stored at 4°C until immunohistochemical processing.

3.3.2 Cellular models

3.3.2.1 Embryonic mouse hypothalamus cell line N42 (mHypoE-N42)

The adherent cell line mHypoE-N42 (CELLutions Biosystems Inc./Cederlane, Ontario, Canada) was obtained from immortalized mouse embryonic day 15-18 (E15-18) hypothalamic primary cultures, through retroviral transfer of Simian Vacuolating Virus 40 T-Antigen (SV40 TAg). This cell line has been found to express an array of neuropeptides (including NPY and AgRP), biologically active receptors for NPY and leptin, among others, and enzymatic markers such as tyrosine hydroxylase. The cell doubling time is approximately 24 h.

Cells were maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM; 4.5 g/L/ D-glucose, Sigma-Aldrich), supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin (all from Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified atmosphere with 95% air and 5% CO_2, in 75 cm^2 tissue culture flasks. When cells reached approximately 90% confluence, they were washed twice with pre-warmed PBS and detached from the flask by trypsinization, for 3-5 min, at 37°C. The trypsin (Life Technologies, from Invitrogen) was then inhibited by adding growth medium. Cells were sedimented by centrifugation, at 900 rotations per minute (rpm), for 5 min, after which the supernatant was discarded and cells ressuspended in pre-warmed fresh growth medium. Cell density was determined with a hemocytometer, by direct counting and cells were seeded in a new tissue culture flask, for passaging. As for plating, for protein extraction purposes, cells were plated and grown for three days, in uncoated 6-well or 60 mm plates, or in 12-well plates, with gelatin coated 16 mm glass cover-slips, for immunocytochemistry purposes.

3.3.2.2 Rat hypothalamic neural cells primary cultures

Floating hypothalamic neurospheres were obtained as previously described with modifications (Sousa-Ferreira et al., 2011a). Hypothalamic tissue was dissected from rats at embryonic days 18-19 (E18-19) in a calcium and magnesium free solution (CMF; 137 mM NaCl; 5.4 mM KCl; 0.45 mM KH_2PO_4; 0.34 mM Na_2HPO_4; 4 mM $NaHCO_3$; 5 mM glucose; pH 7.4). Batches of 10-14 tissue fragments were successively dissociated by means of a Pasteur pipette in a PBS solution with 5.5 mM glucose, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen), generically designated "Solution A", with no enzymatic digestion. The dissociation involved pipetting the tissue up and down, in Solution A, with a Pasteur

pipette and letting the larger particles sediment. The supernatant suspension containing the smaller tissue particles was then collected to a new centrifuge tube. This suspension was then centrifuged at 800 rpm, for 7 min, after which the supernatant was discarded and the pellet ressuspended in 10 mL of DMEM-F12/GlutaMAX™ , supplemented with 10 ng/mL fibroblast growth factor-2 (FGF), 10 ng/mL epidermal growth factor (EGF), 100 U/ml penicillin, 100 g/ml streptomycin and 1% B27® supplement (all from Invitrogen). The floating neurospheres were allowed to growth, in floating conditions, for 7 days, in uncoated 25 cm^2 flask and maintained at 37°C with 95% air and 5% CO_2. To obtain a differentiated hypothalamic neural culture, neurospheres were then collected, centrifuged at 800 rpm for 5 min and, after discarding most of the medium, dissociated by pipetting up and down with a micropipette tip. The neurospheres were then plated in poly-D-lysine-coated (Sigma-Aldrich) 6-well culture plates, for protein analysis, or poly-D-lysine-coated glass cover-slips for immunocytochemistry purposes. The neurospheres were allowed to attach and differentiate for 16 days, in Neurobasal™ Medium, supplemented with 500 µM L-Glutamine, 2% B27®, 100 U/mL penicillin and 100 g/mL streptomycin (all from Invitrogen), at 37°C, 95% air and 5% CO_2. The cell culture medium was replaced twice a week by aspirating one third of total medium volume from each well and replacing it with fresh medium.

3.3.3 Cellular treatments

To evaluate the effect of NPY on autophagy, cells were treated with recombinant NPY, in a time-dependent manner, and with NPY receptors agonists. To assess which NPY receptor subtype modulates the NPY effect in autophagy, different NPY receptors selective antagonists were also used, as well as, the lysosomal protein degradation inhibitor, chloroquine, to confirm the autophagic flux induced by NPY; and different inhibitors of protein kinases to study the signaling pathways involved in NPY effect in autophagy were also used. Chloroquine, NPY receptors antagonists or protein kinase inhibitors were added to cell culture medium 30 min before the addition of NPY or NPY receptors agonists. Rapamycin was used as a positive control of autophagy induction. The following table summarizes all chemical compounds used, its function and final concentrations.

Table 3.1 - Chemical substances used in cellular treatments.

Compound	*Concentration used*	*Function*	*Source*
NPY	100 nM	Evaluate its effect on autophagy	Phoenix Europe GmbH, Karlsruhe, Germany
[Leu^{31}Pro^{34}]NPY	100 nM	NPY Y_1 receptor agonist	Bachem, Bubendorf, Switzerland
NPY_{19-23}(Gly^1, Ser^3, Gln^4, Thr^6, Ala^{31}, Aib^{32}, Gln^{34})PP	100 nM	NPY Y_5 receptor agonist	Bachem, Bubendorf, Switzerland
BIBP3226	1 µM	NPY Y_1 receptor antagonist	Tocris Bioscience, Bristol, UK
BIIE0246	1 µM	NPY Y_2 receptor antagonist	Tocris Bioscience, Bristol, UK
L-152,804	1 µM	NPY Y_5 receptor antagonist	Tocris Bioscience, Bristol, UK
Choloroquine	100 µM	Lysossomal protein degradation inhibitor	Sigma-Aldrich, St. Louis, MO, USA
3-Methyladenine (3-MA)	5 mM	PI3K inhibitor	Merck Millipore, Billerica, MA, USA
LY294002	1 µM	PI3K inhibitor	Merck Millipore, Billerica, MA, USA
U0126	1 µM	ERK inhibitor	Merck Millipore, Billerica, MA, USA
H89	1 µM	PKA inhibitor	Merck Millipore, Billerica, MA, USA
Rapamycin	10 nM	Autophagy inducer	Sigma-Aldrich, St. Louis, MO, USA

3.3.4 Protein expression analysis

3.3.4.1 Cell lysates

After cell treatments, the cell culture plates were immediately placed on ice, the culture media was discarded by aspiration and each well was washed twice with ice-cold PBS. The cells, as well as, whole hypothalamic tissue, were lysed with radio-immunoprecipitation assay (RIPA) buffer [50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA; 1% (v/v) Triton X-100 (TX-100); 0.5% (w/v) deoxycholate 0.1% (w/v) SDS; 200 µM phenylmethylsulfonylfluoride (PMSF); 1 mM dithiothreitol (DTT); 1 mM sodium orthovanadate (Na_3VO_4); 10 mM sodium fluoride (NaF)], supplemented with complete mini protease inhibitor cocktail tablet (Roche Diagnostics). Cells were then collected by scrapping, using a rubber cell-scrapper. The cell lysates were maintained on ice for 15 min, being mixed by vortexing every 5 min and, afterwards, frozen and stored at -20 °C, until use.

Every sample was quantified for its protein content through the bicinchoninic acid (BCA) protein assay (Pierce/Thermo Fisher Scientific, Rockford, IL, USA) after being centrifuged at

16,100 g, for 10 min, at 4 °C and each supernatant collected to a new tube, to cellular debris removal. The bovine serum albumin (BSA) solution (2 mg/mL) was used as standard. After following the manufacturer's instructions for protein quantification, the samples were denatured with 6x concentrated electrophoresis sample buffer (0.5 M Tris-HCl, pH 6.8, 30% (v/v) glycerol, 10.4% (w/v) SDS, 0.6 M DTT, 0.012% (w/v) bromophenol blue), boiled for 5 min at 95 °C and stored at -20 °C until use.

3.3.4.2 Western blotting

Western Blotting technique was used in order to immunodetect the expression of autophagy-related proteins. Equal amounts of total protein were loaded per lane and separated by electrophoresis in 4-12% sodium dodecyl sulphate–polyacrylamide gels (SDS-PAGE). The electrophoresis was run on a Tris-Bicine buffer (25 mM Tris; 25 mM Bicine; 1% (w/v) SDS; pH 8.3), first at 70 V, for 10 min, and then, at 120-140 V, for 55-70 min. The protein samples were then transferred electrophoretically from the gels to previously methanol-activated polyvinylidenedifluoride (PVDF) membranes (Merck Millipore), in CAPS transfer buffer (10 mM CAPS, pH 11.0; 10% (v/v) methanol), at a 750 mA constant current, for three hours, at 4 °C. Afterwards, the membranes were blocked for one hour at room temperature, in 5% (w/v) low-fat milk in a Tris-buffered saline (TBS; 20 mM Tris; 137 mM NaCl; pH 7.6) containing 0.1% (v/v) Tween 20 (TBS-T), and incubated overnight with the primary antibodies, in TBS-T with 5% (w/v) BSA, at 4 °C. The primary antibodies used (all at a dilution of 1:1,000) were: mouse anti-beclin-1 (BECN1; BD Biosciences, San Jose, CA, USA), rabbit anti-LC3B, anti-SQSTM1, anti-MTOR, anti-phospho-MTOR (Ser2448), anti-RPS6K, anti-phospho-RPS6K (Thr389), anti-phospho-AKT(Ser473), anti-AKT, anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), anti-p44/42 MAPK (ERK1/2), anti-phospho-PKA (Thr197) and anti-PKA (all other from Cell Signaling Technology, Beverly, MA, USA). After three washes with TBS-T, the membranes were incubated for one hour, at room temperature, with an alkaline phosphatase-linked secondary antibody, specific to rabbit or mouse immunoglobulin G (IgG; GE Healthcare, Buckinghamshire, UK) in a 1:10,000 dilution in TBS-T with 5% (w/v) BSA. Protein immunoreactive bands were visualized by chemifluorescnce with the ECF substrate (GE Healthcare) in a VersaDoc Imaging System (Bio-Rad).The membranes were reprobed with a monoclonal β-tubulin I (Sigma-Aldrich) immunoreactivity in a 1:10,000 dilution in TBS-T with 5% (w/v) non-fat milk, at 4 °C. After being washed in TBS-T, the membranes were incubated with an alkaline phosphatase-linked secondary antibody, specific to mouse IgG (GE Healthcare), in a 1:10,000 dilution in TBS-T with 5%

(w/v) non-fat milk, for one hour. The optical density of the bands was quantified with the Quantity One Software (Bio-Rad). The results are normalized to β-tubulin and are expressed as the relative amount compared to control.

3.3.4.3 Immunocytochemistry

After treatments, cells were washed twice with pre-warmed PBS and then fixed with ice-cold 4% (w/v) paraformaldehyde/PBS for 15 min. Cells were permeabilized with 0.25% (v/v) TX-100/PBS (mHypoN42) or 0.75% (v/v) TX-100/PBS (rat differentiated hypothalamic neural cells) for 10 min, washed with PBS and then blocked for one hour with 10% goat serum (GS)/PBS (mHypoN42) or 3% (w/v) BSA/PBS (rat differentiated hypothalamic neural cells). The cells were incubated with primary antibodies overnight at 4 °C. After incubation, cells were washed in PBS and incubated for one hour at room temperature with the respective secondary antibodies. The nuclei were stained with Hoechst 33342 (2 µg/mL; Invitrogen) during secondary antibody incubation. The coverslips were washed in PBS and mounted on glass slides with Dako Fluorescence Mounting Medium (Dako S3023, Glostrup, Denmark). The primary antibodies used were: rabbit anti-LC3B (1:400; Cell Signaling Technology), mouse anti-NeuN (1:400; Merck Millipore), anti-glial fibrillary acidic protein (GFAP, 1:500; Merck Millipore), anti-SOX2 (1:200; R&D Systems, Abingdon, UK) and anti-CD68 (ED-1, 1:50; Bio-Rad, Hercules, CA, USA). The secondary antibodies used (at a dilution of 1:200) were: Alexa-Fluor 488-conjugated goat anti-rabbit IgG and Alexa-Fluor 594-conjugated goat anti-mouse IgG (all from Invitrogen). Cells were analyzed on a Zeiss Axiovert fluorescence microscope (Carl Zeiss, Oberkochen, Germany). The procedure was performed for three independent culture preparations.

3.3.4.4 Immunohistochemistry

Hypothalamic NPY and LC3B expression, as well as, the co-localization of GFP with POMC and NPY or AgRP were assessed by immunohistochemistry on brain sections. Briefly, 25 µm brain coronal sections were blocked and permeabilized in PBS with 10% GS and 0.3% (v/v) TX-100, to label NPY, in PBS with 10% GS and 0.5% (v/v) TX-100, to label LC3B, and in 10% GS with 3% BSA and 0.3% (v/v) TX-100, to co-localization assays, for two hours. Brain slices were then incubated with polyclonal rabbit anti-NPY antibody (1:6000; Sigma-Aldrich), polyclonal rabbit anti-LC3B antibody (1:400; Cell Signaling Tecnhology), or polyclonal chicken anti-POMC antibody (1:1000, Abcam, Cambridge, UK) with polyclonal rabbit anti-

NPY antibody (1:6000; Sigma-Aldrich) or polyclonal goat anti-AgRP antibody (1:200, Santa Cruz Biotecnhology, Heidelberg, Germany) in the respective blocking solution, overnight at 4 °C. Sections were then washed in PBS and incubated with goat anti-rabbit Alexa-Fluor 594-conjugated secondary antibody, goat anti-chicken Alexa-Fluor 647-conjugated secondary antibody or donkey anti-goat Alexa-Fluor 594-conjugated secondary antibody (all 1:200, Invitrogen), according to the primary antibodies used, for two hours at room temperature. The nuclei were stained with Hoechst 33342 (2 µg/ml; Invitrogen). After incubation, brain sections were washed and mounted in slides with Mowiol® mounting medium (Sigma-Aldrich) and analyzed on a Zeiss Axiovert fluorescence microscope (Carl Zeiss).

Quantification of NPY immunoreactivity in the mouse hypothalamic ARC

Four weeks after AAV injection, NPY immunohistochemistry and imaging procedures were performed. Coronal sections of approximately equal spacing were sampled over the anterior–posterior extent of the hypothalamic ARC (Bregma -1.34 to -2.54) for NPY immunoreactivity determination (four mice/group). The ARC was defined using The Paxino's Mouse Brain Atlas. For each mouse, the ARC of one hemisphere was delimited and the integrated density (the product of area and mean grey value; arbitrary units) of 12 sections was measured using the Fiji software (National Health Institute, Bethesda, MD, USA). The integrated density values were summed to yield total integrated density values for each animal, and the mean of the total integrated density values was calculated for each experimental group. Analyses were done on one hemisphere from each section. The results are expressed as the relative amount compared to control.

3.3.5 Autophagy assays

There are several assays for measuring autophagy, with microtubule-associated protein 1 light chain 3 beta (MAP1LC3B; hereafter referred as LC3B) detection as central approach for cellular readout of autophagy level. Upon autophagy induction, the cytoplasmic form of LC3B is converted by cleavage and lipidation to a transient, autophagosomal membrane-bound form of LC3B (LC3B-II). LC3B-II is an essential component of autophagosome membrane and a marker of all autophagic structures, immature and mature autophagosomes as well as autolysosomes. To monitor autophagy, others autophagy markers were used namely sequestosome 1 (SQSTM1, also known as p62) and beclin 1 (BECN1). SQSTM1 is an ubiquitin binding protein that binds directly to LC3B-II and is

degraded by autophagy. Thus, the protein levels of SQSTM1 are inversely related to autophagic activity (Pankiv et al., 2007). BECN1 is a component of a large molecular complex involved on autophagy vesicle formation (Wirawan et al., 2012).

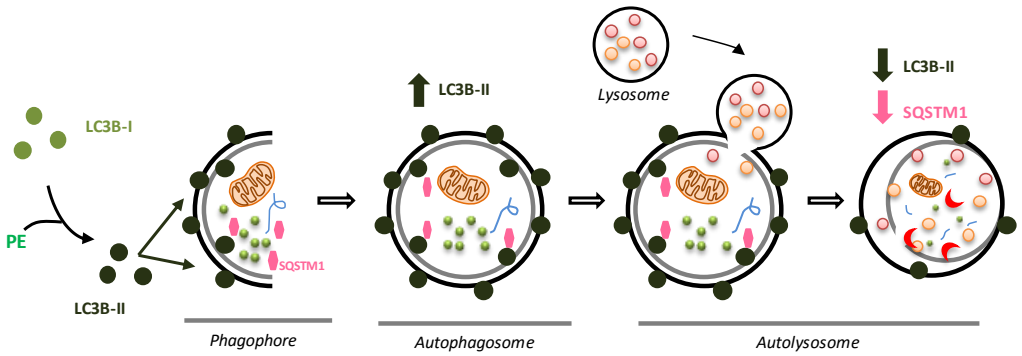

Figure 3.1 - Scheme depicting autophagic flux and common autophagy markers. Autphagic flux is a dynamic process which involves the formation of a crescent-shaped double membrane, the phagophore, which expands around a portion of cytoplasm and fuses to form the autophagosome. The mature autophagosome then fuses with a lysosome to generate an autolysosome, where the cargo is degraded. LC3B is the common autophagy marker used. The cytoplasmic LC3B-I is converted in LC3B-II by lipidation (PE: phosphatidylethanolamine) and it is present in all autophagic strucutures. The increased LC3B-II is correlated with an increase in number of autophagosomes, and then LC3B-II is degraded by lysosomal enzymes. SQSTM1 is also used to monitored autophagy. This protein is selectively incorporated into autophagosomes through direct binding to LC3 and is efficiently degraded by autophagy; thus, the total cellular expression levels of SQSTM1 are inversely correlated with autophagic activity. Adapted from (Mizushima et al., 2010; Klionsky et al., 2012).

However, analyze LC3B-II levels give only a snapshot look at autophagy at any one static point during the process from phagophore to autolysosome; thus measure autophagy requires the analysis of autophagic flux, the rate at which material is cleared from the cell by autophagy. Therefore, assays to detect endogenous LC3 processing in the presence or absence of an inhibitor of lysosomal turnover of autophagosome content, complemented by a more dynamic image-base mcherry-GFP-LC3 reporter of autophagy were used to strength the autophagic changes in our experimental conditions.

3.3.5.1 Determination of autophagic flux by LC3B turnover assay

Increased LC3-II levels can be associated with either enhanced autophagosome synthesis or reduced autophagosome turnover, perhaps due to delayed trafficking to the lysosomes, reduced fusion between compartments or impaired lysosomal proteolytic activity. To better interpret changes in levels of processed LC3B-II, the difference in the amount of LC3B-II in the presence or absence of lysosomal inhibitor, chloroquine, can be used to examine the transit of LC3B-II through the autophagic pathway, and thus, the autophagic

flux. Therefore, the LC3B turnover assay measures the amount of LC3B-II delivered to the lysosomes for degradation by comparing the LC3B-II amounts in the presence and absence of the lysosomal inhibitor, chloroquine (CQ; 100 µM). Autophagic flux, expressed as "LC3B-II net flux", was determined by subtracting the densitometric value of LC3B-II amount in samples non-treated with chloroquine (LC3B-II − CQ) from the corresponding sample treated with chloroquine (LC3B-II + CQ). If autophagic flux is stimulated, the amount of LC3B-II will be higher in the presence of lysosomal inhibitor (CQ), while if LC3B-II protein does not increase in the presence of CQ indicates that autophagic flux is not stimulates and a defect or delay earlier in the process, prior to degradation at the autolysosome, occured.

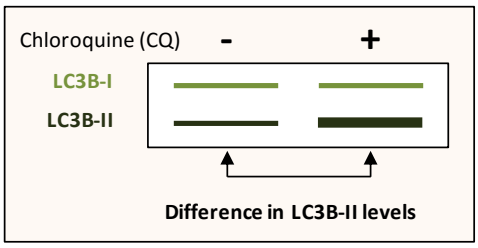

Figure 3.2 - Autophagic flux monitoring by LC3B turnover assay. Degradation of LC3B-II inside the autolysosome is estimated by the comparison of two samples with and without lysosomal inhibitor treatment (chloroquine, CQ). Adapted from (Mizushima et al., 2010).

3.3.5.2 Determination of autophagic flux by live-cell imaging using mCherry-GFP-LC3

Autophagic flux can also be morphologically traced with an mCherry-GFP-LC3 tandem construct. This methodoly to measure autophagic flux explores the difference in the nature of these two fluorescent proteins, once the fluorescent signal of GFP is quenched in the low pH inside the lysosomes and mCherry exhibits more stable fluorescence in acidic compartments. Therefore, autophagosomes and autolysosomes are labeled with yellow (mCherry and GFP) and red (mCherry only) signals, respectively. If autophagic flux is increased, both yellow and red punctae are increased; however, if autophagosome maturation into autolysosomes is blocked, only yellow punctae are increased without a concomitant increase in red punctae.

mHypoN42 cells plated in µ-Slide 8 well ibiTreat imaging chambers (Ibidi, Planegg-Martinsried, Deutschland) were cultured for two days at 37°C with 95% air and 5% CO_2. Cells were then transfected with 0.5 µg mCherry-GFP-LC3 plasmid DNA (kind gift from José Santos Ramalho, from CEDOC, New University of Lisbon) using Torpedo^{DNA} Transfection Reagent (Ibidi) for 24 h, according to the manufacturer's specifications. For mCherry-GFP-

LC3B analysis, imaging was performed on a spinning-disk confocal (Cell Observed SD; Carl Zeiss) with an inverted microscope (Axio Observer Z1; Carl Zeiss) using a Plan-Apochromat 63x, 1,4 NA oil immersion objective (Carl Zeiss) in an environmental chamber at 37°C. Digital images were acquired with an EM charge-coupled device camera (Rolera EM-C2; QImaging) using ZEN software (Carl Zeiss). For each condition, a z-stack in three different fields was imaged in each well and images were taken every 1 min for 30 min, upon the treatment with NPY. Cellular GFP-LC3- and mCherry-LC3-positive puncta were detected and quantified using the Find Peaks plugin and the Analyze Particles tool in Fiji software (National Institute of Health) and averaged per cell. More than 20 cells were analyzed in each condition and data are representative of, at least, three independent experiments.

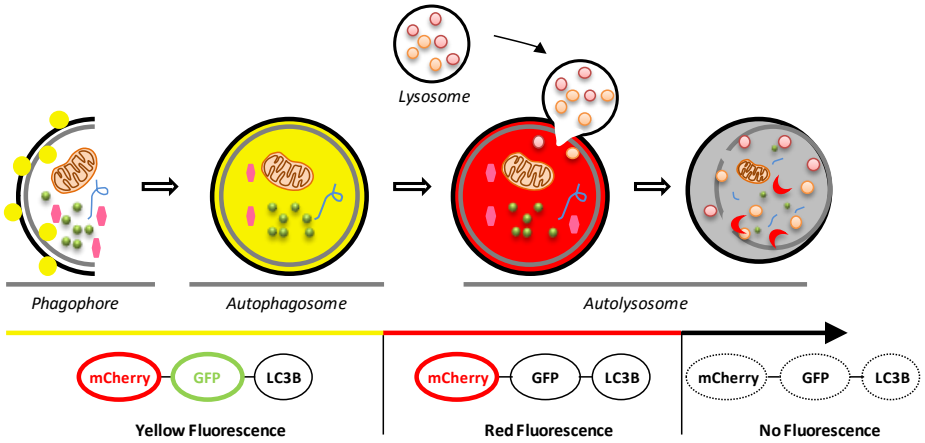

Figure 3.3 - Tracking different stages of autophagic flux with double-tagged LC3B (mCherry-GFP-LC3). A tandem fusion of the red, acid-insensitive mCherry and the acid-sensitive GFP is fused to LC3B, to create a pH-sensitive sensor used to monitor autophagy in live cells. In autophagosomes, both tags emit fluorescent light resulting in a yellow fluorescence. When autophagosomes fuse with lysosomes, the pH decreases, and the acidic autolysosomes are labeled with a red signal, after quenching of GFP fluorescence in the lysosome. Adapted from (Hansen and Johansen, 2011).

3.3.6 Statistics

Results are expressed as mean ± standard error of the mean (SEM). Data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's post test for multiple comparisons or Student's unpaired *t* test with two-tailed *p* value when comparing two groups only. A value of $p<0.05$ was considered significant. Prism 5.0 (GraphPad Software) was used for all statistical analysis.

3.4 Results

3.4.1 Neuropeptide Y induces autophagy in rodent hypothalamic neurons

There is evidence that NPY is strongly upregulated upon caloric restriction, but its role on autophagy regulation and potential interference with the aging process is not known. Therefore, we investigated whether NPY induces autophagy and which NPY receptor(s) modulate this process. For this purpose we monitored autophagy upon NPY treatment using the markers microtubule-associated protein 1 light chain 3 beta (LC3B), sequestosome 1 (SQSTM1) and beclin 1 (BECN1) in a) a mouse embryonic hypothalamic neuronal cell line (mHypoN42) and b) a primary culture of rat differentiated hypothalamic neural cells. Rapamycin was used as a positive control of autophagy stimulation through mechanistic target of rapamycin (MTOR) inhibition (Ravikumar et al., 2004).

Cells were exposed to 100 nM NPY, as described in other studies (Lemos et al., 1997; Rhim et al., 1997; Santos-Carvalho et al., 2013), for 10 and 30 min and 1, 3, 6 and 24 h. As shown by Western blot analysis in Figure 3.4A, NPY increased LC3B-II levels after 10 min of treatment (161.6 ± 21.4% of control) in mHypoN42 hypothalamic neurons. This increase was still observed after 30 min (160.2 ± 24.4% of control), but LC3B-II decreased to basal levels after longer time exposures, suggesting that LC3B-II is being degraded by the lysosome. NPY also induced the activation of autophagy in rat differentiated hypothalamic neural cells after 3 and 6 h of NPY treatment (170.5 ± 21.2% and 181.6 ± 26.7% of control, respectively, Figure 3.4B). Concomitant with the increase in LC3B-II levels, the protein content of SQSTM1 was significantly decreased in mHypoN42 hypothalamic neurons after NPY treatment for 30 min (78.1 ± 3.8% of control; Figure 3.4C) and in rat differentiated hypothalamic neural cells after 6 h of NPY exposure (67.5 ± 6.2% of control; Figure 3.4D). NPY did not change BECN1 levels in both mHypoN42 hypothalamic neurons and rat differentiated hypothalamic neural cells (Figure 3.4E and F). Overall, the increase in the abundance of LC3B-II and decrease in SQSTM1 protein levels suggest that NPY induces autophagy in hypothalamic neurons.

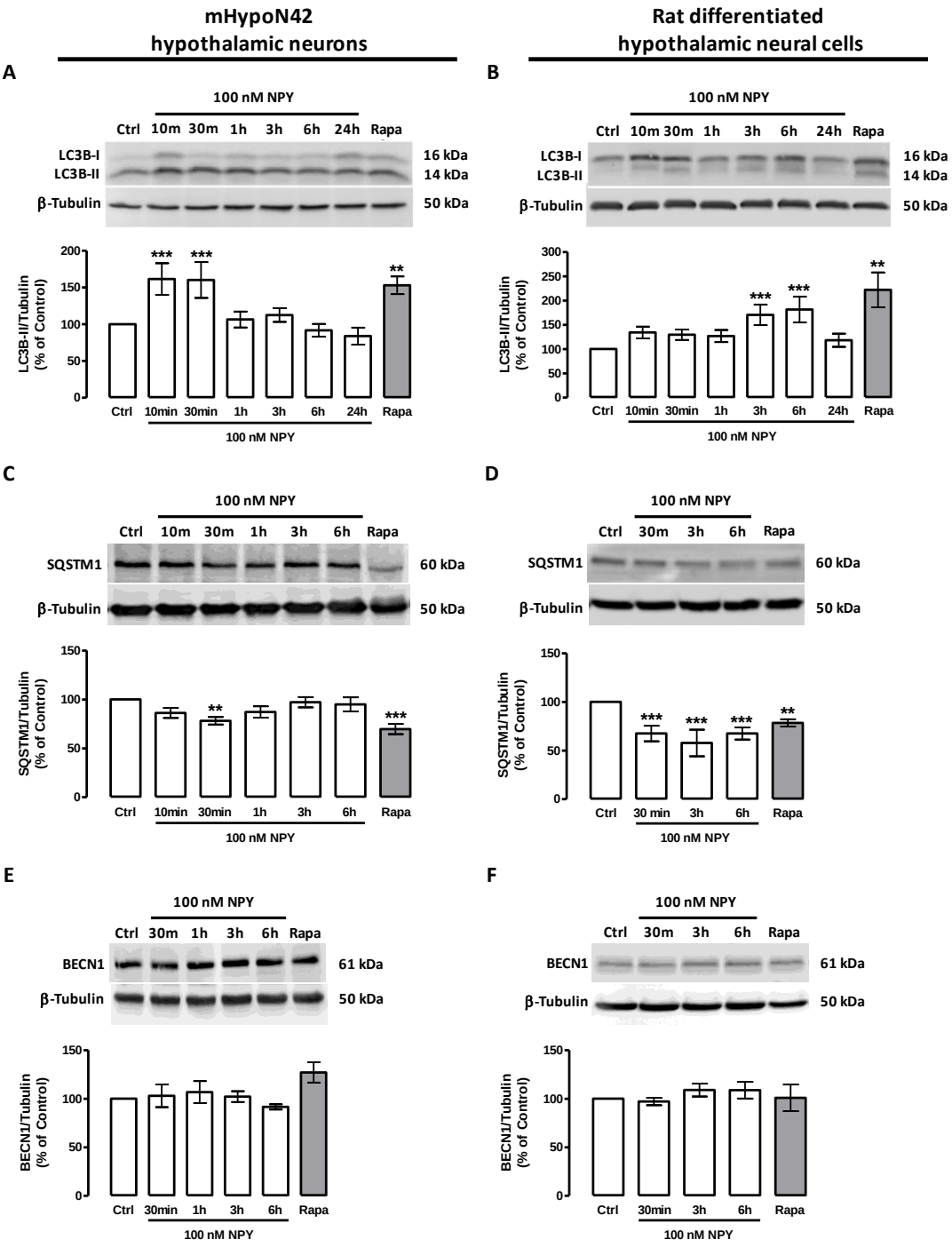

Figure 3.4 - NPY increases LC3B-II steady-state levels in a time-dependent manner in mHypoN42 hypothalamic neurons and in rat differentiated hypothalamic neural cells. mHypoN42 hypothalamic neurons (**A**, **C** and **E**) and rat differentiated hypothalamic neural cells (**B**, **D** and **F**) were exposed to 100 nM NPY in a time-dependent manner (10 min to 24 h). Cells were also treated with 10 nM rapamycin (Rapa) for 1 h as a positive control for autophagy induction. Whole cell extracts were assayed for LC3B (**A** and **B**), SQSTM1 (**C** and **D**), BECN1 (**E** and **F**) and β-tubulin (loading control) immunoreactivity by Western blotting as described in Methods. Representative western blots for each protein are presented above each respective graph. The results represent the mean ± SEM of, at least, five independent experiments and are expressed as the relative amount compared to control (Ctrl). ***p*<0.01, ****p*<0.001, significantly different from control, as determined by ANOVA followed by Dunnett's post test.

Autophagy is characterized by the redistribution of LC3B-II into cytoplasmatic puncta and the formation of autophagosomes. To determine whether the increased LC3B-II steady-state levels mirrored an increase of autophagosome number upon NPY treatment, we analyzed LC3B immunoreactivity by immunocytochemistry in mHypoN42 hypothalamic neurons and rat differentiated hypothalamic neural cells. NPY, similarly to rapamycin, caused the redistribution of LC3B from a diffuse localization, with few aggregates all over the cell, to a cytoplasmatic punctate pattern, localized preferentially in the perinuclear region, in mHypoN42 hypothalamic neurons (Figure 3.5, top panel). Cells exposed to NPY for long periods of time revealed a more diffuse LC3B distribution, with few puncta, suggesting that LC3B-associated structures were undergoing degradation. A similar LC3B redistribution was seen in rat differentiated hypothalamic neural cells (Figure 3.5, bottom panel).

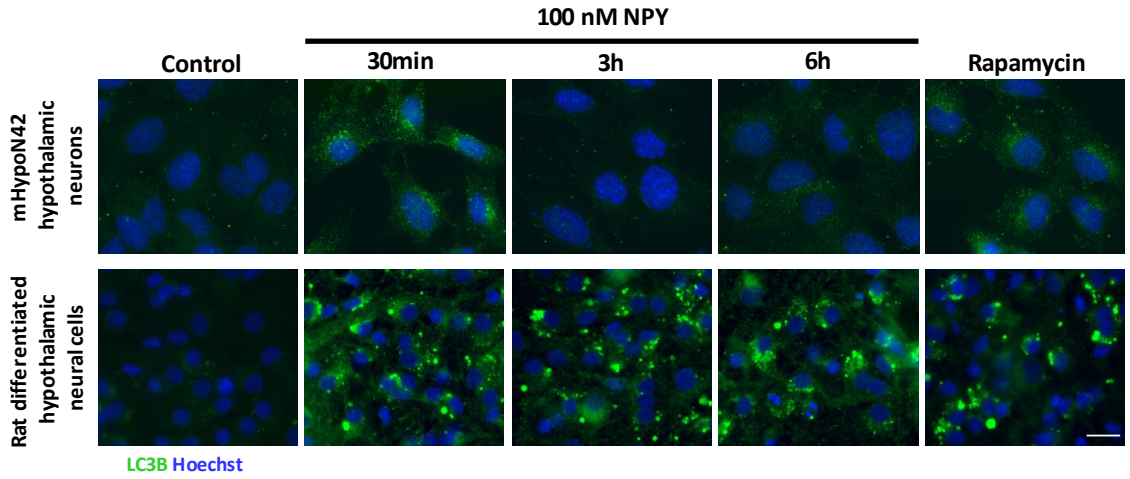

Figure 3.5 - NPY induces LC3B puncta formation in mHypoN42 hypothalamic neurons and rat differentiated hypothalamic neural cells. mHypoN42 hypothalamic neurons (top panel) and rat differentiated hypothalamic neural cells (bottom panel) were exposed to NPY (100 nM; 30 min, 3 h, 6 h) and to 10 nM rapamycin (1 h), a positive control for autophagy induction. Cells were immunolabeled for LC3B (green). Nuclei were stained with Hoechst (blue). Scale bar, 20 µm.

Although rat differentiated hypothalamic neural cell cultures are mainly constituted by mature neurons (Sousa-Ferreira et al., 2011a), other cell types exist in the culture. In order to identify which hypothalamic cells were undergoing autophagy upon NPY treatment, we performed double-labeling immunocytochemistry experiments. To identify different cell types present in the cell culture, several cell markers were used: NeuN (marker of mature neurons), GFAP (marker of astrocyte-like neural progenitor cells and mature macroglial cells), ED-1 (marker of microglia) and SOX2 (marker of neural stem/progenitor cells). As shown in Figure 3.6A, a diffuse cytoplasmatic distribution of LC3B, with few puncta, was

observed in NeuN-, GFAP-, ED-1- and also in SOX2-positive cells in control conditions. Although NPY increased LC3B puncta formation in all the cell types studied upon 6h of treatment, the effect was more pronounced in mature neurons (NeuN-positive cells); as expected. Approximately 60% of the total number of cells were positive for NeuN (57.0±1.2% of NeuN-positive nuclei/Hoechst total nuclei; Figure 3.6B), indicating that rat differentiated hypothalamic neural cell cultures are mainly constituted by differentiated mature neurons. Interestingly, SOX2-positive cells also presented a high number of LC3B puncta when exposed to NPY. In fact, half of the total number of cells was positive for the SOX2 marker (52.5±2.3% of SOX2 positive nuclei/Hoechst total nuclei; Figure 3.6B). The transcription factor SOX2 is crucial for the development of the central nervous system and is present in neural stem cells. SOX2 expression is high in undifferentiated cells and declines with cell differentiation; however, some mature neurons retain SOX2 expression upon differentiation (Cavallaro et al., 2008). Recent studies have shown that autophagy is related with the maintenance of self-renewing capacities of stem cells as well as neural stem cells differentiation (Vázquez et al., 2012; Vessoni et al., 2012) and NPY has also been implicated in neural precursors proliferation and differentiation (Agasse et al., 2008; Decressac et al., 2009). The increase of autophagy in SOX2-positive cells may suggest that NPY is involved in hypothalamic neuronal differentiation.

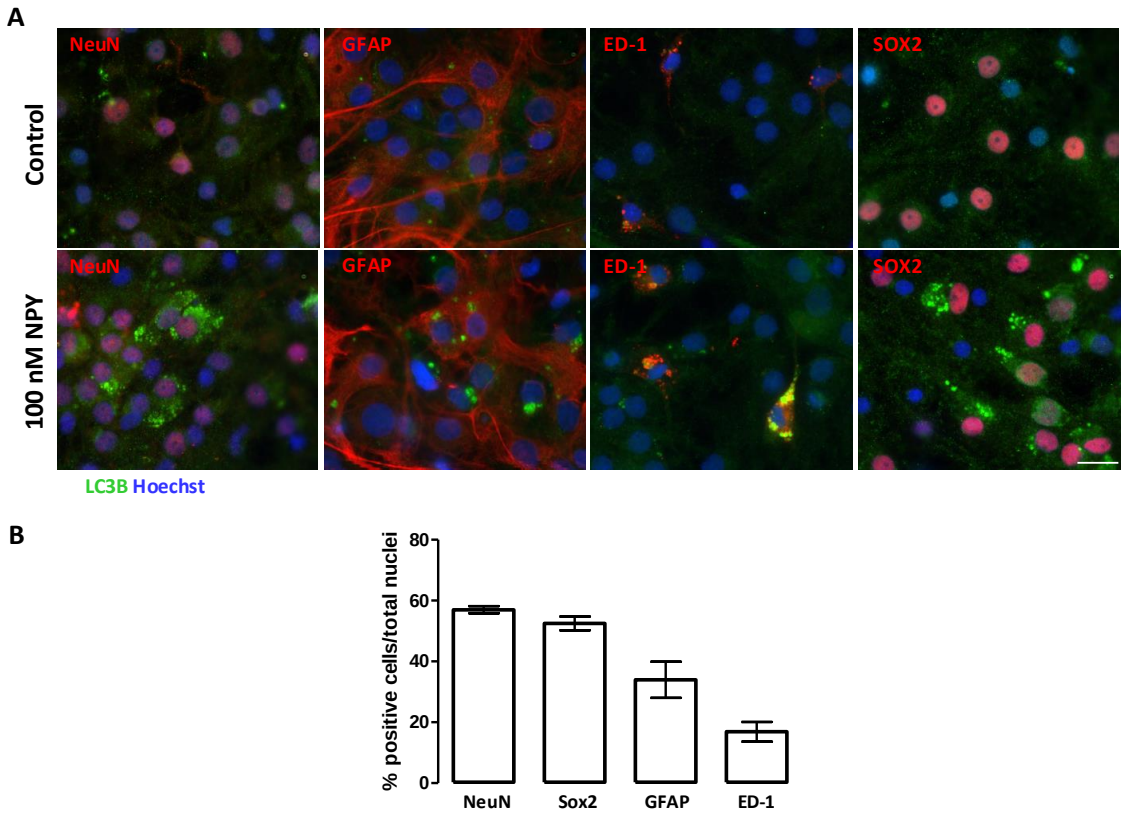

Figure 3.6 - NPY–induced LC3B puncta in different cell types of rat differentiated hypothalamic neural cells. (**A**) Rat differentiated hypothalamic neural cells were exposed to 100 nM NPY for 6 h. Cells were immunolabeled for LC3B (green); NeuN (red; mature neurons), GFAP (red; neural progenitor cells and mature glial cells), ED-1 (red; microglia) and SOX2 (red; neural stem cell/progenitor cell marker). Nuclei were stained with Hoechst (blue). Figures are representative of three independent experiments. Scale bar, 20 µm. (**B**) The rat differentiated hypothalamic neural cells were obtained from the differentiation of hypothalamic neurospheres. More than half of the total nuclei were positive for NeuN marker (57.0 ± 1.2%), indicating that the cells are differentiated to mature neurons. GFAP- and ED-1-positive cells were also detected indicating the presence of astrocyte-like neural progenitor cells and mature glial cells (GFAP-positive, 34.0 ± 6.0%), and microglia (ED-1-positive, 16.9 ± 3.2%), respectively. Additionally, some hypothalamic cells kept the expression of multi-potency marker Sox2 (Sox2-positive, 52.5 ± 2.3%). The results represent the mean ± SEM of, at least, three independent experiments.

An increase in LC3B-II steady state levels or LC3B puncta is not sufficient to guarantee an increase of the autophagic flux upon NPY treatment. LC3B-II can accumulate due to enhanced autophagosome formation or inhibition of autophagic degradation, due to delayed trafficking to the lysosomes, reduced fusion between compartments or impaired lysosomal proteolytic activity (Klionsky et al., 2012). To rule out the possibility that the increase of LC3B-II was due to an inhibited autophagosome degradation rather than autophagosome formation, we evaluated endogenous autophagic flux in the absence and presence of chloroquine, an inhibitor of autophagic degradation (Mizushima et al., 2010; Klionsky et al., 2012; Menzies et al., 2012). mHypoN42 hypothalamic neurons and rat differentiated hypothalamic neural cells were treated with chloroquine (100 µM; CQ) 30 min prior to the incubation with NPY (100 nM; 30 min or 6 h, respectively). As observed

before (Figure 3.4A and B), LC3B-II levels increased in NPY-treated cells (Figure 3.7A and D). However, in the presence of chloroquine, this increase was significantly higher than in cells treated with NPY alone. Functional autophagic flux assays showed an increased lysosomal accumulation of LC3B-II (Figure 3.7B and E) in NPY-treated ells. The decrease of SQSTM1 levels in NPY-treated cells was not observed in the presence of chloroquine (Figure 3.7C and F). In fact, as a consequence of autophagic degradation inhibition by chloroquine, SQSTM1 accumulated upon NPY treatment. These observations corroborate that NPY is enhancing autophagic flux in hypothalamic neurons.

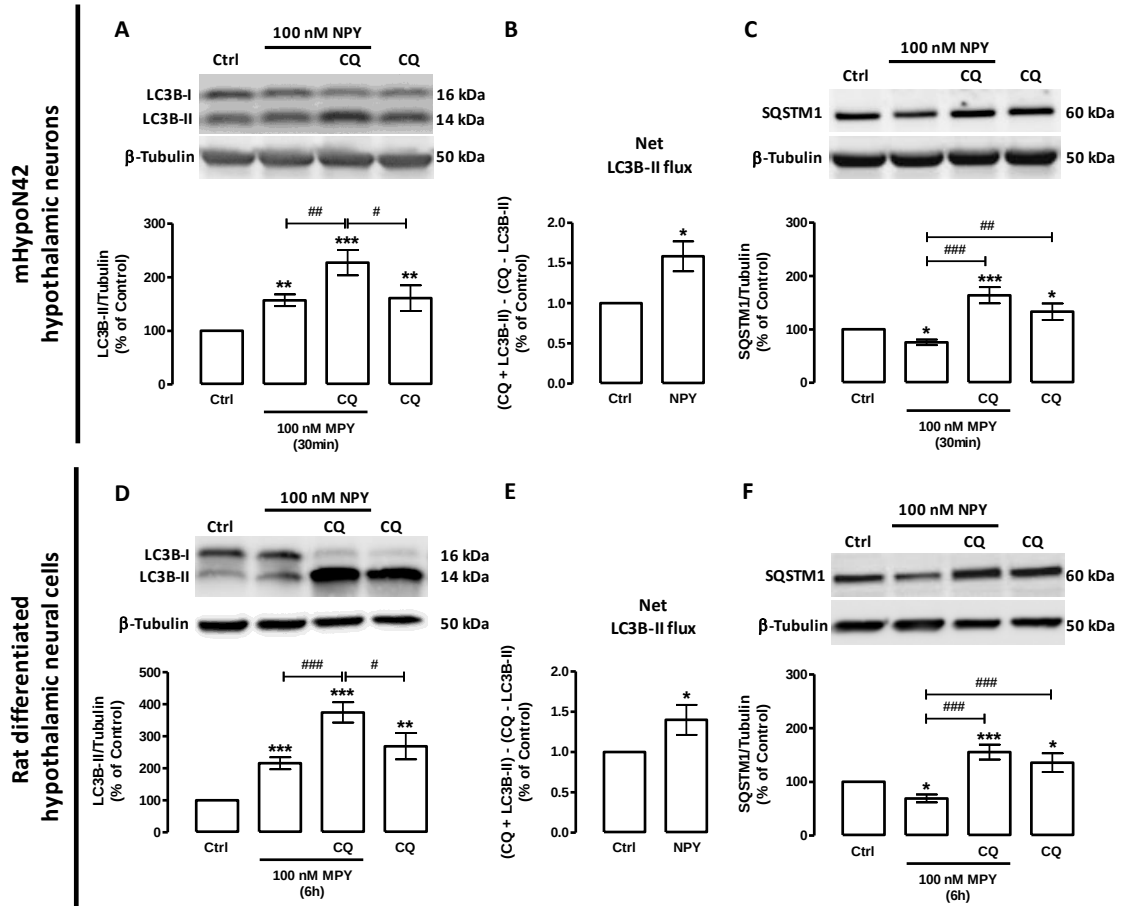

Figure 3.7 - NPY increases autophagic flux in mHypoN42 hypothalamic neurons and in rat differentiated hypothalamic neural cells. mHypoN42 hypothalamic neurons (**A-C**) and rat differentiated hypothalamic neural cells (**D-F**) were treated with 100 µM chloroquine (lysosomal inhibitor; CQ) 30 min before NPY treatment (100 nM) for 30 min or 6h, respectively. Whole cell extracts were assayed for LC3B (**A** and **D**), SQSTM1 (**C** and **F**) and β-tubulin (loading control) immunoreactivity by Western blotting, as described in Methods. Representative Western blots for each protein are presented above each respective graph. The results represent the mean ± SEM of, at least, four independent experiments and are expressed as the relative amount compared to control (Ctrl). LC3B flux analysis in mHypoN42 hypothalamic neurons (**B**) and rat differentiated hypothalamic neural cells (**E**) is shown. Autophagic flux was determined in the presence of the lysosomal inhibitor chloroquine, and expressed as "LC3B-II net flux" calculated by subtracting the densitometric value of LC3B-II amount in samples non-treated with chloroquine (LC3B-II − CQ) from the corresponding sample treated with chloroquine (LC3B-II + CQ). **p*<0.05, ***p*<0.01, ****p*<0.001, significantly different from control; #*p*<0.05, ##*p*<0.01, ###*p*<0.001, significantly different from NPY.

To further support that NPY enhances autophagic flux in hypothalamic neurons, we measured autophagic flux by using the tandem mCherry-GFP-LC3 cell-based assay (Kimura et al., 2007; Klionsky et al., 2012) in mHypoN42 hypothalamic neurons. This assay takes advantage of the differential pH sensitivities of red and green fluorescent proteins. The activation of autophagy is characterized by the redistribution of mCherry-GFP-LC3 reporter to autophagosomes (mCherry-GFP-LC3 puncta; yellow puncta) and autolysosomes (mCherry-LC3 puncta; red-only puncta), and loss of GFP fluorescence in the autolysosomes. As shown in Figure 3.8A and B, NPY increased the autophagic flux in mHypoN42 hypothalamic neurons. NPY significantly increased the number of autophagosomes (yellow puncta), autolysosomes (red-only puncta), and consequently, it also increased the total number of LC3 puncta (yellow+ red-only puncta). Overall, the observations gathered with both the western blot analysis of endogenous LC3B, showing an increase of the LC3B turnover and LC3B net flux, and the real-time image-based quantification of autophagic flux using the tandem mCherry-GFP-LC3 reporter, showing an increase in both LC3 puncta and autophagic flux, clearly demonstrate that NPY enhances autophagic flux in hypothalamic neurons.

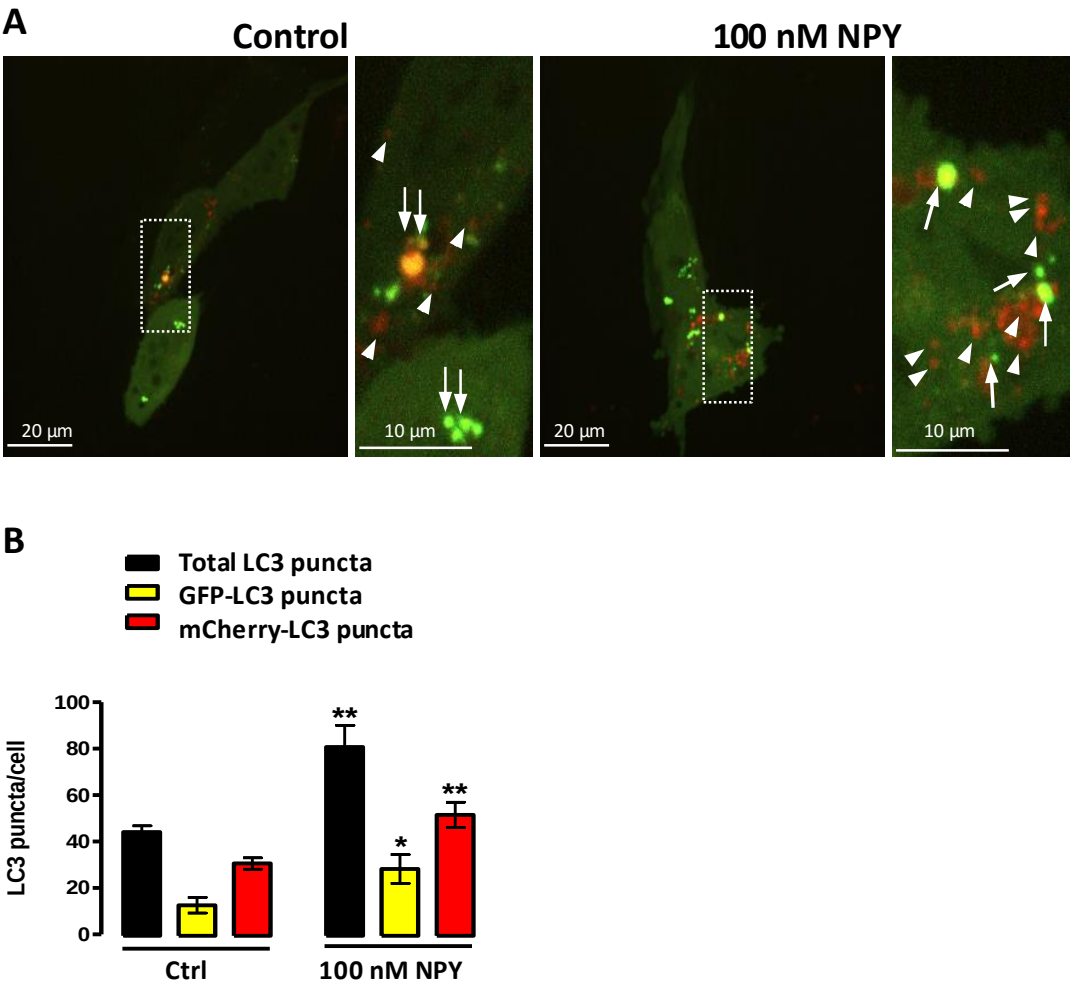

Figure 3.8 - NPY increases autophagic flux in hypothalamic neurons – mCherry-GFP-LC3 assay. mHypoN42 hypothalamic neurons were transfected with mCherry-GFP-LC3 plasmids for 24 h and then exposed to 100 nM NPY for 30 min. (**A**) Representative images of autophagosome (yellow puncta, arrows) and autolysosome (red puncta; arrowheads) formation in mHypoN42 hypothalamic neurons plasmids are shown. Right images are a higher magnification from the boxed areas in left. (**B**) Quantification of the number of mCherry-GFP-LC3 (yellow) and mCherry-only LC3 (red-only) puncta per cell in each condition (>30 cells per group). **p*<0.05, ***p*<0.01, significantly different from control (Ctrl) as determined by Student's unpaired *t* test.

3.4.2 Neuropeptide Y induces autophagy through NPY Y_1 and Y_5 receptors activation

To evaluate the specificity of the NPY effect and investigate which NPY receptor(s) subtype(s) modulate NPY-induced autophagic flux, cells were exposed to different NPY receptors selective antagonists, NPY Y_1 antagonist (BIBP3226; 1 µM), NPY Y_2 antagonist (BIIE0246; 1 µM) or NPY Y_5 antagonist (L-152,804; 1 µM), 30 min before NPY incubation, in the absence and presence of chloroquine (LC3B-II net flux). In mHypoN42 hypothalamic neurons, only NPY Y_1 and Y_5 receptor antagonists blocked the lysosomal accumulation of LC3B-II in NPY-treated cells (Figure 3.9A). Along with the increase of LC3B-II steady state

levels, NPY induced a decrease in SQSTM1 levels in mHypoN42 hypothalamic neurons (76.9 ± 4.9% of control), which was inhibited by NPY Y_1 and Y_5 receptors antagonists but not by the NPY Y_2 receptor antagonist (Y_1, 103.5 ± 7.4%; Y_2, 85.2 ± 4.8%; Y_5, 102.3 ± 8.3% of control; Figure 3.9B). This observation is in accordance with the fact that NPY increases the autophagic flux in mHypoN42 cells only through the activation of NPY Y_1 and Y_5 receptors.

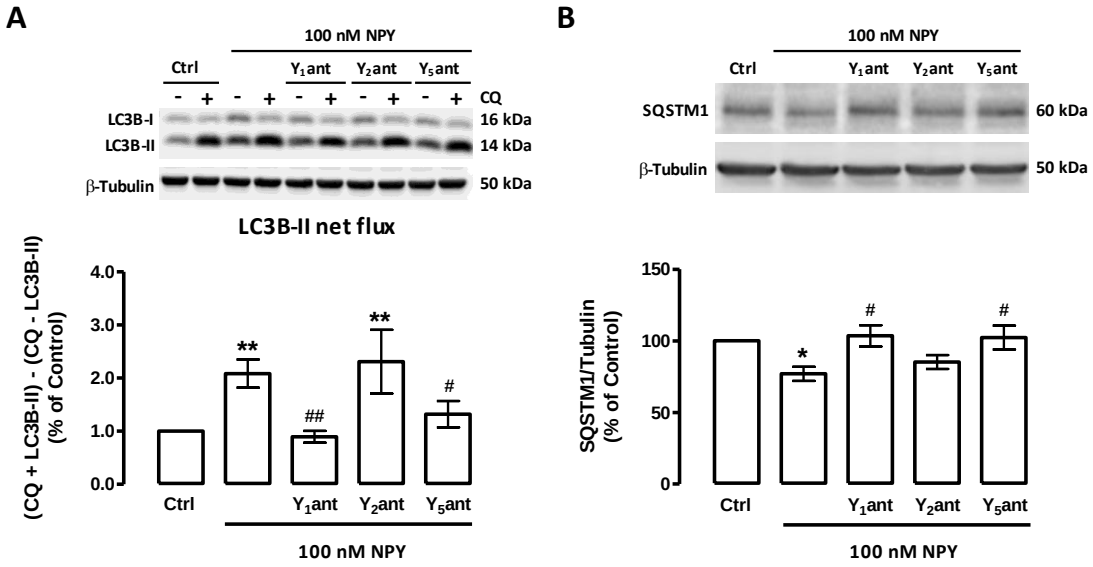

Figure 3.9 - NPY enhances autophagy through NPY Y_1 and Y_5 receptors activation in mHypoN42 hypothalamic neurons. mHypoN42 hypothalamic neurons were treated with NPY Y_1 receptor antagonist (Y_1ant; BIBP3226; 1 μM); Y_2 receptor antagonist (Y_2ant; BIIE0246; 1 μM), or Y_5 receptor antagonist (Y_5ant; L-152,804; 1 μM) 30 min before NPY treatment (100 nM, 30 min), in the absence or presence of chloroquine (100 μM; CQ). Whole cell extracts were assayed for LC3B, SQSTM1 (**B**) and β-tubulin (loading control) immunoreactivity by Western blotting, as described in Methods section. Representative Western blots for each protein are presented above each respective graph. LC3B-II net flux (**A**) was determined by subtracting the densitometric value of LC3B-II amount in samples non-treated with chloroquine (LC3B-II − CQ) from the corresponding sample treated with chloroquine (LC3B-II + CQ). The results represent the mean ± SEM of, at least, five independent experiments and are expressed as the relative amount compared to control (Ctrl). *p<0.05, **p<0.01, significantly different from control; $^{\#}p$<0.05, $^{\#\#}p$<0.01, significantly different from NPY, as determined by ANOVA followed by Bonferroni's post test.

In rat differentiated hypothalamic neural cells, NPY Y_1, Y_2 and Y_5 receptors antagonists inhibited NPY-induced autophagic flux (Figure 3.10A). In addition, NPY Y_1, Y_2 and Y_5 receptor antagonists (Y_1, 101.9 ± 4.6%; Y_2, 101.3 ± 4.2%; Y_5, 109.0 ± 5.9% of control; Figure 3.10B) inhibited the decrease of SQSTM1 levels upon NPY treatment. These results suggest that NPY increases autophagic flux in hypothalamic neurons through NPY Y_1 and Y_5 receptors activation, and NPY Y_2 receptor may be relevant for autophagy regulation in other hypothalamic neural cells.

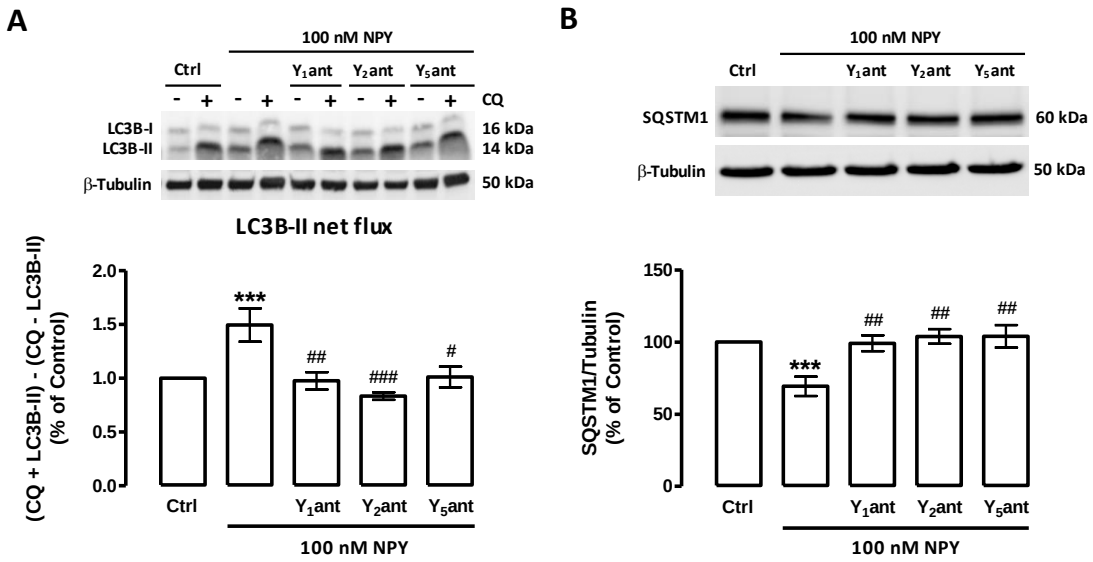

Figure 3.10 - NPY enhances autophagy through NPY Y_1, Y_2 or Y_5 receptors activation in rat differentiated hypothalamic neural cells. Rat differentiated hypothalamic neural cells were exposed to NPY (100 nM) for 6h, in the presence of NPY Y_1 receptor antagonist (Y_1ant; BIBP3226; 1 μM); Y_2 receptor antagonist (Y_2ant; BIIE0246; 1 μM), or Y_5 receptor antagonist (Y_5ant; L-152 804; 1 μM), in the absence or presence of chloroquine (100 μM; CQ). Whole cell extracts were assayed for LC3B, SQSTM1 (**B**) and β-tubulin (loading control) immunoreactivity by Western blotting, as described in Methods section. Representative Western blots for each protein are presented above each respective graph. LC3B-II net flux (**A**) was determined by subtracting the densitometric value of LC3B-II amount in samples non-treated with chloroquine (LC3B-II − CQ) from the corresponding sample treated with chloroquine (LC3B-II + CQ). The results represent the mean ± SEM of, at least, five independent experiments and are expressed as the relative amount compared to control (Ctrl). ***p<0.01, significantly different from control; $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001, significantly different from NPY, as determined by ANOVA followed by Bonferroni's post test.

3.4.3 NPY induces autophagy in hypothalamic neurons through PI3K, ERK, and PKA activation.

One of the molecular switches for autophagy induction is the inhibition of mechanistic target of rapamycin complex 1 (mTORC1) (He and Klionsky, 2009; Jung et al., 2010). mTORC1 activity may be assessed by analysis of phosphorylated MTOR (Ser2448), which is the active kinase form, and by the analysis of phosphorylated ribosomal protein S6 kinase (RPS6K) (Thr389), which is a substrate of active MTOR (Dunlop et al., 2009). In order to evaluate if mTORC1 was being inhibited upon NPY treatment, mHypoN42 hypothalamic neurons were treated with NPY and whole cell extracts were assayed for phospho-MTOR and phospho-RPS6K, by Western blotting. Rapamycin, known to inhibit MTOR activity, was used as positive control (Ravikumar et al., 2004). Though rapamycin treatment led to a significant decrease in both phospho-MTOR (63.3 ± 13.8% of control; Figure 3.11A and C) and phospho-RPS6K (34.9 ± 4.9% of control; Figure 3.11A and E), NPY did not alter the phosphorylation status of both MTOR and RPS6K proteins (Figure 3.11A, C and E). Similar

results were obtained in rat differentiated hypothalamic neurons. As expected, rapamycin inhibited MTOR, leading to a decrease in phospho-MTOR (54.2 ± 6.3% of control; Figure 3.11B and D) and phospho-RPS6K (22.6 ± 5.4% of control; Figure 3.11B and F) levels. Conversely, NPY had no effect on phospho-MTOR and phospho-RPS6K (Figure 3.11B, D and F). These results suggest that NPY does not induce autophagy through the inhibition of mTORC1.

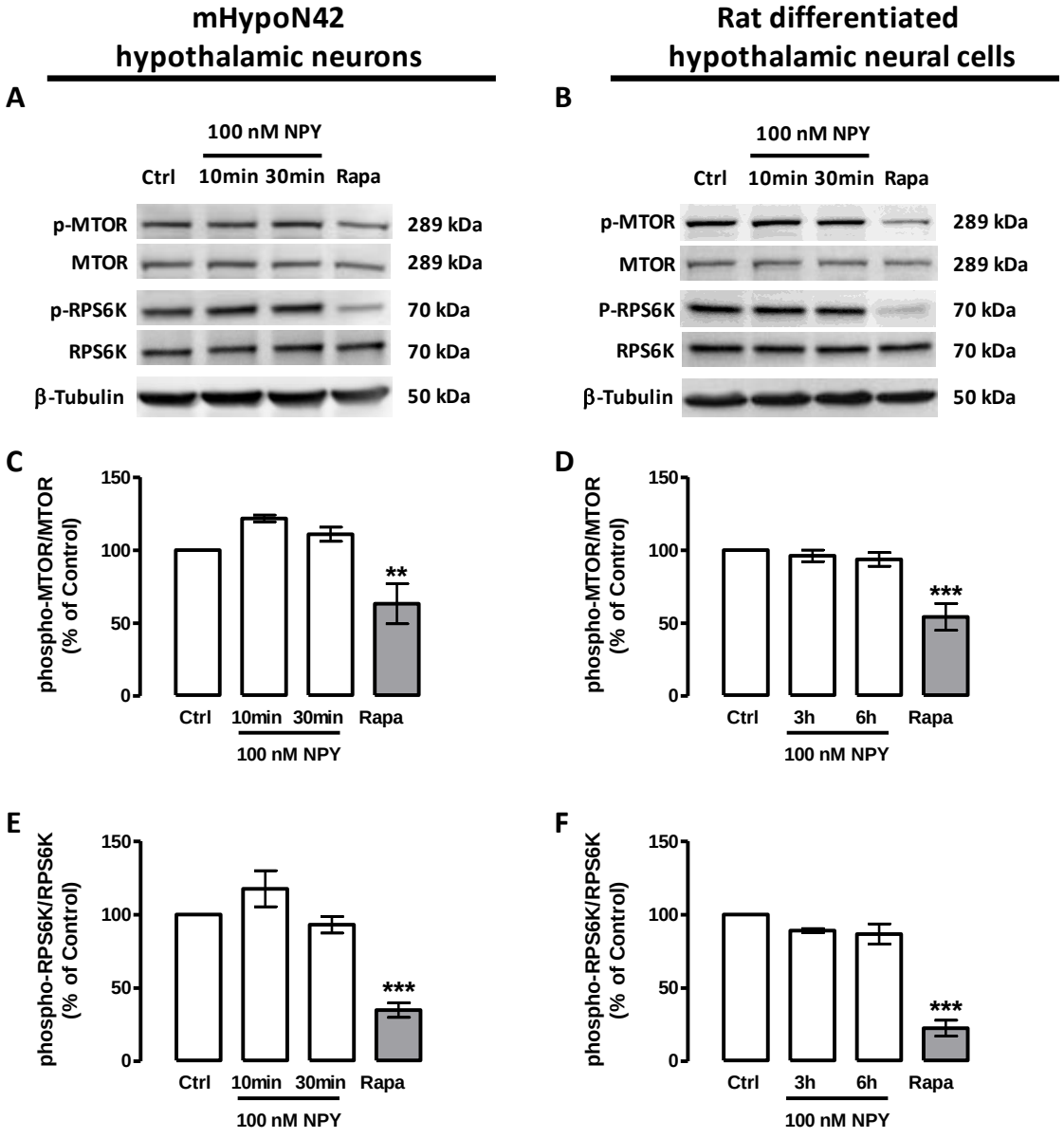

Figure 3.11 - NPY enhances autophagy through a MTOR-independent pathway. mHypoN42 hypothalamic neurons (**A, C** and **E**) and rat differentiated hypothalamic neural cells (**B, D** and **F**) were treated with 100 nM NPY for 30 min or 6 h, respectively. Cells were also treated with 10 nM rapamycin (Rapa) for 1 h as a positive control for autophagy induction. Whole cell extracts were assayed for phospho-MTOR (p-MTOR), MTOR, phospho-RPS6K (p-RPS6K), RPS6K and β-tubulin (loading control) immunoreactivity by Western blotting as described in Methods section. Representative Western blots for each protein are presented (**A** and **B**). The results represent the mean ± SEM of, at least, five independent experiments and are expressed as the relative amount compared to control (Ctrl). ***p*<0.01 and ****p*<0.001, significantly different from control, as determined by ANOVA followed by Dunnett's post test.

However, several other pathways are known to regulate autophagy in mammalian cells (Ravikumar et al., 2010). To further investigate other possible pathways involved in NPY-induced autophagic flux, mHypoN42 hypothalamic neurons were treated with NPY in the presence of PI3K inhibitors (3-methyladenine, 3-MA; 5 mM or LY294002; 1 μM), MEK/ERK inhibitor (U0126; 1 μM) or PKA inhibitor (H89; 1 μM), in the presence or absence of chloroquine. The increase of LC3B-II lysosomal accumulation (LC3B-II net flux; Fig. 3.12A), and the decrease of SQSTM1 protein content (Figure 3.12B) induced by NPY were inhibited by all of the protein kinase inhibitors tested. These results suggest that NPY induces autophagy in mHypoN42 hypothalamic neurons through PI3K, MEK/ERK, and PKA signaling pathways.

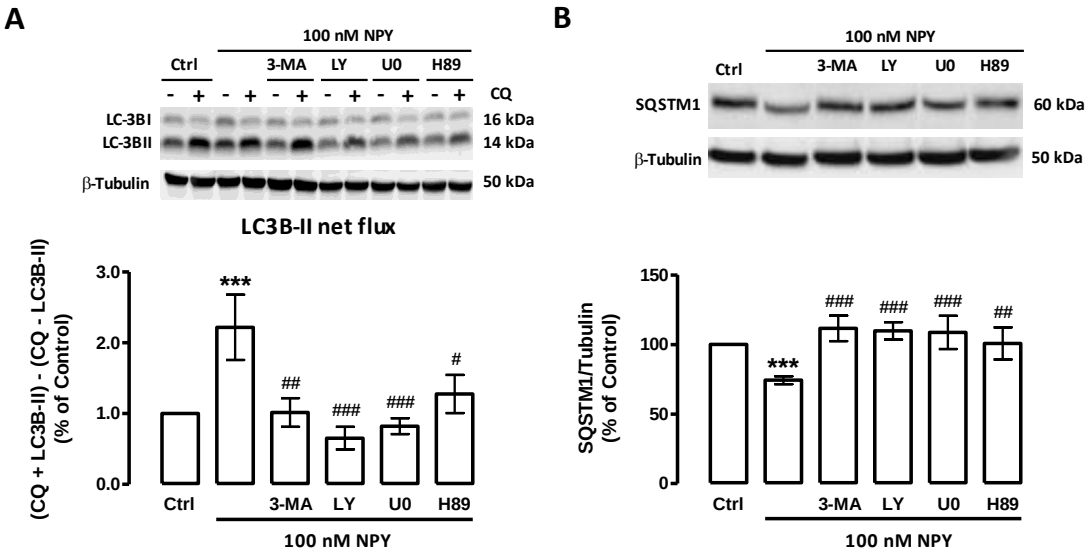

Figure 3.12 - The stimulatory effect of NPY on autophagy in mHypoN42 hypothalamic neurons is mediated by PI3K, MEK/ERK, and PKA signaling pathways. mHypoN42 hypothalamic neurons were treated with NPY in the presence of PI3K inhibitors (3-methyladenine, 3-MA; 5 mM or LY294002; 1 μM); MEK/ERK inhibitor (U0126; 1 μM), or PKA inhibitor (H89; 1 μM), in the absence or presence of chloroquine (CQ; 100 μM). Whole cell extracts were assayed for LC3B, SQSTM1 (**B**) and β-tubulin (loading control) immunoreactivity by Western blotting, as described in Methods section. Representative Western blots for each protein are presented above each respective graph. LC3B-II net flux (**A**) was determined by subtracting the densitometric value of LC3B-II amount in samples non-treated with chloroquine (LC3B-II − CQ) from the corresponding sample treated with chloroquine (LC3B-II + CQ). The results represent the mean ± SEM of, at least, five independent experiments and are expressed as the relative amount compared to control (Ctrl). ***p<0.001, significantly different from control; $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001, significantly different from NPY, by ANOVA followed by Bonferroni's post test.

Since NPY increases autophagic flux in mHypoN42 hypothalamic neurons through the activation of NPY Y_1 and Y_5 receptors (Figure 3.9A and B), to further elucidate the signaling pathways coupled to one or both of these NPY receptors, we evaluated the effect of NPY Y_1 and Y_5 receptor stimulation, by using the respective receptor agonist, on AKT, a

downstream effector of class I PI3K, MEK/ERK and PKA signal transduction cascades by Western blot using phospho-specific antibodies. mHypoN42 hypothalamic neurons were treated with PI3K inhibitor (LY294002; 1 μM), MEK/ERK inhibitor (U0126; 1 μM) or PKA inhibitor (H89; 1 μM) 30 min before the incubation with NPY Y_1 receptor agonist ([Leu^{31}Pro^{34}]NPY, 100 nM) or NPY Y_5 receptor agonist [NPY$_{19–23}$(Gly^{1}, Ser^{3,22}, Gln^{4,34}.Thr^{6}, Ala^{31}, Aib^{32})PP, 100 nM]. As shown in Figure 3.13A, NPY Y_1 receptor agonist increased AKT phosphorylation in mHypoN42 hypothalamic neurons and this effect was blocked by NPY Y_1 receptor antagonist, as expected, and also by the PI3K inhibitor. NPY Y_1 receptor agonist increased the phosphorylation of PKA and this effect was abolished by the NPY Y_1 receptor antagonist, the PI3K inhibitor and the PKA inhibitor (Figure 3.13C). NPY Y_1 receptor agonist had no effect on ERK phosphorylation (Figure 3.13B). The NPY Y_5 receptor agonist significantly increased both ERK (Figure 3.13E) and PKA (Figure 3.13F) phosphorylation in mHypoN42 hypothalamic neurons, and NPY Y_5 receptor antagonist blocked these effects. The NPY Y_5 receptor agonist-mediated ERK and PKA activation was blocked by the MEK/ERK inhibitor and the PKA inhibitor, respectively. NPY Y_5 receptor agonist did not increase AKT phosphorylation. These results suggest that whereas the NPY Y_1 receptor agonist activates both AKT and PKA signaling pathways in a PI3K-dependent manner, the NPY Y_5 receptor agonist activates both MEK/ERK and PKA signaling pathways in mHypoN42 hypothalamic neurons.

Because PI3K, MEK/ERK and PKA signaling pathways are involved in NPY-induced autophagy, as shown in Figure 3.12, we next evaluated the involvement of each signaling pathway on NPY Y_1 and Y_5 receptor agonists-induced autophagic flux. For that, mHypoN42 hypothalamic neurons were pretreated with PI3K inhibitor, MEK/ERK inhibitor or PKA inhibitor 30 min before the incubation with NPY Y_1 or NPY Y_5 receptor agonists in the absence or presence of chloroquine. As expected, NPY Y_1 (158.2 ± 17.5% of control; Figure 3.13G) and Y_5 (137.5 ± 1.8% of control; Figure 3.13H) receptor agonists increased LC3B-II flux in mHypoN42 hypothalamic neurons and this effect was abolished in the presence of the respective NPY receptor antagonist (Figure 3.13G and H, respectively). In addition, NPY Y_1 receptor induced LC3B-II flux was inhibited in the presence of PI3K and PKA inhibitors (Figure 3.13G). However, PI3K, MEK/ERK, or PKA inhibitors inhibited the stimulatory effect of NPY Y_5 receptor activation on autophagic flux. Therefore, whereas NPY Y_1 receptor activation increased autophagic flux through PI3K and PKA signaling pathways, NPY Y_5 receptor activation increased autophagic flux through PI3K, MEK/ERK, and PKA. Overall, by using pharmacological tools, we show that, in mHypoN42 hypothalamic neurons, NPY

enhances autophagic flux through the activation of NPY Y_1 and Y_5 receptors, and this effect is mediated by the concerted activation of PI3K, MEK/ERK, and PKA signaling pathways.

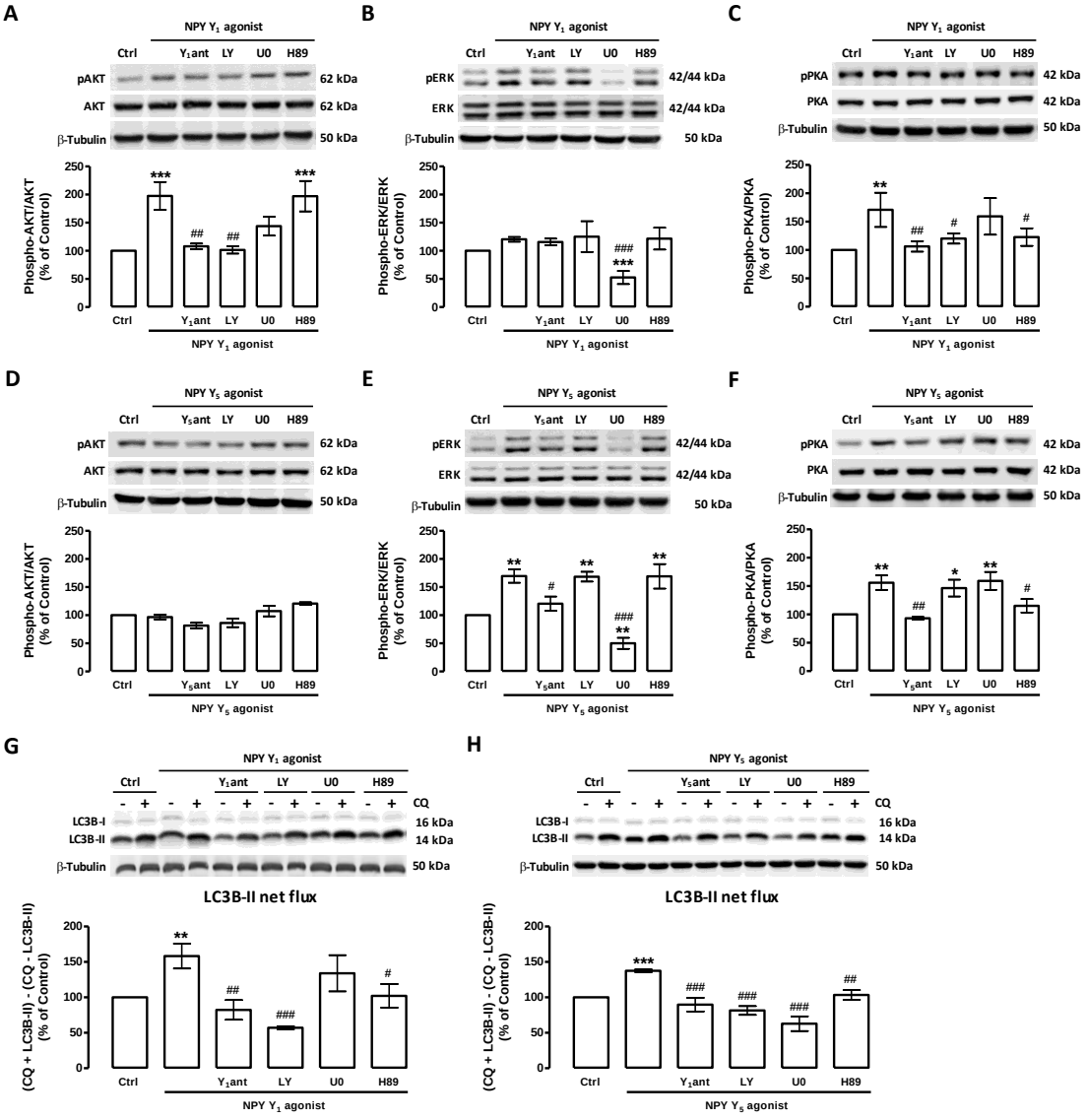

Figure 3.13 - NPY Y_1 and Y_5 receptors activation increases autophagic flux in hypothalamic neurons through PI3K, MEK/ERK and PKA signaling pathways. mHypoN42 hypothalamic neurons were treated with PI3K inhibitor (LY294002; 1 µM); MEK/ERK inhibitor (U0126; 1 µM) or PKA inhibitor (H89; 1 µM) 30 min before NPY Y_1 receptor agonist ([Leu^{31}Pro^{34}]NPY, 100 nM; **A-C** and **G**) or NPY Y_5 receptor agonist [NPY$_{19–23}$(Gly^1, Ser^{3,22}, Gln^{4,34}.Thr^6, Ala^{31}, Aib^{32})PP; 100 nM; **D-F** and **H**], in the absence or presence NPY Y_1 receptor antagonist (Y_1ant; BIBP3226; 1 µM; **A-C** and **G**), NPY Y_5 receptor antagonist (Y_5ant; L-152 804; 1 µM; **D-F** and **H**) or chloroquine (CQ; 100 µM; **G** and **H**). Whole cell extracts were assayed for anti-phospho-AKT (pAKT), anti-AKT (**A** and **D**), anti-phospho-p44/42 (pERK), anti-p44/42 (ERK) (**B** and **E**), anti-phospho-PKA (pPKA), anti-PKA (**C** and **F**), anti-LC3B and β-tubulin (loading control) immunoreactivity by Western blotting as described in Methods section. Representative Western blots for each protein are presented. LC3B-II net flux (**G** and **H**) was determined by subtracting the densitometric value of LC3B-II amount in samples non-treated with chloroquine (LC3B-II − CQ) from the corresponding sample treated with chloroquine (LC3B-II + CQ). The results represent the mean ± SEM of, at least, five independent experiments and are expressed as the relative amount compared to control (Ctrl). *p<0.05, **p<0.01, ***p<0.001, significantly different from control (Ctrl); $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001, significantly different from NPY Y_1 or Y_5 receptor agonist, as determined by ANOVA followed by Bonferroni's post test.

3.4.4 NPY induces autophagy in mouse hypothalamus: an *in vivo* study

In order to evaluate the effect of NPY on hypothalamic autophagy modulation *in vivo*, NPY was overexpressed in hypothalamic arcuate nucleus (ARC), by gene transfer using adenoassociated viral vectors (AAV), in male C57BL/6 mice. Mice were injected with AAV encoding either GFP (AAV-GFP, control group) or NPY (AAV-NPY), under a neuronal-specific promoter (Sousa-Ferreira et al., 2011b), by bilateral stereotaxic injection in each ARC. After 4 weeks, brains were isolated and processed for immunohistochemistry to verify the expression of NPY in the hypothalamus. In control AAV-GFP mice brains, higher NPY immunoreactivity is mostly restricted to the ARC (Figure 3.14A). On the other hand, NPY-overexpressing mice (AAV-NPY mice) brains displayed a stronger, widespread expression of NPY through the several hypothalamic areas, but still more pronounced in the ARC (Figure 3.14A). NPY immunoreactivity was quantified through the anterior-posterior length of the ARC. As shown in Figure 3.14B, an increase of ~70% of NPY immunoreactivity was achieved in NPY-overexpressing mice 4 weeks upon infection compared to control mice (AAV-GFP; 172.5 ± 21.6% of control). We and others previously reported that hypothalamic NPY overexpression led to hyperphagia, increased body weight gain and several serum alterations, consistent with obesity phenotypes, such as high glucose and high cholesterol (Sousa-Ferreira et al., 2011b). In order to avoid this type of alterations that could potentially influence autophagy regulation (Ost et al., 2010; Las et al., 2011), NPY overexpressing mice were submitted to pair feeding, being given only the same daily amount of food that AAV-GFP mice consumed. The body weight of control (AAV-GFP) and NPY-overexpressing mice (AAV-NPY) before AAV injection was similar (22.7±0.2 g and 22.7±0.4 g, respectively). Four weeks upon AAV infection, NPY-overexpressing mice (AAV-NPY) had a body weight gain similar to control mice (27.8 ± 0.4 g and 26.4 ± 0.6 g, respectively). In addition, the levels of glucose, cholesterol, and triglycerides levels of AAV-NPY mice (223.3 ± 28.3 mg/dL; 82.2 ± 2.6 mg/dL and 100.0 ± 14.2 mg/dL, respectively) were not statistically different from the control group (214.8 ± 11.2 mg/dL; 68.4 ± 3.3 mg/dL and 89.8 ± 7.6 mg/dL, respectively). Although cholesterol levels were 1.2-fold higher in AAV-NPY mice, this increase was very far from the 6.2-fold increase previously observed in AAV-NPY rats (Sousa-Ferreira et al., 2011b). Although the pair feeding could not completely prevent the referred effects of NPY overexpression, it successfully prevented the obese phenotype.

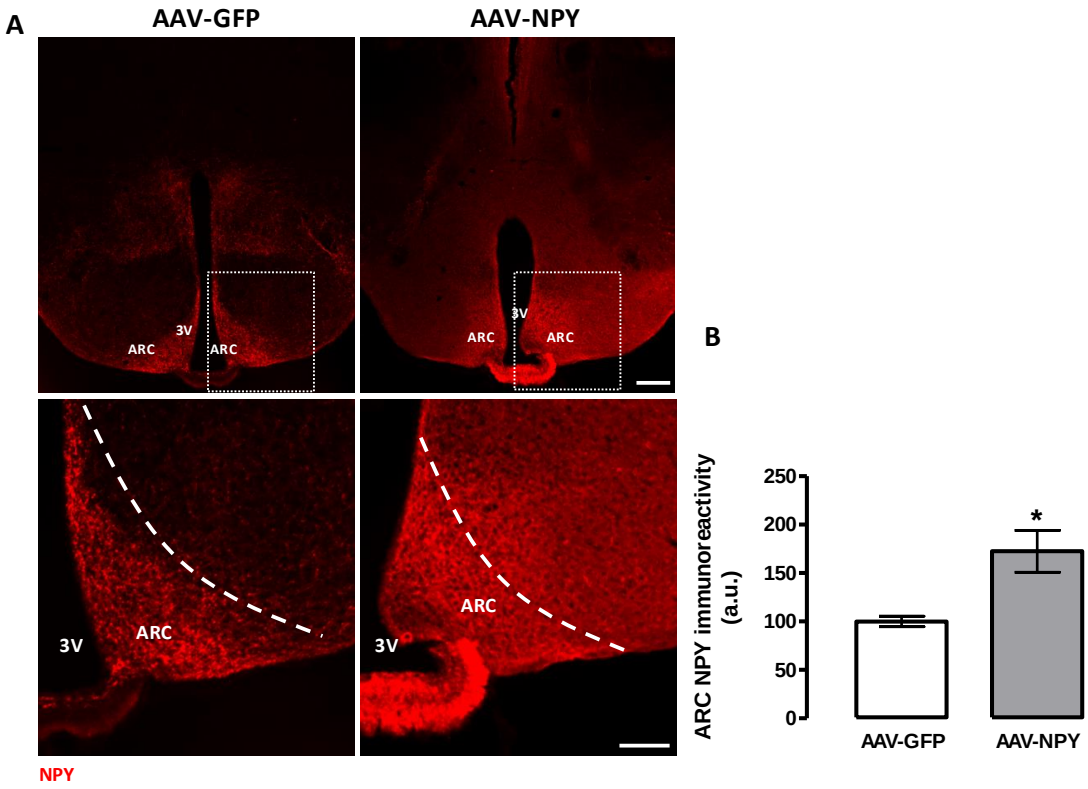

Figure 3.14 - NPY expression in mouse arcuate nucleus was modulated via bilateral injection with AAV. (**A**) Representative images of NPY immunoreactivity in the mouse arcuate nucleus (ARC) one month after AAV injection, in the control group (AAV-GFP; left panel) and ARC NPY overexpressing group (AAV-NPY; right panel). Bottom panel: high magnification of representative images of ARC NPY immunoreactivity modulated by AAV injection. Scale bar, 100 µm. (**B**) Quantification of NPY immunoreactivity through the anterior-posterior length of the mouse ARC, one month after AAV injection. The results represent the mean ± SEM and are expressed as the relative amount compared to control group (AAV-GFP). *p<0.05, significantly different from control group as determined by Student's unpaired t test. n=4 mice per group.

AAV particles contain recombinant plasmids with NPY cDNA under the human synapsin promoter, which guarantees that AAV particles infect mature neurons and only these cells can express the transgene. To confirm that the infection only occurs in mature neurons, AAV particles with recombinant plasmid with GFP cDNA under the human synapsin promoter were injected in our mice control group. Therefore, POMC neurons within the hypothalamic ARC expressed the GFP (Figure 3.15B), as well as, NPY/AgRP neurons and other neurons. In spite of NPY and AgRP stainning is punctated and does not label cell bodies (Figure 3.15C and D); several GFP-positive neurons were encircled by AgRP or NPY staining (Figure 3.15C and D, respectively), and they were not positive for POMC labeling (Figure 3.15B, arrowhead); this indicates that AAV particles can also infect NPY/AgRP neurons.

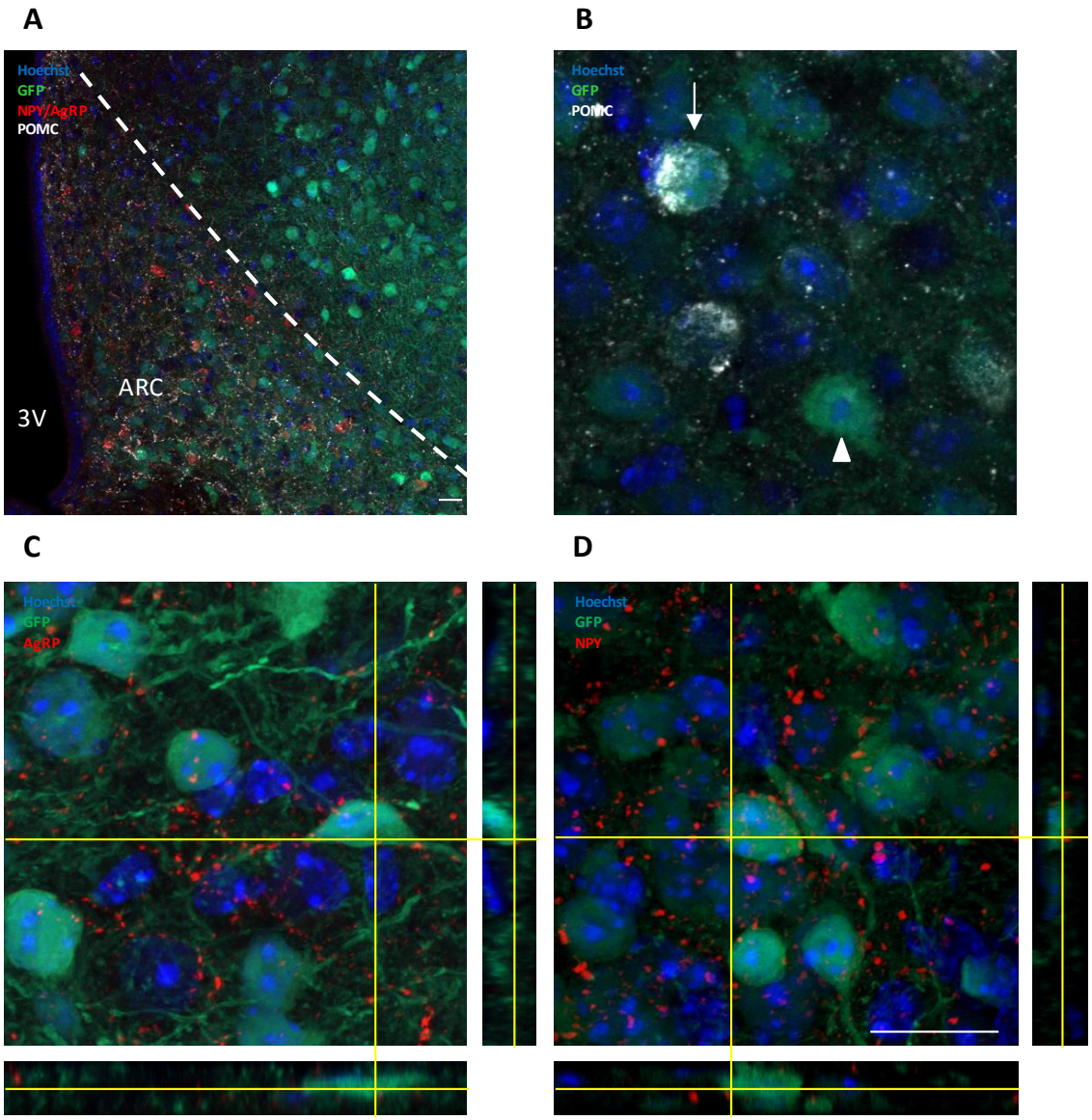

Figure 3.15 - Mature neurons in the hypothalamic arcuate nucleus infected with AAV particles containing recombinant plasmids with GFP cDNA under the human synapsin promoter. (**A**) Representative images of the immunohistochemical colocalization of GFP with POMC and NPY or AgRP, in the arcuate nucleus (ARC) of the hypotalamus. (**B**) High magnification of representative image of POMC and GFP immunoreactivity in hypothalamic ARC; arrow indicates co-localization of GFP and POMC immunoreactivity, and arrowhead shows GFP-positive neurons that are not positive for POMC labeling**.** (**C**) Representative images of GFP and AgRP immunohistochemistry localization (**C**), and GFP and NPY (**D**), with respective orthogonal reconstruction (using 0.6 µm thick optical slices). Nuclei were stained with Hoechst (blue). Scale bar, 20 µm.

We then evaluated the effect of hypothalamic NPY overexpression on autophagy in the mouse hypothalamus. For that, whole hypothalamic lysates from AAV-GFP and AAV-NPY mice were assayed for the protein content of the autophagy markers LC3B-II, SQSTM1 and BECN1. Mice overexpressing ARC NPY showed a decrease of LC3B-II (77.4 ± 6.9% of control; Figure 3.16A) and SQSTM1 protein content (70.3 ± 4.9% of control, Figure 3.16B) in the

hypothalamus, compared to control mice. Similar to mHypoN42 and rat differentiated hypothalamic neurons, BECN1 protein levels were not altered in the hypothalamus of AAV-NPY mice (Figure 3.16C). The decrease of the LC3B-II and SQSTM1 protein levels may indicate faster protein degradation in these mice, consistent with an increase of the autophagic flux in the hypothalamus. LC3B puncta in the ARC were also evaluated by immunohistochemical staining. As shown in Figure 3.16D, in the control group, LC3B puncta are small and diffusely distributed in the cytoplasm, while in the ARC of NPY-overexpressing mice (AAV-NPY) LC3B puncta are larger due to the increase of LC3B recruitment to autophagosomes, resulting in a less diffuse cytoplasmatic staining.

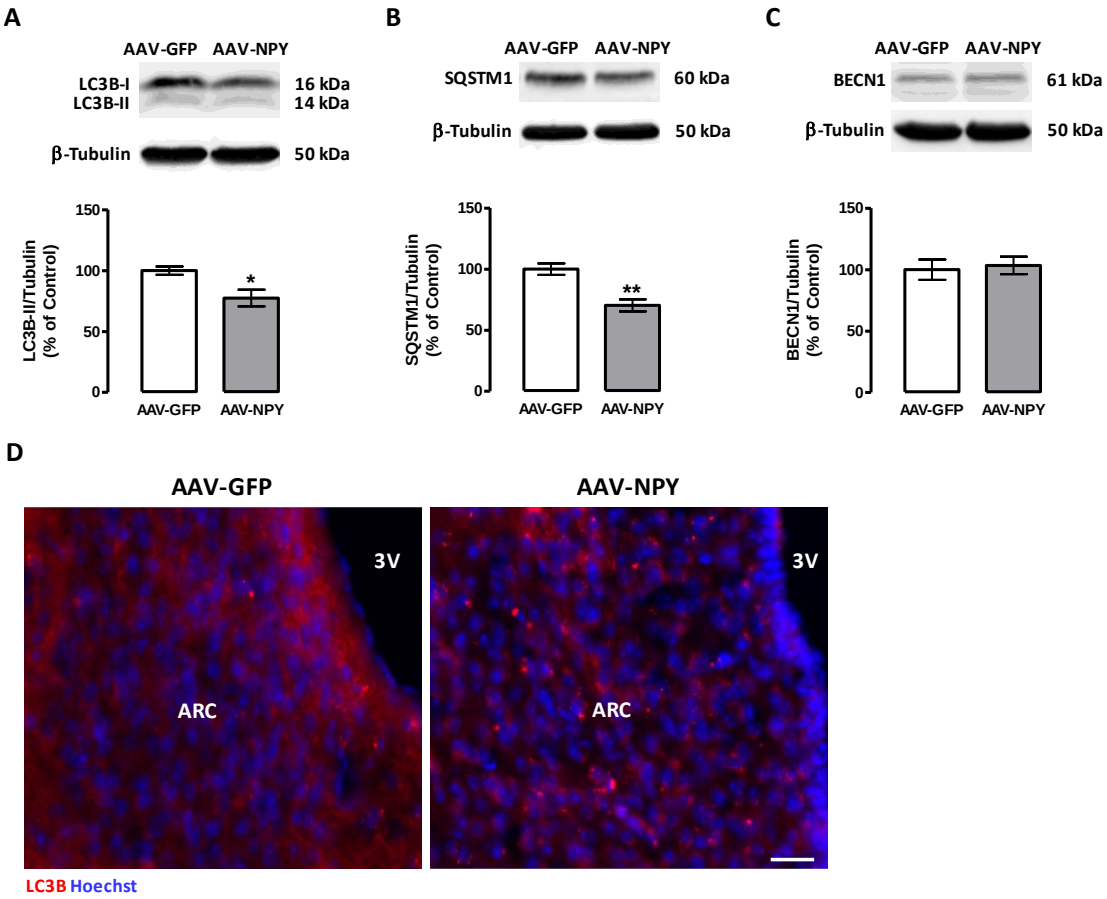

Figure 3.16 - Overexpression of ARC NPY increases autophagy in mouse hypothalamus. NPY expression in mouse ARC was modulated via bilateral injection with AAV. Whole hypothalamic lysates from control group mice (AAV-GFP) and ARC NPY overexpressing mice group (AAV-NPY) were assayed for LC3B (**A**), SQSTM1 (**B**), BECN1 (**C**) and β-tubulin (loading control) immunoreactivity by Western blotting as described in Methods. Representative Western blots for each protein are presented above each respective graph. The results represent the mean ± SEM and are expressed as the relative amount compared to control (AAV-GFP). n=6/7 mice per group. **p*<0.05, ***p*<0.01, significantly different from control group (AAV-GFP) as determined by Student's unpaired *t* test. (**D**) Representative images of LC3B immunoreactivity in the mouse ARC one month after AAV injection, in the control group (AAV-GFP) and ARC NPY overexpressing group (AAV-NPY). Scale bar, 20 µm. n=3 mice per group.

3.5 Discussion

In the present study we show that NPY increases autophagy in both hypothalamic neuronal cellular models and mice overexpressing NPY in the hypothalamus. This conclusion is supported by the fact that NPY increased LC3B steady-state levels, autophagosome number and autophagic flux in hypothalamic neurons. In addition, the decrease of both LC3B-II and SQSTM1 in the hypothalamus of mice overexpressing NPY also suggests that NPY induces a fast degradation of these autophagic substrates.

The ability of hypothalamic neurons to stimulate autophagy in response to NPY is consistent with the roles of hypothalamic neurons in feeding and energy homeostasis (Kaushik et al., 2011, 2012; Quan et al., 2012). NPY-mediated activation of autophagy in the hypothalamus is particularly relevant as compromising autophagy in orexigenic agouti-related peptide (AgRP) neurons, which co-express NPY, in mice resulted in decreased body weight, reduced total body fat content, and decreased food intake in response to fasting (Kaushik et al., 2011). Moreover, recent studies have shown that the selective loss of autophagy in anorexigenic proopiomelanocortin (POMC) neurons in mice increased food intake and body weight (Kaushik et al., 2012; Quan et al., 2012).

Growing evidence indicate that basal autophagic activity decreases with age, thus contributing to the aging phenotype and to the aggravation of age-related disease (Cuervo, 2008), and that hypothalamic autophagy is impaired in normal aged mice. Aging reduced hypothalamic Atg7 levels, decreased steady- state levels of LC3B-II, indicating reduced autophagosome content, and decreased lysosomal accumulation of LC3B-II and SQSTM1 (Kaushik et al., 2012), that might contribute to the metabolic dysregulation observed with age. The aging process is also related to alterations in neuroendocrine control of energy homeostasis, namely a decrease of hypothalamic NPY levels (Higuchi et al., 1988; Gruenewald et al., 1994; Chiodera et al., 2000; Vela et al., 2003). Because autophagy declines with aging (Cuervo, 2008), it is plausible that decreased hypothalamic autophagy might contribute to the metabolic deregulation observed with age and that such dysfunction may result from decreased NPY levels. Therefore, it is tempting to speculate that autophagy stimulation by NPY in hypothalamic neurons could restore the metabolic homeostasis, and therefore, the role of hypothalamic NPY on food intake regulation might depend on autophagy activation.

In the present study, we also observed that NPY increased autophagic flux in rat differentiated hypothalamic neural cells and this effect was inhibited by NPY Y_1, Y_2 or Y_5 receptor antagonists. In mHypoN42 hypothalamic neurons only the inhibition of NPY Y_1 or

Y_5 receptor blocked the increase in autophagic flux induced by NPY (Figure 3.17). Since rat differentiated hypothalamic neural cells is a culture constituted mainly by neurons but also with other cell types, namely astrocytes and microglia, these observations suggest that NPY Y_2 receptor may be relevant for autophagy regulation in other hypothalamic neural cells. Although NPY Y_2 receptor is not involved in the increase of autophagic flux in mHypoN42 hypothalamic neurons, the activation of this receptor could contribute to the first step of autophagy induction. However, autophagy impairment in NPY/AgRP or POMC hypothalamic neurons has opposite effects on food intake and body weight, as mentioned above, and the contribution of both NPY receptors on autophagy may represent a synergic, redundant, or compensatory mechanism among NPY receptors localized in different neurons. Further studies are needed to dissect this issue, and the use of NPY receptors knockout mice could elucidate which NPY receptor is critical.

We further investigated which intracellular pathways are involved in the stimulatory effect of NPY on autophagy. Several signaling pathways seem to regulate autophagy in mammalian cells. Interestingly, contrarily to rapamycin, NPY increased autophagy in hypothalamic neurons through a MTOR-independent pathway. Although autophagy is required for the recycling of cellular compartments and nutrients, its regulation depends on the metabolic state of the cell and nutrient availability. Activation of MTOR kinase activity in response to rich nutrient conditions and insulin/growth factor signaling suppresses autophagy, while inhibition of MTOR by starvation induces autophagy. Since in the cell culture models used, cells are under a rich nutrient condition, NPY may induce autophagy in a MTOR-independent pathway, activating other signaling pathways. In fact, we observed that NPY enhances autophagic flux in mHypoN42 hypothalamic neurons through the concomitant activation of PI3K, MEK/ERK and PKA signaling pathways. PI3K inhibitors, including 3-methyladenine, wortmannin, and LY294002 have been used as autophagy inhibitors. These inhibitors inhibit class I as well as class III PI3K; however, some studies have suggested 3-methyladenine as specific class III PI3K inhibitor of autophagy (Blommaart et al., 1997; Petiot et al., 2000). While class III PI3K, also known as VPS34, is essential for autophagosome formation and therefore, autophagy induction, class I PI3K suppresses autophagy via indirect activation of MTOR complex. The net effect of these inhibitors is typically to inhibit autophagy because the class III enzymes that are required to activate autophagy act downstream of the negative regulatory class I enzymes. ERK, a mitogen-activated protein kinase has also been implicated in autophagy regulation (Wang et al., 2009a; Cagnol and Chambard, 2010). Several studies involving pharmacological inhibition of ERK demonstrated that it mediates starvation- and TNF-α-induced autophagy (Ogier-Denis

et al., 2000; Sivaprasad and Basu, 2008). Consistent with these reports, we showed in this study that NPY induced autophagy in hypothalamic neurons via the ERK pathway, as showed by a decline in LC3B-II protein content and autophagic flux by MEK/ERK inhibitor. In addition to PI3K and ERK, our results also suggest that NPY enhances autophagic flux in hypothalamic neurons via PKA pathway. However, it is described that PKA negatively regulates autophagy either by directly phosphorylating LC3B or by activating mTORC1, which inhibits autophagy (Mavrakis et al., 2006). Since NPY can increase cAMP levels and consequently activate PKA in hypothalamic neurons (Dhillon et al., 2009; Hong et al., 2012), this discrepancy may be due to cell type specificity.

Then, we examined the activation status of each signaling component upon NPY Y_1 and Y_5 receptor agonist treatment, since NPY increased autophagic flux in these cells only through the activation of NPY Y_1 and Y_5 receptor. NPY Y_1 receptor agonist increased AKT and PKA phosphorylation and this effect was class I PI3K-dependent. On the other hand, NPY Y_5 receptor agonist activated directly MEK/ERK and PKA. Therefore, PI3K, MEK/ERK and PKA activation in fact contribute to NPY-mediated effects on mHypoN42 hypothalamic neurons. However, to further support the involvement of all signaling pathways on autophagy regulation by NPY, we then determined the effect of each signaling pathway on NPY Y_1 and Y_5 receptor agonist-induced autophagic flux. NPY Y_1 receptor-induced LC3B-II flux was inhibited in the presence of PI3K or PKA inhibitors. Since NPY Y_1 receptor agonist increased both AKT and PKA phosphorylation and LY294002 can inhibit both class I and class III PI3K, these results suggest that NPY Y_1 receptor increases the autophagic flux through both class I and class III PI3K and PKA. NPY Y_5 receptor agonist-induced LC3B-II flux was inhibited by PI3K, MEK/ERK or PKA inhibitors. As NPY Y_5 receptor agonist increased ERK and PKA, but not AKT phosphorylation, these results suggest that NPY Y_5 receptor increases autophagic flux through MEK/ERK, PKA and class III PI3K (Figure 3.17).

We also evaluated the role of NPY on autophagy in an *in vivo* model, by overexpressing NPY in mouse hypothalamic arcuate nucleus (ARC), using AAV gene transfer technology. Mice overexpressing NPY in the ARC showed a lower levels of LC3B-II and SQSTM1 steady state levels in the hypothalamus, compared to controls (AAV-GFP) which indicates that these autophagic substrates are undergoing a rapid degradation process, consistent with an increase of the autophagic flux in the hypothalamus. Therefore, we demonstrated that a sustained upregulation of NPY levels in the ARC of adult mouse is sufficient to induce autophagy in the hypothalamus. Thus, the regulation of hypothalamic autophagy, through the modulation of NPY levels, could become an effective intervention in conditions where hypothalamic function is compromised.

Besides reduced levels of NPY observed with aging in several brain areas, such as hypothalamus, hippocampus and cortex; neurodegenerative diseases are also associated with reduced NPY (Rose et al., 2009; Decressac et al., 2010, 2012). Since NPY has a well-established neuroprotective effect, autophagy activation by NPY could be a relevant potential strategy to investigate in neurodegenerative diseases characterized by impaired autophagy.

In addition, the increase in NPY can lead to several physiological modifications similar to those induced by CR. Central administration of NPY has been shown to induce hyperphagia (Stanley et al., 1986; Beck et al., 1992), lower blood glucose levels in both humans and rats (Ahlborg and Lundberg, 1994; Marks and Waite, 1997; Bischoff and Michel, 1998), and reduce core body temperature (Billington et al., 1991; Kotz et al., 2000). Interestingly, transgenic rats overexpressing NPY show improved resistance to stress and increased mean lifespan (Michalkiewicz et al., 2003). In addition, it has been shown recently that CR does not increase lifespan in NPY knockout mice, enlightening NPY role as a lifespan and aging regulator (Chiba et al., 2014). In humans, increased NPY levels may also be correlated with lifespan benefits because long-lived female centenarians have higher NPY plasma levels compared with younger women (Schoch et al., 1996). Moreover, this study shows that NPY induces autophagy in the hypothalamus, and our research group also demonstrated that the inhibition of NPY receptors blocks CR-induced autophagy in hypothalamic neurons (Aveleira and Botelho, et al., 2015). As autophagy is a key mechanism underlying CR's beneficial effects and considering that it is difficult to implement and sustain a CR regimen in humans, increasing research interest has focused on identifying agents that can mimic the beneficial effects of CR. Increasing evidence, including our study, suggest that NPY is a putative CR mimetic candidate.

Since autophagy and NPY levels decrease with age in the hypothalamus, a brain region recently described as crucial for the progress of whole-body aging (Zhang et al., 2013); and that NPY regulates autophagy in the hypothalamus, as shown in our study; modulation of NPY levels may be manipulated to produce protective effects against hypothalamic impairments associated with age. Therefore, further studies on the role of NPY in the regulation of hypothalamic autophagy are expected to provide new therapeutic avenues to extend longevity and ameliorate age-related deteriorations.

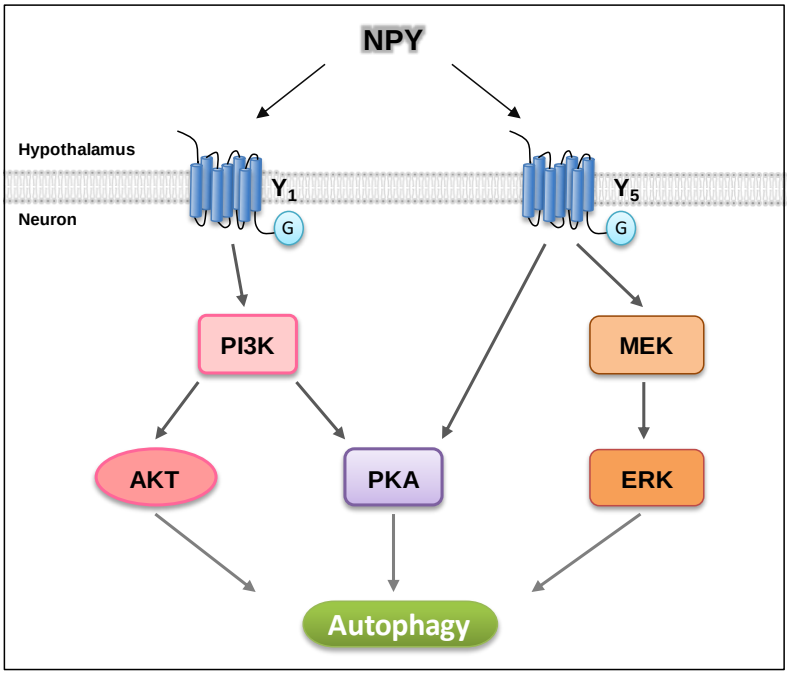

Figure 3.17 - NPY induces autophagy in hypothalamic neurons through NPY Y_1 and Y_5 receptors activation through a combination of intracellular pathways. NPY Y_1 receptor activates both AKT and PKA signaling pathways in a PI3K-dependent manner and NPY Y_5 receptor activation increases both MEK/ERK and PKA phosphorylation.

CHAPTER 4

Autophagy modulates synaptic plasticity of hypothalamic POMC neurons

4.1 Abstract

NPY/AgRP and POMC neuronal populations of the hypothalamic arcuate nucleus are responsible for energy homeostasis, regulating anabolic and catabolic state. The hypothalamus receives and integrates peripheral nutritional and hormonal signals and adapts to the changes to maintain energy balance. It was demonstrated that synaptic plasticity in feeding circuits are important in this central process. Autophagy, a degradation process of intracellular compartments, in the hypothalamus was also identified as a player metabolic regulation. However, a role for autophagy in plasticity of feeding circuits has not been interrogated.

The aim of this study was to interrogate the role of autophagy in synaptic input organization on POMC neurons under standard diet and food deprivation. Using POMC neuron-selective Atg7-deficient mice, we observed that Atg7 deletion in POMC neurons resulted in increased cell area and perimeter, and nematosomes accumulation. We also found a distinct difference in the quantitative and qualitative synaptology of POMC cells between Atg7-deficient mice and respective control, with a significantly greater number of inhibitory inputs in POMC neurons of Atg7-deficient mice. POMC neurons with no functional Atg7 also show an impairment of neuronal activation in arcuate nucleus, after an overnight fasting.

The results reveal a role of autophagy in control of synaptic plasticity of POMC neurons, extending the importance of this cellular mechanism as feeding-related mechanism acting in the arcuate nucleus to energetic balance control.

4.2 Introduction

Energy balance is maintained by homeostatic system involving the brain and the periphery. A key component of this system is the hypothalamus that regulates food intake and energy expenditure regulation, integrating peripheral hormonal and nutritional signals (Horvath and Diano, 2004; Williams, 2012). The melanocortin system, in the arcuate nucleus (ARC) of the hypothalamus, is responsible for energy homeostasis and comprises two major neuronal populations – agouti-related protein (AgRP)/neuropeptide Y (NPY) expressing neurons and cocaine- and amphetamine-related transcript (CART) and pro-opiomelanocortin (POMC) expressing neurons (Horvath and Diano, 2004; Simpson et al., 2009). There is a reciprocal neuronal connectivity between these two neuronal populations and other extrahypothalamic neuronal systems, involved in control of energy balance (Cowley et al., 2001; Horvath et al., 2010; Spanswick et al., 2012).

POMC neurons decrease food intake and increase energy expenditure by releasing α-melanocyte stimulating hormone (α-MSH), an orexigenic peptide derived from POMC precursor protein. The α-MSH activates melanocortin-3 (MC3R) and melanocortin-4 (MC4R) receptors on neuronal populations located in arcuate, paraventricular and dorsomedial nucleus and in the lateral hypothalamus. By contrast, NPY/AgRP neurons release AgRP and NPY with an orexigenic effect (Kalra et al., 1999; Belgardt et al., 2009; Simpson et al., 2009). AgRP antagonizes anorexigenic melanocortin cells at their targeted sites where MC3R and MC4R are located. In addition, NPY/AgRP system also directly inhibits POMC perykarya through NPY action as well as the inhibitory amino acid neurotransmitter, γ-aminobutyric acid (GABA), which is present in NPY/AgRP cell terminals that innervate POMC cells (Horvath et al., 1997; Cowley et al., 2001; Horvath, 2005; Vong et al., 2011).

Moreover, NPY/AgRP and POMC neurons receive excitatory and inhibitory synaptic inputs; and recent studies revealed that synaptic vesicle release and continual plasticity in the feeding circuits are an important component in hypothalamic energy balance control (Dietrich and Horvath, 2009; Zhang et al., 2011). The two neurotransmitters that account for most of the synaptic activity in the hypothalamus are the amino acids glutamate and GABA (Delgado, 2013). GABA represents the main inhibitory neurotransmitter in the central nervous system (CNS), and exerts a early inhibitory transmission through $GABA_A$ receptors, and through $GABA_B$ receptors, as a late component of inhibition (Meister, 2007). GABA is able to induce feeding and positive energy balance after intracerebroventricular injections (Baldwin et al., 1990; Ebenezer and Baldwin, 1990). On the other hand, glutamate exerts its effect through ionotropic AMPA, kainate, NMDA, and delta receptors, and through

metabotropic receptors, and represents the main excitatory neurotransmitter in hypothalamic neuroendocrine regulation (Delgado, 2013). In addition, glutamate is expressed in POMC/CART neurons, suggesting a glutamatergic phenotype for this neuronal population (Jarvie and Hentges, 2012).

Therefore, leptin, ghrelin and other afferent signals regulate AgRP/NPY and POMC neurons on transcriptional level and by altering neuronal firing. Several evidence have shown that hypothalamic neuronal populations exhibit a neuronal connectivity between them which is implicated in the endocrine regulation of energy balance and changes in response to metabolic hormones, such as leptin and ghrelin (Pinto et al., 2004; Horvath and Gao, 2005; Sternson et al., 2005; Gao et al., 2007; Andrews et al., 2008). Moreover, in obesity models, the stimulatory and inhibitory input organization into hypothalamic neurons is associated with the endocrine effects of the melanocortin system to peripheral signals, and with the control of body weight (Horvath and Diano, 2004; Horvath et al., 2010). Thus, these studies allow propose that the perikaryal inputs of orexigenic NPY/AgRP neurons are dominated by inhibitory connections when circulating leptin levels are high and ghrelin levels are low (Horvath, 2006). When leptin levels decrease and ghrelin levels increase, stimulatory synapses dominate over inhibitory inputs. On POMC perikarya, the changes occur in the opposite direction described for the NPY/AgRP inputs. Some of the inhibitory inputs on POMC cells are likely to originate from NPY/AgRP neurons, and some of the stimulatory inputs on both cell types originate in the lateral hypocretin neurons (Horvath, 2006). Thus, the synaptic input organization of hypothalamic neurons is able to rapidly rewiring the afferents neurons in response to metabolic hormones, even before any changes in feeding behavior and body weight, and this synaptic plasticity is a critical component in regulation of energy homeostasis and metabolic phenotype (Horvath, 2006).

Macroautophagy (hereafter referred to as autophagy) is a catabolic process in which parts of cytoplasm and intracellular organelles are engulfed in a double-membrane vesicle (autophagosome) and then delivered to the lysosome, where the sequestered cargo is degraded and recycled (Klionsky, 2007). This process is mediated by a group of autophagy-related proteins (Atg), and it is necessary for maintenance of cellular homeostasis (Donati, 2006).

Beyond cellular turnover, autophagy is also involved in control of energy homeostasis, by its action on the liver, pancreas, adipocytes, and more recently in the hypothalamus (Komatsu et al., 2005; Jung et al., 2008; Singh et al., 2009). A decrease in autophagy in hypothalamic neurons contributes to the metabolic deregulation (Meng and Cai, 2011). The specific deletion of Atg7, an essential autophagy gene encoding E1-like enzyme in the two

ubiquitin-like conjugation system, in orexigenic AgRP/NPY neurons in mice resulted in a decrease on body weight, on total fat content and on food intake in response to fasting (Ohsumi and Mizushima, 2004; Kaushik et al., 2011). On the other hand, loss of autophagy by deletion of Atg7 in anorexigenic POMC neurons increases mice body weight, food intake, and induces an abnormal development of POMC neuronal projections (Coupé et al., 2012; Kaushik et al., 2012; Quan et al., 2012). Although these studies show the importance of autophagy on energy balance, the autophagic role on synaptic plasticity in the hypothalamus remains unknown. In the present study, we investigated how Atg7 deletion in POMC neurons interferes with the synaptic organization in these neurons, under a normal diet or food deprivation.

4.3 Materials and Methods

4.3.1 Animals

Male mice were housed individually in an environmental temperature of 22 °C with a 12:12h light-dark cycle, and provided *ad libitum* access to water and standard laboratory chow. Animal usage was in compliance with and approved by the Institutional Animal Care and Use Committee of the Saban Research Institute of the Children's Hospital of Los Angeles. POMC-specific Atg7 knockout mice (Pomc-Cre; $Atg7^{loxP/loxP}$) were generate as described by Coupé et al. (2012). Briefly, Pomc-Cre mice with C57BL/6 background were crossed with mice carrying a loxP-flanked Atg7 allele ($Atg7^{loxP/loxP}$) in a C57BL/6 background. Breeding colonies were maintained by mating Pomc-Cre; $Atg7^{loxP/+}$ mice with $Atg7^{loxP/loxP}$ mice. We used as controls Cre-negative $Atg7^{loxP/loxP}$ mice. Animals were maintained during 10 weeks with standard chow diet or last night fasting, then, intracardially perfused with 4% (w/v) paraformaldehyde fixative. Brains were dissected out, washed in phosphate buffer saline (PBS; 137 mM NaCl; 2.7 mM KCl; 10 mM Na_2PO_4; 1.8 mM KH_2PO_4; pH 7.4) and cut on vibratome (LEICA Vibratome 1000 Class Microtome, Leica Biosystems, Buffalo Grove, IL, USA). Slices (50 µm) containing the ARC of the hypothalamus were collected and stored in glass flasks sections floating in PBS supplemented with 0.12 µmol/L sodium azide. The slices were stored at 4 °C until immunohistochemical processing.

4.3.2 Transmission electron microscopy

Electron microscopy protocol was performed as described before (Dietrich et al., 2013). Brains slices containing arcuate nucleus of the hypothalamus were washed in PBS several times, cryoprotected, and frozen and thawed three times in liquid nitrogen. Slices were washed again and blocked with normal goat serum for 30 min. After, sections were incubated with a rabbit polyclonal anti-POMC (1:4000; Phoenix Pharmaceuticals, Burlingame, CA, USA) for 48 hours at 4 °C. Sections were extensively washed, incubated with biotinylated goat anti-rabbit IgG (1:250; Vector Laboratories, Burlingame, CA, USA) for 2 hours at room temperature. Then, the slices were incubated with avidin–biotin complex (ABC Elite kit, Vector Laboratories), for 30 min, and the immunoreactivity developed with diaminobenzidine (DAB). After extensive washing, 1% osmium tetroxide was added to the sections for 30 min, and then, they were dehydrated in increasing ethanol concentrations. During the dehydration, 1% uranyl acetate was added to the 70% ethanol to enhance ultrastructural membrane contrast. Dehydration was followed by flat embedding in

Durcupan. Ultrathin sections were cut on a Leica ultra-microtome (Reichert-Jung Ultramicrotome, Leica Microsystems, Buffalo Grove, IL, USA), collected on Formvar-coated (Electron Microscopy Sciences, Ft. Washington, PA, USA) single-slot grids, and analyzed with a Tecnai 12 Biotwin (FEI, Hillsboro, USA) electron microscope.

4.3.3 Fluorescence microscopy

Coronal brain slices were washed with PBS, and incubated in 1% (v/v) H_2O_2/PBS solution to block endogenous peroxidase activity for 15 min, at room temperature. After washing, sections were incubated with 0.2% (v/v) TritonX-100(TX-100)/PBS, for 30 min, washed and incubated with primary antibodies: rabbit polyclonal anti-POMC (1:1000, Phoenix Pharmaceuticals) and guinea-pig polyclonal anti-AgRP (1:2000, Abcam, Cambridge, MA, USA). Sections were then washed and incubated with the secondary antibodies Alexa Fluor 488 goat anti-rabbit IgGs and Alexa Fluor 568 goat anti-guinea pig IgGs (1:500, Invitrogen, Carlsbad, CA, USA), for two hours at room temperature. Sections were mounted with Vectashield Mouting Medium (Vector Laboratories).

4.3.4 Light microscopy

Coronal brain slices were washed with PBS, and incubated in 1% (v/v) H_2O_2/PBS solution for 15 min, at room temperature. After washing, sections were incubated with 0.2% (v/v) TX-100/PBS, for 30 min, washed and incubated with rabbit polyclonal anti-c-Fos (1:10 000; Millipore, Billerica, MA, USA) at room temperature. After washing, sections were incubated with biotinylated goat anti-rabbit secondary antibody (1:250; Vector Laboratories), for 2 hours. Then, sections were incubated with ABC Elite kit (Vector Laboratories), washed, and immunoreactivity for c-Fos was developed with nickel-DAB reaction. Brain sections were then mounted with Permount mounting medium (Fisher Scientific, Pittsburgh, PA, USA).

4.3.5 Cell analysis and quantitative synaptology

Random sections containing POMC cells with a visible nucleus were analyzed. Trans-sectional area and perimeter of each neuron were measured using Fiji software (National Health Institute, Bethesda, MD, USA). Nematosomes were counted in POMC cells and the data are expressed as number of nematosome *per* 100 μm^2 of cytosol area. The analysis of synapse number is presented as number of synapses per 100 µm of cell membrane.

Inhibitory synapses were identified by their symmetric morphology and excitatory synapses by their asymmetric morphology as previously described (Pinto et al., 2004). Their distinct morphology is correlated with the content, and the glutamate is related with asymmetric synapses and GABA with symmetric synapses (Pinto et al., 2004).

4.3.6 Mitochondria and mitochondria-endoplasmic reticulum (ER) quantification

The mitochondria profiles were traced using Fiji software (National Health Institute). Trans-sectional area of each mitochondrion was measured and the data were expressed as total mitochondria trans-sectional area per cell. Mitochondria density was estimated by dividing the number of mitochondria profiles by the cellular areas. Mitochondria coverage was estimated by dividing the total area of mitochondria by the cellular areas. Differences in mitochondria density and coverage were tested using Student's unpaired *t* test. For mitochondria-ER interaction, the number of mitochondria in contact with ER profiles was scored in high-magnification images.

4.3.7 Statistics

All values were expressed as mean± SEM. Data were analyzed by using Student's unpaired *t* test or two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test for multiple comparisons. A value of $p<0.05$ was considered significant. Prism 5.0 (GraphPad Software) was used for all statistical analysis.

4.4 Results

4.4.1 Lack of Atg7 in POMC neurons alters morphometric POMC features

To study the physiological role of autophagy on morphometric features of POMC cells, we used POMC-specific autophagy-deficient mice, namely mice with a deletion of Atg7 (POMC-Cre, $Atg7^{loxP/loxP}$). Analysis of the trans-sectional cell area showed that POMC neurons area of the POMC-Cre, $Atg7^{loxP/loxP}$ group is higher compared with control group - $Atg7^{loxP/loxP}$ (103.1±6.5 *vs* 87.91±5.3 μm^2, p=0.0357; Figure 4.1A). Also, non-autophagic neurons showed bigger perikarya than $Atg7^{loxP/loxP}$ neurons (40.3±1.3 *vs* 36.6±1.1 µm, p<0.05; Figure 4.1B). Analyzing the intracellular ultrastructure of these neurons, we observed cytoplasmatic structures named by nematosomes or stigmoid bodies (García-Ovejero et al., 2001) in POMC neurons of $Atg7^{loxP/loxP}$ (6 in 35 cells/5 mice) and POMC-Cre, $Atg7^{loxP/loxP}$ mice (11 in 27 cells/4 mice). However, these non-membrane-bound structures presented a different distribution between the two groups: the nematosomes were present in a significant higher number on POMC cells deficient in Atg7 (1.168±0.332 *vs* 0.417±0.185 nematosome *per* 100 μm^2 cytosol area, p<0.05; Figure 4.1 C and D).

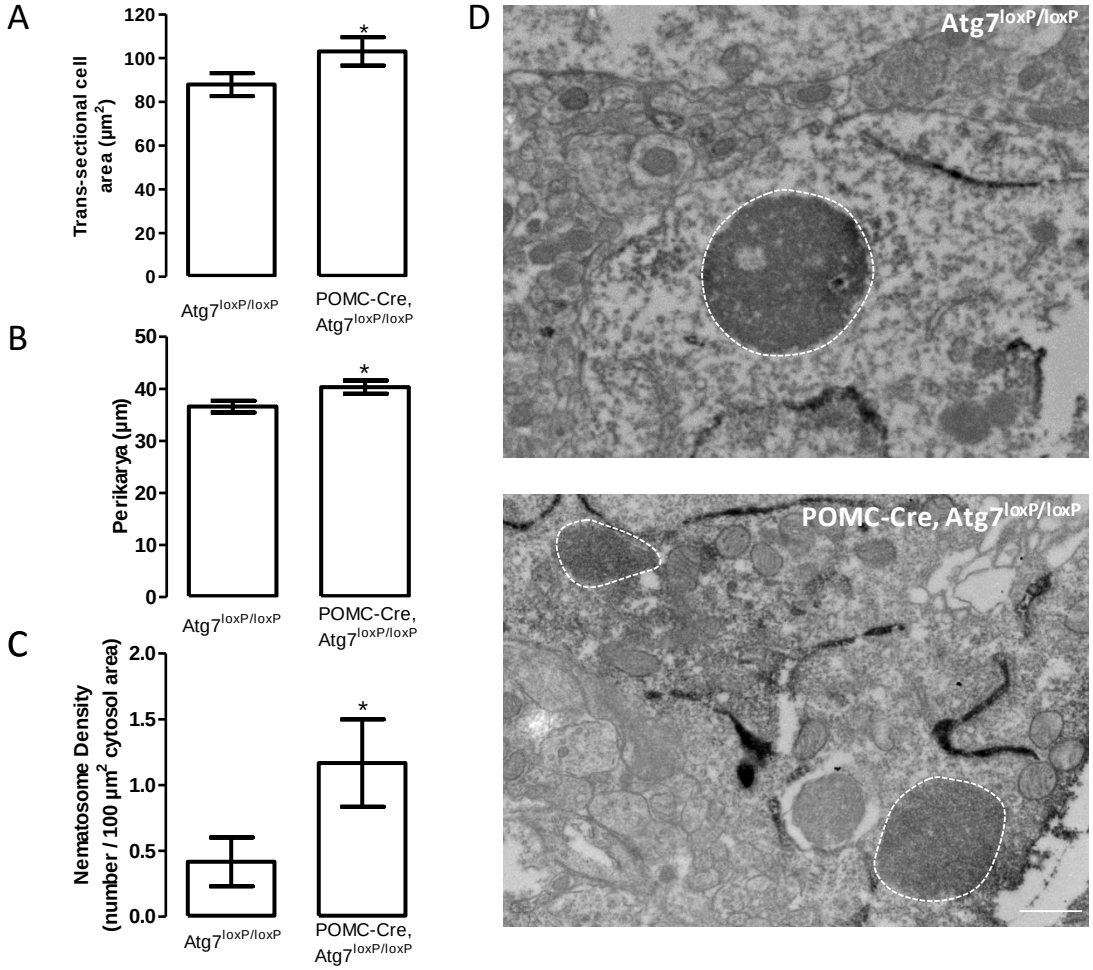

Figure 4.1 - Lack of Atg7 in POMC neurons alters morphometric POMC features. (A and B) Atg7 deletion increased trans-sectional area (**A**) and perykarya (**B**) of POMC neurons. (**C**) Atg7 deficiency affected nematosome (dashed white circles) density in POMC neurons. Data are mean ± SEM; *n*=26-35 cells; **p*<0.05, significantly different from control group as determined by Student's unpaired *t* test. (**D**) Representative electron micrographs showing nematosome structure taken from POMC-imunolabeled cytosol of $Atg7^{loxP7loxP}$ and POMC-Cre, $Atg7^{loxP7loxP}$ mouse. Scale bar, 500 nm.

4.4.2 POMC-specific ablation of Atg7 alters mitochondria-endoplasmic reticulum (ER) contacts

Since, mitochondrial and endoplasmic reticulum (ER) dysfunction have been associated with the development of metabolic alteration, we also assessed potential alterations in mitochondria and mitochondria-ER contacts in POMC neurons caused by POMC-specific deletion of Atg7. Ultrastructure analysis by electron microscopy showed similar mitochondrial densities in POMC neurons between both animal groups (POMC-Cre, $Atg7^{loxP/loxP}$= 43.84±3.357, $Atg7^{loxP/loxP}$=49.66±4.05 number/100 µm^2 cell area; Figures 4.2A and D), and also no differences were found in trans-sectional mitochondria area (POMC-Cre, $Atg7^{loxP/loxP}$= 0.094±0.004, $Atg7^{loxP/loxP}$=0.097±0.003 µm^2; Figures 4.2B). On the other

hand, the mitochondria coverage of the cell area was decreased in POMC neurons lacking Atg7 compared to control (3.682±0.290 *vs* 4.813±0.353 µm^2/100 µm^2 cell area, *p*=0.0218; Figures 4.2C). This change is due to enlarged of trans-sectional area of POMC neurons from POMC-Cre, Atg7$^{loxP/loxP}$ mice compared to control mice group (Figure 4.1A), resulting a decreased ratio. Interestingly, the number of mitochondria-ER contacts was significantly increased in POMC neurons from POMC-Cre, Atg7$^{loxP/loxP}$ mice compared with values in control POMC neurons (22.06±2.17 *vs* 13.69±1.57 %, *p*=0.0022; Figures 4.2D and E).

Collectively, these results indicate that autophagy may have no relevant impact on mitochondria morphology. However, the lack of Atg7 in hypothalamic POMC neurons led to changes in mitochondria-ER interactions. More studies are needed to confirm these results.

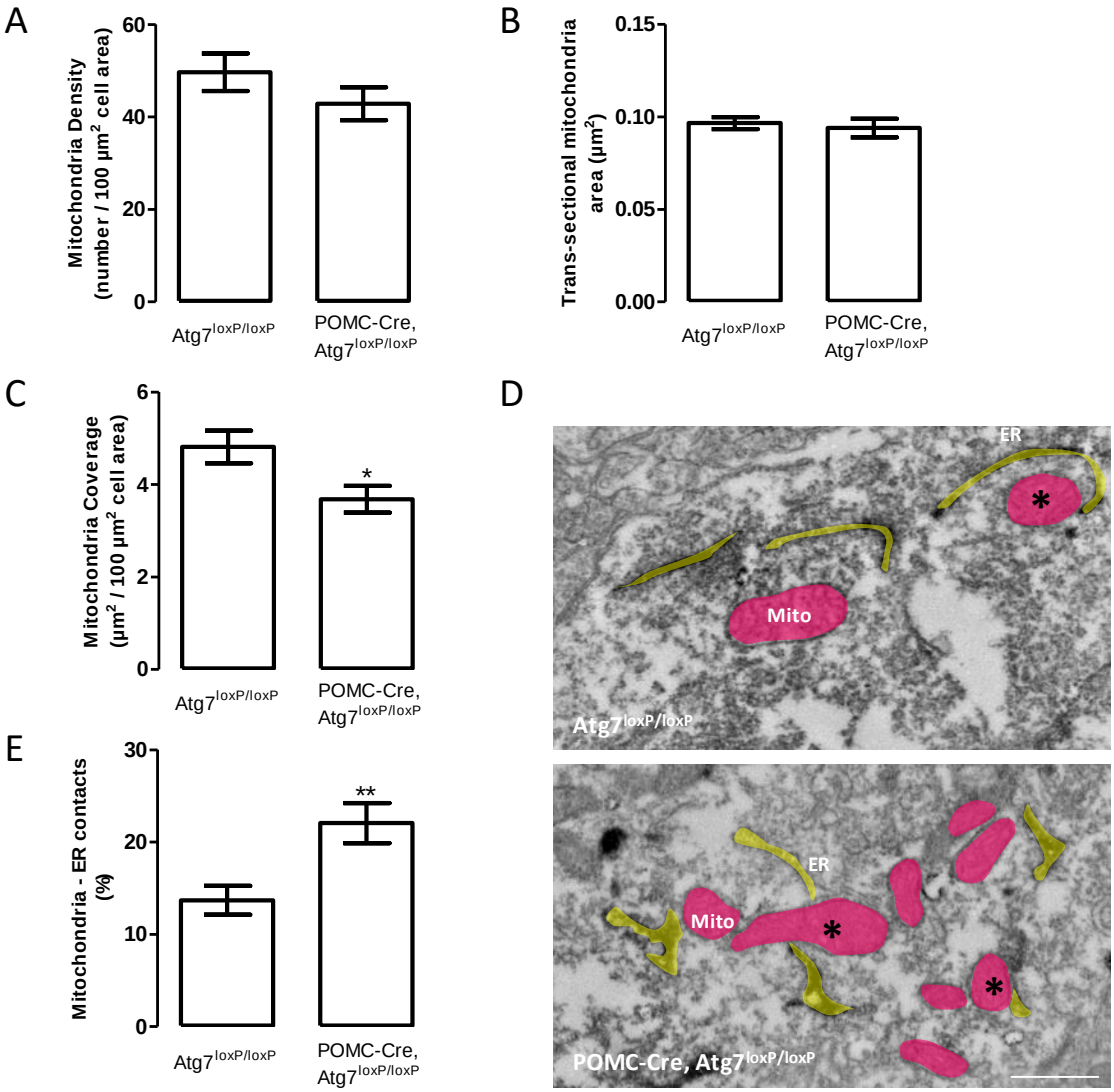

Figure 4.2 - Mitochondria coverage and mitochondria-ER contacts are altered in POMC neurons of POMC-Cre, Atg7$^{loxP/loxP}$ mice. Mitochondria density (**A**), trans-sectional mitochondria area (**B**) and mitochondria coverage (**C**) in POMC neurons. (**D** and **E**) Representative electron microscopy images (**D**) and quantification of mitochondria-ER contacts in POMC neurons from male Atg7$^{loxP/loxP}$ and POMC-Cre, Atg7$^{loxP/loxP}$ mice (**E**).

Mitochondria (Mito: pink areas) and endoplasmic reticulum (ER: yellow areas) are shown. Black asterisks show mitochondria in contact with ER. Data are mean ± SEM; *n*=26-35 cells; **p*<0.05; ***p*<0.01, significantly different from control group as determined by Student's unpaired *t* test. Scale bar, 500 nm.

4.4.3 Specific deletion of Atg7 in POMC neurons affects synaptic input organization of hypothalamic POMC neurons

To investigate whether Atg7 deficiency in POMC neurons affects the synaptology of these hypothalamic neurons, we analyzed the synaptic input organization, particularly the number and type of synaptic inputs on POMC perikarya of $Atg7^{loxP/loxP}$ and POMC-Cre, $Atg7^{loxP/loxP}$ mice. Under fed conditions, the total number of synaptic inputs onto POMC neurons was higher in POMC-Cre, $Atg7^{loxP/loxP}$ mice than $Atg7^{loxP/loxP}$ mice (10.24±0.92 *vs* 6.27±0.81 synapses *per* 100 µm perikarya, *p*=0.0019; Figure 4.3A). After an overnight fasting, no differences were found between these both feeding status in $Atg7^{loxP/loxP}$ animal group. However, in POMC-Cre, $Atg7^{loxP/loxP}$ mice, the total number of synapses was significant decreased after an overnight fasting (Fed=10.24±0.92, Fasting=6.23±0.70 synapses *per* 100 µm perikarya), to values similar to fasted $Atg7^{loxP/loxP}$ mice group (6.75±0.89 synapses *per* 100 µm perikarya). Regarding the type of synapses implicated in these differences, both POMC perikarya of $Atg7^{loxP/loxP}$ and POMC-Cre, $Atg7^{loxP/loxP}$ mice had symmetrical, putatively inhibitory synapses and asymmetrical, putatively excitatory synapses, in fed (Figure 4.3B) and fasting conditions. Nevertheless, there was a significant increase in the number of inhibitory synaptic inputs onto the satiety-promoting POMC neurons in POMC-Cre, $Atg7^{loxP/loxP}$ mice compared with the $Atg7^{loxP/loxP}$ mice on the standard chow diet (7.24±0.70 vs 3.81±0.53 synapses *per* 100 µm perikarya; *p*=0.0002). The number of excitatory inputs *per* 100 µm POMC perikarya did not differ significantly between the mice groups (POMC-Cre, $Atg7^{loxP/loxP}$= 3.01±0.53, $Atg7^{loxP/loxP}$=2.46±0.41 synapses *per* 100 µm perikarya; Figure 4.3C). An increase in the inhibitory synaptic density in POMC neuron-specific Atg7 deficiency suggests an increase of the inhibitory tonus triggered by autophagy deficiency.

The synaptic input organization of POMC neurons was also evaluated after overnight fasting and compared to fed conditions. In $Atg7^{loxP/loxP}$ group, no significant differences were found on the number of excitatory and inhibitory inputs onto POMC neurons between fed and fasted conditions (Figure 4.3C). Nevertheless, an increased number of inhibitory synapses was observed (Fed=3.81±0.53, Fasting=4.86±0.70 synapses *per* 100 µm perikarya) in POMC neurons of $Atg7^{loxP/loxP}$ mice after overnight fast, although not significant, it complies with previous reported (Horvath, 2005; Horvath and Diano, 2004; Pinto et al.,

2004). However, Atg7 deficiency altered the synaptic distribution on POMC-Cre, Atg7$^{loxP/loxP}$ mice (Figure 4.3C). The excitatory synapses number was not altered but the inhibitory inputs on this neuronal population significantly decreased under fasted condition (Fed=7.24±0.70, Fasting=4.01±0.57 synapses *per* 100 µm perikarya, p<0.001). After an overnight of food deprivation, the inhibitory organization shows a different pattern of reorganization, when we compare both animal models. The NPY/AgRP perikarya could not be assessed in mice, because the labeling of NPY is limited to axonal processes.

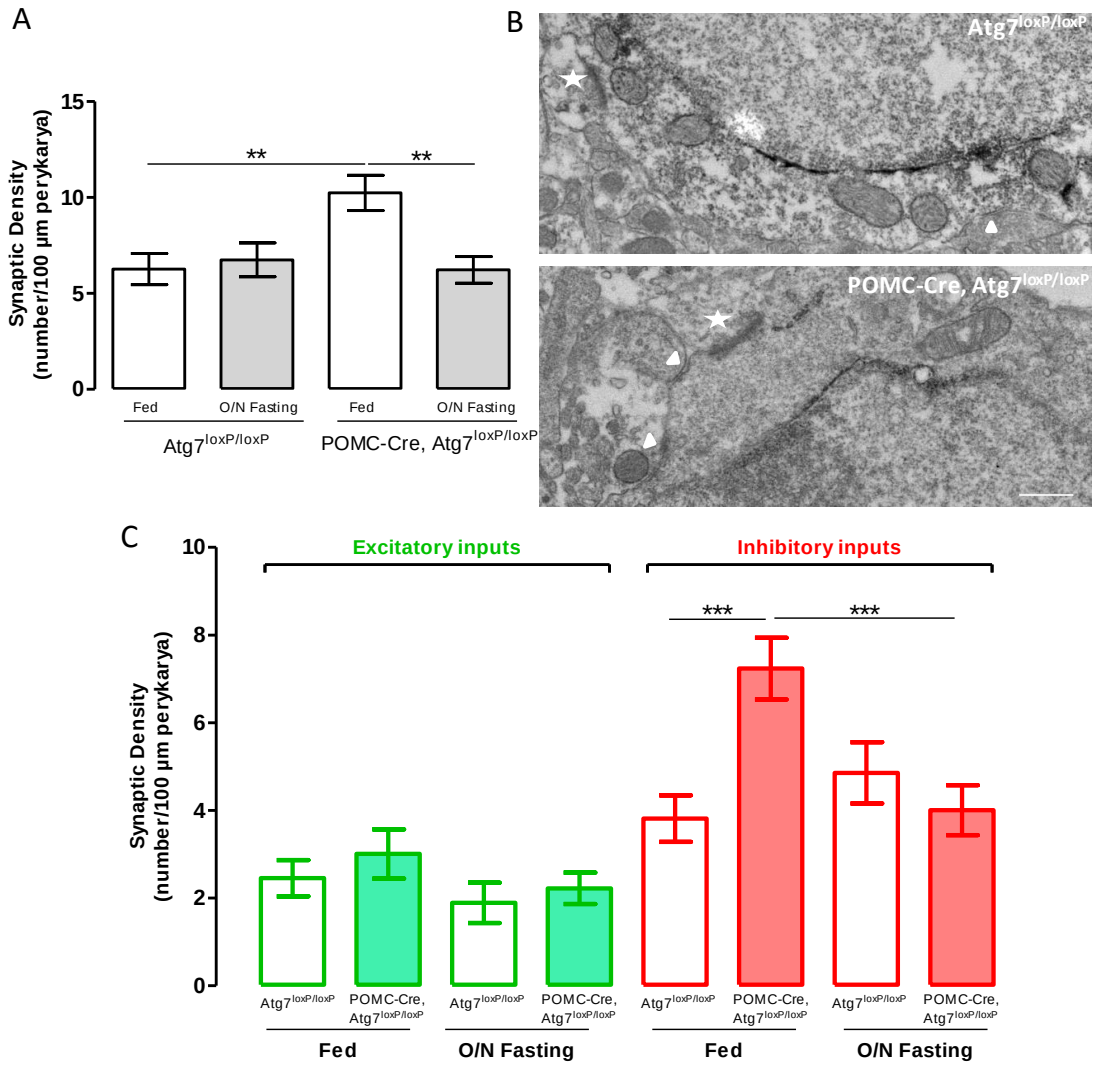

Figure 4.3 - Specific deletion of Atg7 in POMC neurons affects synaptic input organization of hypothalamic POMC neurons. (**A**) Atg7 deficiency increased the total number of synapses onto POMC neurons under fed conditions. (**B**) Representative electron micrographs showing POMC-labeled perikarya of Atg7^{loxP7loxP} and POMC-Cre, Atg7^{loxP7loxP} mice. The stars indicate the asymmetric, putatively excitatory synapses, and arrows point to the symmetric, putatively inhibitory synapses. Scale bar, 500 nm. (**C**) Under fed conditions, the increase of total number of synapses onto POMC neurons in Atg7 deficiency mice is due to an increase in number of symmetric, putatively inhibitory inputs and not asymmetric, putatively excitatory connections. An overnight fasting (O/N Fasting) decreased the number of the total synapses (**A**) and the total number of inhibitory synapses (**C**) in

POMC-Cre, Atg7^{loxP7loxP} mice. The results represent mean ± SEM; *n*=27-35 cells per group; ***p*<0.01, ****p*<0.001 as determined by two-way ANOVA, followed by Bonferroni's Multiple Comparison Test.

As NPY/AgRP neuronal population is able to antagonize POMC neurons effects directly by GABA and NPY release (Cowley et al., 2001), then we studied the contacts between these two neuronal populations of the melanocortin system by immunofluorescence studies. Staining AgRP terminals connections (red) and POMC neurons (green) and count the red puncta in contact with external limit of the green POMC cellular body (Figure 4.4A), we found no significant differences between the two experimental groups under a standard chow diet (POMC-Cre, Atg7$^{loxP/loxP}$=0.140±0.013, Atg7$^{loxP/loxP}$=0.113±0.014 AgRP contact points *per* µm POMC perikarya, p=0.1394), neither in each animal group (Atg7$^{loxP/loxP}$ and POMC-Cre, Atg7$^{loxP/loxP}$) after an overnight fasting (Figure 4.4B). This result suggests that the inhibition of POMC neurons through inhibitory contacts is provided by other cell types than NPY/AgRP neurons, through GABA release.

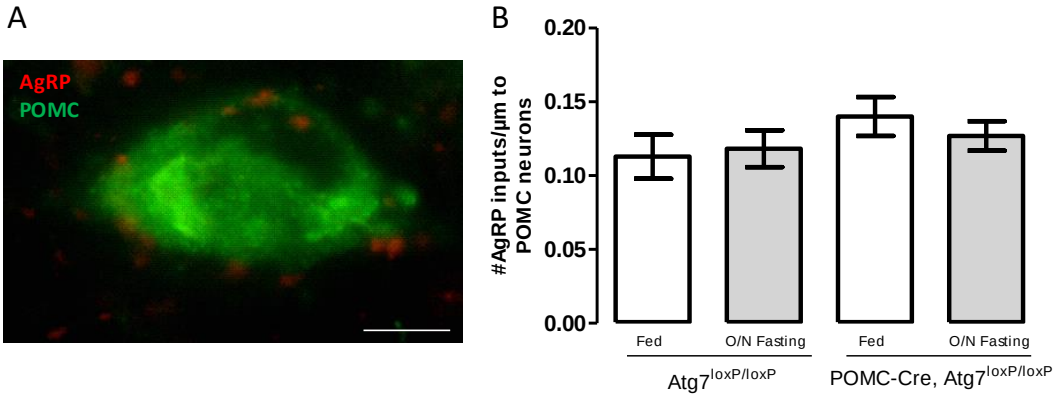

Figure 4.4 - Lack of Atg7 in POMC neurons no change the AgRP inputs on POMC perikarya. (**A**) Representative image of AgRP puncta (red) in contact with perikarya of POMC cell body (green). Scale bar, 5 µm. (**B**) No significant differences were found between the animal groups, taking into account the fed and fasting status (O/N Fasting). The results represent mean ± SEM; *n*=31-48 cells *per* group; two-way ANOVA, followed by Bonferroni's Multiple Comparison Test.

4.4.4 Mice with Atg7 deficiency in POMC neurons causes no normal neuronal activity to fasting conditions

Then, we evaluated whether autophagy deficiency-induced rearrangements in the synaptic organization of POMC neurons alters the neuronal activity of hypothalamic arcuate nucleus after an overnight fasting. To analyze the neuronal activity, we analyzed the c-Fos expression, a marker of neuronal activation in hypothalamic arcuate nucleus, in both Atg7$^{loxP/loxP}$ and POMC-Cre, Atg7$^{loxP/loxP}$ mice, under standard diet and food deprivation. In

Atg7$^{loxP/loxP}$, there was a significant increase of c-Fos immunoreactivity after an overnight of fasting (Fed=181.0±26.91; Fasting=286.7±24.04, c-Fos positive cells on hypothalamic arcuate nucleus/animal; Figure 4.5). However, no changes of c-Fos expression were observed in POMC-Cre, Atg7$^{loxP/loxP}$ (Fed=159.3±32.8; Fasting=196.7±47.58, c-Fos positive cells on hypothalamic arcuate nucleus/animal, $p<0.05$). Additionally, c-Fos expression was significant decreased after fasting in POMC-Cre, Atg7$^{loxP/loxP}$ mice compared to the controls in same conditions (Figure 4.5). These results suggest that activation of hypothalamic neurons by fasting is an autophagy-dependent process.

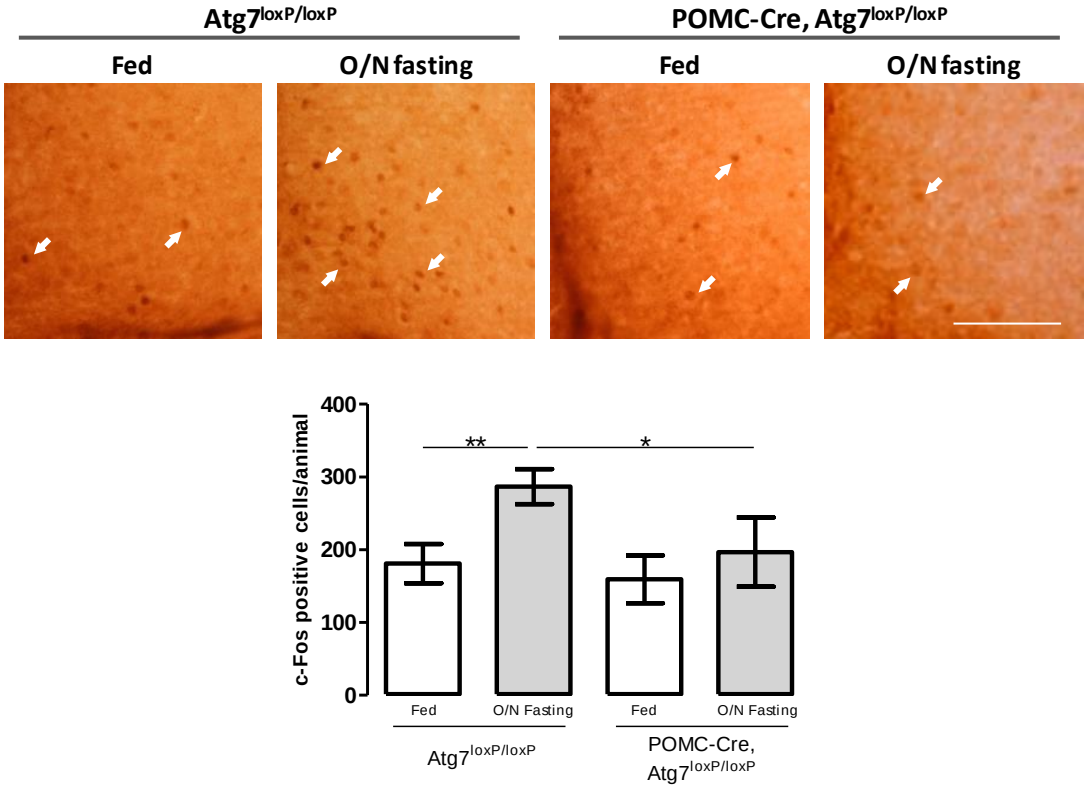

Figure 4.5 - Mice with Atg7 deficiency in POMC neurons causes abnormal adaptation to fasting conditions. (**A**) Representatitve photos of imunohistochemistry for c-Fos (arrows) for both Atg7$^{loxP/loxP}$ and POMC-Cre, Atg7$^{loxP/loxP}$ animal groups, under fed and overnight fasting (O/N Fasting). Scale bar, 50 µm. (**B**) Histogram bars represent the quantification of c-Fos-labeled cells in the arcuate nucleus of Atg7^{loxP7loxP} and POMC-Cre, Atg7^{loxP7loxP} mice. The results represent mean ± SEM; *n*=3-4 mice/group; **p*<0.05; ***p*<0.01, two-way ANOVA, followed by Bonferroni's Multiple Comparison Test.

4.5 Discussion

In the present study we show that the loss of constitutive autophagy in POMC neurons alters the morphometric features and the synaptic input organization of these cells, as well as, compromises the neuronal activation in hypothalamic arcuate nucleus, after a fasting period.

Autophagy has been shown to be important to energetic balance regulation (Rubinsztein et al., 2012). Beside loss of autophagy causes neurodegeneration (Hara et al., 2006; Komatsu et al., 2006), some studies also show that it has impact on central control of energy metabolism, feeding and energy balance. Kaushik et al. (2011) observed that the specific deletion of Atg7, an essential autophagy gene for the autophagosome biogenesis, in AgRP neurons decreases AgRP levels, body weight and total fat mass (Kaushik et al., 2011). In agreement, other studies show that deletion of Atg7 in POMC neurons change central metabolic regulation (Coupé et al., 2012; Kaushik et al., 2012; Quan et al., 2012). Loss of constitutive autophagy in POMC neurons reduces POMC fiber density (Coupé et al., 2012), and increases POMC trans-sectional cell area and perimeter, as showed by our morphometric analysis. Since autophagy is required to generate α-MSH (Kaushik et al., 2012), a relationship between the high cell area in POMC neurons, observed in this study, with lack of constitutive autophagy and POMC/ACTH accumulation in these cells, confirming the role for autophagy in the neurotransmitters modulation, and consequently, in the control of cell size.

In the present study we also observed that autophagy deficiency in POMC neurons caused an accumulation of nematosomes, also designated by stigmoid bodies. These structures were found in 14.1% of POMC cells analyzed from control group (in 35 POMC cells, 6 contained nematosomes) and in 40.7% of POMC cells analyzed from POMC-Cre, $Atg7^{loxP/loxP}$ mice (in 27 POMC cells, 11 contained nematosomes).

The first description of a nematosome-like structure was in 1962 by Rosenbluth (Rosenbluth, 1962), as granular inclusion having no limiting membrane in the cochlear ganglion of rats. In the hypothalamus, these “nucleolus-like bodies” were referenced in 1965 and ‘Nematosome’ name, firstly described as structures typically located in the perinuclear cytoplasm in neurons of rat sympathetic ganglia, was used by several authors (Rosenbluth, 1962; Shimizu, Nobuo, Ishii, 1965; Grillo, 1970; Hinfelang-Gertner, Colette, Stoeckel, Marie-Elisabeth, Porte, Aimé, Dellmann, Horst-Dieter, Madarász, 1974). More recently, the term nematosomes or stigmoid bodies, was used to name nonmembrane-bound cytoplasmic body structures (García-Ovejero et al., 2001; Komatsu et al., 2006).

The nature and physiologic significance of nematosomes are far to be completely known. The presence of nucleic acid, imunoreactivity for huntingtin-associated protein 1 (HAP1), aromatase-associated human placental antigen X-P2 (hPAX-P2) and 5-hydroxytryptamine 7 (5-HT7), androgen and glucocorticoid receptors in the nematosome have been described (Grillo, 1970; Ockleford et al., 1987; Gutekunst et al., 1998; Li et al., 1998; Muneoka and Takigawa, 2003; Fujinaga et al., 2009, 2011). Moreover, nematosome has been described to be involved in protein synthesis and transport (Shinoda et al., 1993; Gustafson et al., 1996). A similar nematosome structure was found in $Atg7^{flox/flox}$; nestin-Cre hypothalamic neurons, and described as circular or elliptical body present in the perikarya, with ubiquitin staining (Komatsu et al., 2006). In addition, loss of autophagy in POMC neurons causes SQSTM1 and ubiquitin accumulation on arcuate nucleus of hypothalamus (Coupé et al., 2012; Quan et al., 2012), indicating a possible commitment of protein degradation for the origin of nematosomes.

Endoplasmic reticulum (ER) and mitochondria are able to sense nutrient fluctuations and integrate the signals, to maintain cell homeostasis (Baltzer et al., 2010; Youle and van der Bliek, 2012), through the establishment of contact sites between both organelles. ER and mitochondria participate in essential bidirectional communication processes (Rowland and Voeltz, 2012), suggesting that a tight coordination between mitochondrial dynamics and interorganelle interactions are crucial to cellular integrity. Regarding this, in the present study we observed that Atg7 deletion in POMC neurons does not alter the mitochondria density neither trans-sectional mitochondria area; however, the Atg7 deletion increased the number of mitochondria-ER contacts. Given that the defective mitochondria-ER contacts may underlie leptin resistance and obesity development (Schneeberger et al., 2013); with the metabolic alterations observed in POMC-Cre, $Atg7^{loxP/loxP}$ mice (Coupé et al., 2012; Quan et al., 2012), the results were unexpected. This may be related to the fact that ER could be critical for autophagosome formation and the dynamic exchange between ER and mitochondria is required for the function of the autophagic machinery (Hamasaki et al., 2013); thus we have the autophagic process blocked, and the accumulation of mitochondria-ER contacts may be a consequence. However, ER stress markers should be performed in order to better clarify whether ER stress could underlie the autophagy impairment and consequently, contributing to leptin resistance observed in this mouse model.

The molecular and cellular processes behind the plasticity and flexibility of neuronal interactions in hypothalamic arcuate nucleus are not fully understood. The present study reveals that autophagy regulates anatomical synaptic organization of POMC neurons of the

hypothalamic arcuate nucleus. Specifically, Atg7 deletion induced significant differences in the wiring of POMC neurons. POMC-Cre, Atg7$^{loxP/loxP}$ mice have more synaptic inputs on POMC neurons than Atg7$^{loxP/loxP}$ mice. These synaptic input changes are associated with an increase of inhibitory inputs on POMC neurons of POMC-Cre, Atg7$^{loxP/loxP}$ mice, favoring an increased inhibitory tone, when compared with the pattern seen in Atg7$^{loxP/loxP}$ mice. This pattern of neural connections predicted the vulnerability to obesity of POMC-Cre, Atg7$^{loxP/loxP}$ mice, once it is associated with a decrease anorexigenic tone, that can be responsible for the increased food intake and body weight gain that mice exhibited (Coupé et al., 2012; Kaushik et al., 2012; Quan et al., 2012). The alteration in the ratio of symmetric to asymmetric inputs onto POMC neurons led us to explore the origin of these projections. POMC cells can be inhibited by direct GABA and NPY release from projections of NPY/AgRP neurons (Horvath et al., 1997; Cowley et al., 2001; Pinto et al., 2004). Since, no differences in the contacts number between these two types of cells were observed, other hypothalamic regions may be responsible for inhibitory innervations of POMC neurons, once POMC neurons also receive inhibitory inputs from ventromedial hypothalamic nucleus and non-NPY labeled cells within the arcuate nucleus (Sternson et al., 2005).

Hypothalamic circuits are able to support the rapid synaptic perikaryal remodeling in response to metabolic changes. During food deprivation, which is characterized by low levels of circulating leptin and high levels of ghrelin, NPY/AgRP neurons are activated and POMC neurons are inhibited (Horvath and Diano, 2004; Horvath, 2005). According to synaptic plasticity of melanocortin system, our data suggest that POMC neurons show no normal adaptation to fasted conditions when Atg7 expression is blocked. After an overnight fasting, Atg7$^{loxP/loxP}$ mouse follow the pattern described above, with an increased POMC tone inhibition. However, loss of autophagy leads to a decrease in the number of inhibitory synapses, under fasting condition. This synaptic input organization might be a manifestation of physiological compensation efforts in an attempt to promote POMC tone under fasting conditions. The abnormal synaptic rearrangement is accompanied by inappropriate response of the wiring of the melanocortin system with a POMC neuron-specific autophagy deficiency. Fasting induced an increase of neuronal c-Fos expression in the arcuate nucleus in Atg7$^{loxP/loxP}$ mice, which is not observed after overnight fasting in POMC-Cre, Atg7$^{loxP/loxP}$ mice. A similar response pattern was described for late-onset obesity animals, where there is a reduced fasting-induced activation of hypothalamic arcuate neurons associated with hyperleptinemia, as well as, less STAT3 phosphorylation after leptin injection in fasted mice (Becskei et al., 2009a, 2009b, 2010). POMC-Cre, Atg7$^{loxP/loxP}$ mice show resistance to leptin and POMC neurons also exhibit an impaired leptin-induced

STAT3 phosphorylatin (Quan et al., 2012). Taking into account that leptin promotes autophagy and obesity down-regulates the autophagy in the hypothalamus (Malik et al., 2011; Meng and Cai, 2011), it would be important to explore whether physiologic effects of leptin, or even, leptin resistance, might be mediated by autophagy regulation.

While autophagy effect on synaptic plasticity of satiety-promoting melanocortin neurons emerge as a potential field to explain feeding behavior and body weight changes triggered by hypothalamus, it would be important to better understand interneuronal signaling. It is already known that in leptin-deficient *ob/ob* mice, more inhibitory synapses are formed on POMC neurons and more excitatory synapses accompanied by fewer inhibitory synapses are presented on NPY neurons The resulting synaptic profile may in part account for the increased food intake in these mice (Pinto et al., 2004). Moreover, the synaptic input organization of POMC neurons on standard diet predicts the vulnerability to weight gain on high fat diet. However, in spite of some intercellular mechanisms begin to be unravel (Dietrich and Horvath, 2013b), the role and plasticity of glutamatergic and GABAergic inputs to POMC and NPY/AgRP neurons is not completely known. Moreover, in AgRP neurons, NMDA receptors reveal to play a critical role in energy balance control, suggesting that that fasting-induced activation of AgRP-releasing neurons is associated with markedly increased glutamatergic input (Liu et al., 2012). Additionally, food deprivation elevates excitatory input in NPY/AgRP neurons, and ghrelin increases glutamate release through the activation of presynaptic receptors, and activate orexigenic neurons through ionotropic glutamate receptors (Yang et al., 2011). However, in POMC neurons, the few studies only report that NMDA receptors do not play a fundamental role on energy balance control. Furthermore, only few reports studied the autophagy effect on synaptic remodeling. In *Caenorhabditis elegans*, endocytosed $GABA_A$ receptors, but not acetylcholine receptors, are targeted to autophagosomes (Rowland et al., 2006); and autophagy promotes synapse growth in *Drosophila melanogaster* (Shen and Ganetzky, 2009). In rat hippocampal neurons, chemical long-term depression induced AMPA receptor degradation through autophagy (Shehata et al., 2012).

Therefore, the role of autophagy effect on neuronal wiring and its impact on pre- and/or postsynaptic sites, as well as, the parent cells of origin of the altered inputs should be further determined. Moreover, it will be interesting to analyze glial cells, because they play a pivotal role in allowing normal activity and have been implicated in the regulation of synaptic plasticity in POMC and NPY/AgRP neurons (Coppola et al., 2007; Mobbs, 2007; Horvath et al., 2010).

In conclusion, our study extends the widespread importance of autophagy as feeding-regulated process acting in the arcuate nucleus to control of energetic balance. This catabolic process plays a critical role on cell morphological features, nematosomes accumulation, mitochondria-ER contacts and synaptic plasticity of POMC neurons, altering the input organization of hypothalamic circuits even in fasted conditions. Therefore, modulating autophagy for control of synaptic plasticity in satiety-promoting melanocortin neurons in the hypothalamus might offer strategies to the body weight regulation, even to develop pharmacological tools against obesity.

CHAPTER 5

Concluding Remarks

5. Concluding remarks

Autophagy impairment is a hallmark of aging, contributing to the aging phenotype and to the aggravation of age-related diseases. Since hypothalamic autophagy decreases with age, strategies to promote autophagy in the hypothalamus may be relevant to control the aging process. In spite of there are no interventions or gene manipulations that stop or reverse the aging process, caloric restriction (CR) is the most robust non-pharmacological anti-aging intervention known to increase maximal lifespan and healthspan from yeast to mammals (Bergamini et al., 2007; Fontana et al., 2010b). CR anti-aging effects are intimately tied to increased autophagy (Donati, 2006) and alterations in the neuroendocrine system, particularly the increase of neuropeptide Y (NPY) in the hypothalamus (Minor et al., 2009). Moreover, the hypothalamic autophagy stimulated by CR is blocked by NPY Y_1, Y_2 and Y_5 selective antagonists, suggesting that endogenous NPY mediates CR-induced autophagy in hypothalamic neurons, as already described by our group (Aveleira and Botelho, et al., 2015).

Therefore in the present work we studied the role of NPY on autophagy in hypothalamic cells. We show, for the first time, that NPY increases autophagic flux in two hypothalamic neuronal *in vitro* models, rat hypothalamic neural cell primary cultures and mouse hypothalamic cell line. Moreover, the *in vivo* experiments show that NPY overexpression in mouse hypothalamic arcuate nucleus, by gene transfer technology, also stimulates autophagy in hypothalamus. Moreover, we observed that in hypothalamic neurons NPY stimulates autophagy by activating NPY Y_1 or Y_5 receptors through PI3K, ERK and PKA signaling pathways. Since NPY increases autophagy as CR does, we may suggest that NPY is a putative CR mimetic candidate, as already proposed by others (Minor et al., 2009; Chiba et al., 2010). Further studies are needed to confirm this hypothesis.

The metabolic regulation provided by the hypothalamus also involves the input organization and plasticity of the hypothalamic arcuate nucleus. However, some synaptic-related proteins, as well as, basal autophagy are decreased in the hypothalamus with aging process (Jiang et al., 2001; Kaushik et al., 2012). Therefore, to understand the role of the autophagy in the link between the synaptic input organization and metabolic phenotype, the input organization on POMC neurons, which Atg7 expression was specifically knockout, was also studied in present work. We observed that the autophagy suppression in anorexigenic POMC neurons alters some neuronal morphometric features and causes accumulation of nematosomes. Moreover, POMC neuron-specific autophagy deficiency present more symmetric, putatively inhibitory synapses than controls; and after an

overnight fasting, the abnormal synaptic rearrangement observed in POMC neurons from Atg7-deficiency mice is accompanied by inappropriate neuronal response of the hypothalamic actuate nucleus. The results suggest that autophagy has a role in synaptic modulation of POMC neurons. Additional studies are needed to understand the mechanisms involved on synaptic changes induced by autophagy.

As autophagy controls the plasticity and the synaptic organization of POMC neurons and it is impaired in aging and in dietary induced obesity (Meng and Cai, 2011; Kaushik et al., 2012), modulation of autophagy could bring beneficial effects in age-associated impairments and metabolic disorders. This possible effect could also involve a synaptic remodeling in hypothalamic circuits, since it was already shown that synaptic alterations occur in aged hypothalamus and in *ob/ob* mouse model (Jiang et al., 2001; Pinto et al., 2004). Moreover, taking into account that NPY levels decrease with age (Higuchi et al., 1988; Gruenewald et al., 1994; Vela et al., 2003), and that the dysfunction of hypothalamic autophagy might contribute to the metabolic deregulation observed with age, such dysfunction may result from decreased NPY levels. Therefore, modulation of NPY levels in the hypothalamus could restore metabolic homeostasis by both autophagy activation and synaptic remodeling (Figure 5.1).

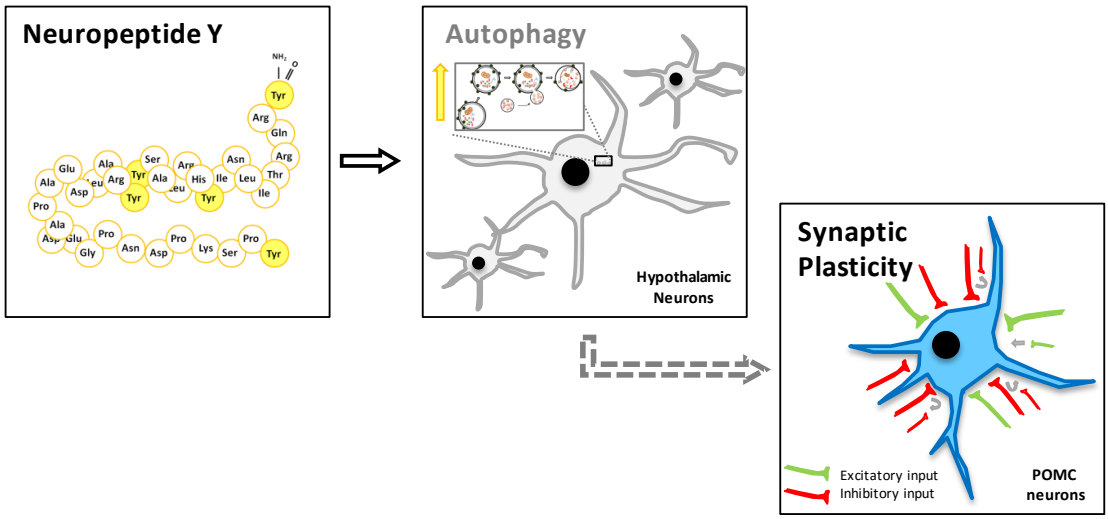

Figure 5.1 - NPY stimulates autophagy in the hypothalamic neurons. Autophgay has a role in synaptic plasticity modulation of hypothalamic POMC neurons. Since hypothalamic NPY levels decrease with aging, the modulation of NPY could rescue age-induced hypothalamic dysfunction, and also restore/improve energy balance homeostasis.

In resume, the **major conclusions** of the present work are:

1) NPY induces autophagy in hypothalamic neurons through NPY Y_1 and Y_5 receptors activation through the concerted actions of PI3K, MEK/ERK, and PKA signaling pathways;
2) Specific inhibition of autophagy in POMC neurons increases soma area and perimeter, and induces nematosomes accumulation in these cells;
3) Specific inhibition of autophagy alters the synaptic input organization of hypothalamic POMC neurons, and impairs the neuronal activation in the hypothalamic arcuate nucleus.

CHAPTER 6

References

6. References

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