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Contents

List of abbreviations

AC	Adenylate cyclase
AD	Alzheimer disease
AHP	After hyperpolarizing potential
AMP	Adenosine monophosphate
AMPK	AMP-dependent protein kinase
ANOVA	Analysis of variance
AP-P	Aminopeptidase P
ARC	Arcuate nucleus of hypothalamus
ATG	Autophagy-related genes
ATP	Adenosine triphosphate
AV	Autophagic vacuoles
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CALERIE	Comprehensive assessment of long term effects of reducing caloric intake
CaMKK	Calmodulin-dependent kinase kinase
CAMKK-β	Calmodulin-dependent kinase beta
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CMA	Chaperone mediated-autophagy
CNS	Central nervous system
COX-2	Cyclooxigenase-2
CPE	Carboxypeptidase E
CPON	C-Terminal flanking peptide of NPY
CR	Caloric restriction
DAG	Diacylglicerol
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DPP-IV	Dipeptidyl-peptidase-IV
ECF	Enhanced chemifluorescence
ER	Endoplasmatic reticulum
ERK	Extracellular signal-regulated kinases
eNOS	Endothelial nitric oxide synthase
Epac	Exchange protein activated by cAMP
FBS	Fetal bovine serum
GAP	GTPase activating protein
GAIP	G-protein subunit α(Gα)-interacting protein
GDP	Guanosine diphosphate
GFAP	Glial fibrillary acidic protein
GH	Growth hormone
GHS-R	Growth hormone secretagogue receptor
GPCR	G-coupled protein receptor
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase

HCl	Hydrogen chloride
HD	Huntington disease
HIF	Hypoxa-inducible factor
H_2O_2	Hydrogen peroxide
IGF-1	Insulin like growth factor-1
IgG	Immunoglobulin G
IL-1β	Interleukin-1 beta
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IM	Isolation membrane
IMPase	Inositol monophosphatase
iNOS	Inductible nitric oxide synthase
IP_3	Inositol triphosphates
IRS	Insulin receptor substrates
LAMP-2A	Lysosomal-associated membrane protein 2
LC-3B	Light chain-3 B
LepR	Leptin receptors
LKB-1	Liver kinase B-1
MAPK	Mitogen-activated protein kinase
MAP4K2	Mitogen-activated protein kinase kinase kinase kinase 2
MAP2	Microtubule-associated protein 2
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rampamycin
mTORC	mTOR complex
NAD	Nicotinamide adenine dinucleotide
NaF	Sodium fluoride
NeuN	Neuronal nucleic protein
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
NPC	Neural progenitor cells
NPY	Neuropeptide Y
NPY-ir	NPY-immunoreactivity
OD	Optical density
ORTO	Sodium orthovanadate
PAM	Peptidylglycine α-amidating monoxygenase
PAS	Phagophore assembly site
PBS	Phosphate buffered saline
PC	Prohormone convertase
PD	Parkinson disease
PDK1	Phosphotidylinositide-dependent protein kinase 1
PE	Phosphatidylethanolamine
PFC	Prefrontal cortex
PGC-1α	Peroxisome proliferative-activated receptor-gamma coactivator-1alpha
PIP2	Phosphatidylinositol 4,5-diphosphate

PI_3K	Phosphatidylinositol-3-kinase
PI(3)P	Phosphatidylinositol-3-phosphate
$PI(3,4,5)P_3$	Phosphatidylinositide-3,4,5-triphosphate
$PI(4,5)P_2$	Phosphatidylinositide-4,5-biphosphate
PI-PLC	Phosphatidylinositol-specific phospholipase C
PKA	Protein kinase A
PKB	Protein kinase B
PLC-ε	Phospholipase C-ε
PMSF	Phenylmethylsulfonyl fluoride
PP	Pancreatic polypeptide
PP-fold	Pancreatic polypeptide fold
PPAR-γ	Peroxisome proliferative-activated receptor-gamma
Pre-Pro-NPY	Pre-pro-neuropeptide Y
Pro-NPY	Pro-neuropeptide Y
PTEN	Phosphatase and tensin homologue
PVDF	Polyvinylidene fluoride
PVN	Paraventricular nucleus
PYY	Peptide YY
p62/SQSTM1	p62/Sequestosome1
qRT-PCR	Quanitative real time polymerase chain reaction
REDD	Regulated in development on DNA damage
RIPA	Radio-immunoprecipitation assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Ser3	Serine residue
SIRT	Sirtuin
SOD	Super oxide dismutase
TBS	Tris-buffered saline
TBS-T	Tris-Buffered Saline and Tween 20
TUJ1	Neuron-specific class III beta-tubulin
TMs	Transmembrane domains
TNF-α	Tumor necrosis factor alpha
TNF-β	Tumor necrosis factor beta
TSC	Tuberous sclerosis complex
ULK	Unc-51 like kinase
UPS	Ubiquitin-proteasome system

Abstract

Aging is determined by complexes and multifactorial processes, whose molecular basis remains poorly understood. The brain is the most affected organ by the aging process, and neurodegenerative diseases are directly related with the increased age. Since average human life expectancy has increased, and consequently, the prevalence of cognitive decline and dementia, aging research is now focused in finding strategies that increase both lifespan and healthspan.

Caloric restriction (CR) is one of the few non-pharmacological manipulations that has been reported to consistently extend lifespan of different organisms. Increasing evidence shows that autophagy, a highly regulated intracellular process involved in the turnover of most cellular constituents and in the maintenance of cellular homeostasis, is involved in anti-aging mechanisms of CR. Furthermore, the anti-aging effects induced by CR were related to changes in the neuroendocrine system, namely the increase of neuropeptide Y (NPY), a potent neuroprotective agent in several brain areas, in the hypothalamic arcuate nucleus (ARC). Evidence suggests that NPY might play an important role in CR anti-aging effects, and in our group was found that NPY not only induces autophagy in hypothalamic neurons, but also mediates nutrient deprivation-induced autophagy in these cells, suggesting that NPY might mediate CR's effects on autophagy. However, the effect of NPY in autophagy in the whole brain, namely in the cortical neurons, as its potential role in lifespan extension, is currently not known. CR is also known to increase the levels of ghrelin, an orexigenic peptide secreted by the stomach. Ghrelin, similarly to NPY, shares some physiological effects induced by CR, possibly acting as an important metabolic mediator in its anti-aging effects. However, the effect of ghrelin in nutrient deprivation induced autophagy has not been studied yet.

The first goal of this study was to evaluate the effect of CR on autophagy in rat cortical neurons, scrutinizing the involvement of NPY and its receptors in this process, as the signaling pathways involved. With this study it was also intended to evaluate the effect of NPY on the regulation of autophagy in cortical neurons, investigating which NPY receptors subtypes and signaling pathways are involved in NPY-induced autophagy. It was also investigated the potential involvement of ghrelin and its receptor on CR-induced autophagy, as well as ghrelin's effect in the modulation of autophagy in cortical neurons.

This study showed that nutrient deprivation induces autophagy in rat cortical neurons. Upon nutrient deprivation it was observed an autophagy induction in a time dependent manner, as proved by the enhancement of the autophagic flux, using chloroquine, a lysosomal degradation inhibitor. The autophagy induction was mediated by NPY Y_1, Y_2 and Y_5 receptors, and involves different signaling pathways. Concomitant with autophagy activation, NPY levels

content increased when cortical neurons undergo nutrient deprivation, supporting the involvement of NPY in nutrient deprivation-induced autophagy.

We next evaluated the effect of NPY *per se* on autophagy regulation in cortical neurons. NPY, similarly to nutrient deprivation, increased autophagic flux in a time dependent manner, and this effect was mediated by NPY Y_1, Y_2 and Y_5 activation.

Besides NPY, CR also increases the levels of ghrelin, an orexigenic peptide secreted by the stomach, which has been suggested to act as a metabolic regulator signal during CR. Therefore we investigated whether ghrelin, similarly to NPY, could be involved in nutrient deprivation-induced autophagy in cortical neurons. Ghrelin not only induces autophagy in cortical neurons but also contributes to the increase in the autophagic flux induced by nutrient deprivation.

This study showed, for the first time, that NPY and ghrelin, both considered caloric restriction mimetics, enhance autophagy in cortical neurons. Furthermore, in response to low nutrient availability, these neurons express NPY and ghrelin. Given that autophagy impairment underlies aging and age-related neurodegenerative diseases, NPY and ghrelin synergistic effect on the regulation of autophagy can be considered a stepping stone for the development of new strategies to delay the aging process and promote healthy lifespan.

Keywords: Aging, Caloric restriction, Autophagy, Neuropeptide Y, Ghrelin, Cortex

Resumo

O envelhecimento é um processo complexo e multifatorial, cujos mecanismos moleculares permanecem ainda desconhecidos. O cérebro é o órgão mais afetado pelo processo de envelhecimento, estando as doenças neurodegenerativas diretamente relacionadas com o aumento da idade. O aumento da esperança média de vida, e o consequente aumento da prevalência de declíneo cognitivo, impulsionou a investigação na área do envelhecimento, a fim de desenvolver estratégias que contribuam para o aumento da esperança de vida com qualidade.

A restrição calórica é uma das estratégias não farmacológicas que promove o aumento da esperança de vida em diferentes organismos. A autofagia, um processo intracelular altamente regulado, envolvido na reciclagem de constituintes celulares e na manutenção da homeostase celular, está envolvida nos mecanismos anti-envelhecimento da restrição calórica. Além disso, os efeitos anti-envelhecimento induzidos pela restrição calórica foram relacionados com alterações no sistema neuroendócrino, nomeadamente o aumento do neuropeptídeo Y (NPY) no núcleo arqueado do hipotálamo. O NPY é um dos peptídeos mais abundantes no sistema nervoso central, tendo um efeito neuroprotector em diversas áreas cerebrais. Estudos sugerem que o NPY pode ser um mediador dos efeitos anti-envelhecimento promovidos pela restrição calórica; no nosso grupo demonstrámos que o NPY não só induz autofagia em neurónios hipotalâmicos, como também medeia a autofagia induzida pela privação de nutrientes, sugerindo que este neuropeptídeo possa estar envolvido nos efeitos benéficos induzidos pela restrição calórica. No entanto, o efeito do NPY na autofagia em outras regiões cerebrais, nomeadamente no córtex, bem como o seu potencial papel no aumento da esperança de vida, é desconhecido. Um outro efeito neuroendócrino promovido pela restrição calórica é o aumento dos níveis de grelina, um peptídeo orexigénico secretado pelo estômago. A grelina, de forma similar ao NPY, mimetiza muitos dos efeitos induzidos pela restrição calórica, atuando possivelmente como um importante mediador metabólico nos seus efeitos anti-envelhecimento. Contudo, o efeito da grelina na autofagia induzida pela restrição calórica ainda não foi investigado.

Neste estudo avaliou-se o efeito da privação de nutrientes na autofagia em neurónios corticais de rato bem como o possível envolvimento do NPY e seus recetores neste processo. Neste trabalho também se estudou o efeito do NPY *per se* na regulação da autofagia e as vias de sinalização envolvidas. O potencial envolvimento da grelina e do seu recetor na autofagia induzida por restrição calórica, tal como, o efeito da grelina na modulação da autofagia em neurónios corticais também foi avaliado.

Este estudo mostrou que a privação de nutrientes induz autofagia em neurónios corticais, de uma forma dependente do tempo de exposição. A indução da autofagia foi mediada pelos recetores Y_1, Y_2 e Y_5 do NPY, envolvendo diferentes vias de sinalização. Paralelamente à promoção da autofagia, a privação de nutrientes induziu um aumento dos níveis de NPY em neurónios corticais, reforçando o envolvimento do NPY na autofagia mediada por privação de nutrientes.

Posteriormente, avaliou-se o efeito do NPY *per se* na regulação da autofagia em neurónios corticais. À semelhança da privação de nutrientes, o NPY também aumentou o fluxo autofágico, de uma forma dependente do tempo de exposição, sendo este efeito mediado pela activação dos recetores Y_1, Y_2 e Y_5 do NPY.

Para além do NPY, a restrição calórica também aumenta os níveis de grelina. Deste modo, estudou-se o envolvimento deste peptídeo na indução da autofagia mediada por privação de nutrientes. A grelina não só induziu a autofagia em neurónios corticais, como também contribuiu para o aumento do fluxo autofágico induzido pela privação de nutrientes.

Este estudo mostrou, pela primeira vez, que o NPY e a grelina, considerados miméticos da restrição calórica, promovem a autofagia em neurónios corticais. Adicionalmente, em resposta a uma baixa disponibilidade de nutrientes, estes neurónios expressam NPY e grelina. Tendo em conta que os processos autofágicos estão comprometidos no envelhecimento e nas doenças neurodegenerativas associadas à idade, o efeito sinergístico do NPY e da grelina na regulação da autofagia pode ser considerado um ponto de partida para o desenvolvimento de novas estratégias para retardar o envelhecimento e aumentar a longevidade aliada a uma melhor qualidade de vida.

Palavras-chave: Envelhecimento, Restrição Calórica, Autofagia, Neuropeptideo Y, Grelina, Cortex

Chapter I

Introduction

1.1. Aging

Population aging is a process that has been observed in most developed countries over the last century (Kirkwood 2008). In 2050, it is predicted that more than two billion people will be over the age of 60 (United Nations) (Figure 1.1).

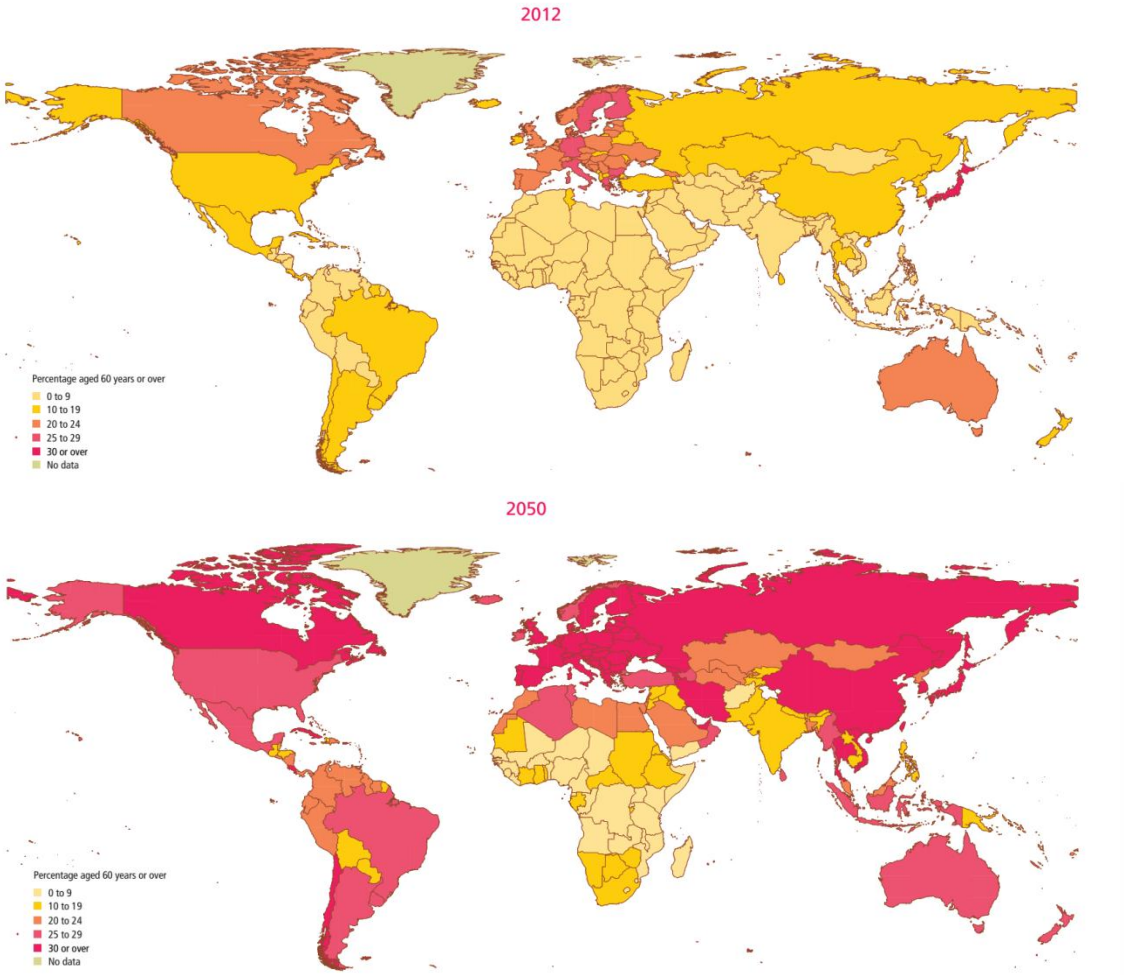

Figure 1.1 - Percentage of the total population aged 60 years or over, from 2012 to 2050. Data from (United Nations, 2012).

The increase in life expectancy can be seen as a result of the success of public health policy and socioeconomic development; however, it also challenges the society to adapt and promote better health and functional capacity of the elderly people, and to understand the biological bases (i.e., processes and their mechanisms) of aging, as well as, morphological and molecular aspects underlying various age-related diseases (Rezzani *et al.*, 2012). Aging is characterized by the progressive and irreversible loss of physiological integrity and is an extremely complex process whose molecular basis remains incompletely understood (Kirkwood 2005, Tripathi 2012). In addition, aging is the major risk factor for all of the predominant killer diseases, including cardiovascular disease, cancer and neurodegenerative diseases, and the main burden of ill health is now falling on the older section of the population

(Partridge *et al.*, 2011). In this context, the study of aging has become one of the most important challenges to modern science. Some theories have been proposed to explain aging in order to identify the major causes of aging and physiological changes that are associated with increasing age (Holliday 2006). However, the contribution of each theory to identify a primary cause of aging has been difficult to establish. Aging, is, therefore, 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).

1.1.1. The aging brain

The effects of aging on the brain and cognition are widespread and have multiple etiologies, exerting on the molecules, cells, vasculature, gross morphology, and cognition. As we age our brains shrink in volume, particularly in the prefrontal cortex (PFC) (Drachman 2006). Brody, in 1955, was the first to suggest that age-related reductions in brain weight were due, in part, to a decline in neuron number in all cortical layers (Brody 1955). Afterward, his work was corroborated by Coleman's studies, that described a 10–60 % decline in cortical neuron density between late childhood and old age (Coleman *et al.*, 1987). In addition to these facts, profound cell loss was found in the hippocampus of aging humans (Ball 1977) and in the hippocampus and PFC of non-human primates (Brizee *et al.*, 1980). However, various technical and methodological issues, such as tissue processing and sampling design, confounded the data obtained from these early reports and their accuracy was called into question later (Morrison *et al.*, 1997). In the 1980s, it became possible to identify and eliminate many of the confounding factors of the previous studies that had indicated a profound decline in neuron number occurring in advanced age, due to developing new stereological principles (West 1993). Studies in humans (West *et al.*, 1994, Pakkenberg *et al.*, 1997), non-human primates (Peters *et al.,* 1994, Gazzaley *et al.,* 1997, Merrill *et al.,* 2000) and rodents (Rapp *et al.*, 1996, Rasmussen *et al.*, 1996, Merrill *et al.*, 2001) have shown that significant cell death in the hippocampus and neocortex is not characteristic of normal aging. Nevertheless, a notable exception to this idea has recently been demonstrated. In aged non-human primates, there is a ~30 % reduction in neuron number in all layers in area 8A of the dorsolateral PFC, which it is significantly correlated with impaired performance on a working memory task, whereas 46 % of the PFC shows conservation of neuron number (Smith *et al.*, 2004). In addition to early reports of a decline in neuronal density with aging, early studies on neuronal dendritic branching suggested massive deterioration in the human entorhinal cortex and hippocampus (Scheibel *et al.*, 1976, Scheibel *et al.*, 1979). The morphology of PFC neurons seems to be more

vulnerable to the effects of aging than hippocampal neurons. In rats, dendritic branching of pyramidal neurons decreases with age for both apical and basal dendrites in superficial cortical layers (Grill *et al.*, 2002). A reduction in dendritic branching with age has also been observed in anterior cingulate layer V of the rat (Markham *et al.*, 2002) and in the human medial PFC (de Brandander *et al.*, 1998, Uylings *et al.*, 2002).

Many of the electrophysiological properties of neurons in the PFC remain unaltered during normal aging, including resting membrane potential; membrane time constant; threshold to elicit an action potential; and rise time and duration of an action potential (Chang *et al.*, 2005). There is some evidence of a small increase in the input resistance in PFC neurons of aged monkeys as well as a decrease in the amplitude and fall time of action potentials (Chang *et al.,* 2005). However, cognitive performance is not related to action potential amplitude, action potential fall time or input resistance (Chang *et al.*, 2005). Neurons in the PFC of aged monkeys also have a significantly larger after hyperpolarizing potential (AHP) compared with young neurons, which suggests that Ca^{2+} homeostasis might also be disrupted in PFC neurons in advanced age (Chang *et al.*, 2005).

1.1.2. Molecular mechanisms underlying the brain aging

As mentioned above, the brain is the most affected organ by the aging process. Aging is determined by a multifactorial and complex processes whose molecular basis remains poorly understood (Kirkwood 2005); however several mechanisms have been proposed to explain the aging process such as increased amount of mitochondrial dysfunction (Ames 2004), oxidative stress (Serrano *et al.*, 2004, Zecca *et al.*, 2004), and accumulation of damaged proteins (Gray 2003, Trojanonski 2003).

The decreased of mitochondrial function has often been associated with aging in general, and aging of the central nervous system (CNS), in particular (Melov 2004). Many studies of genes expression profiling have clearly shown a progressive degeneration in mitochondrial function, which could contribute to the accelerated aging, particularly in brain and muscle, since both are more susceptible to mitochondrial dysfunction. Mitochondrial oxidative phosphorylation is the key source of energy intensive ion fluxes and axonal transport in the projection neurons of cerebral cortex, which degenerate in most neurodegenerative diseases (Yankner *et al.*, 2008). These neurons, therefore, are highly vulnerable to mitochondrial dysfunction (Yankner *et al.*, 2008).

Due to irregularities in the electron transport chain in mitochondria during progressive aging, many super oxides are generated as a byproduct, which may cause damage to respiratory chain proteins (Wallace 2005). In normal course, mitochondria possesses sufficient

machinery to counter these reactive oxygen species (ROS) in form of antioxidant enzymes, including Cu-Zn super oxide dismutase (SOD), cytochrome oxidase, and redox reactions mediated by cytochrome C (Yankner *et al.*, 2008). In aging, the action of these antioxidants is diminished, resulting in local oxidative damage to mitochondrial proteins and deoxyribonucleic acid (DNA). SOD reacts with superoxide radicals and converts them in to hydrogen peroxide (H_2O_2), which is a stable molecule and may diffuse into cytoplasm where it is enzymatically neutralized by cytoplasmatic glutathione peroxidase and peroxisomal catalase. In addition to the generation of superoxide and hydrogen peroxide, the availability of redox-active iron is a major determinant of ROS-mediated cellular damage (Yankner *et al.*, 2008).

The accumulation of damaged molecules within the cells is one of the most widely documented and obvious alterations that occur in neurons during aging (Mattson *et al.*, 2006). It is known that the lifespan is determined by the balance between metabolism, which leads to the accumulation of damage, thus causing aging, and compensatory responses (Rattan 2006). The changes are due to the accumulation of oxidized, misfolded, cross-linked or aggregated macromolecules that are morphologically not normal and so, they cannot properly function (Rezzani *et al.*, 2012). 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, Keller *et al.*, 2002, Nixon *et al.*, 2003). Moreover, it is possible that these macromolecules interfere with other molecules and organelles, or their aggregates, compromising cellular functions sending also erroneous signals. Thus, the cells need to eliminate them for survival. It is also known that cellular damage is associated to aging-related pathologies, including cancer, neurodegeneration, infection and muscle atrophy (Guarente 2008, Kirkwood 2008).

Understanding the biological bases of aging and the molecular mechanisms that underlie various age-related pathologies is utmost important to future genetic and pharmacological interventions to increase lifespan with life quality (Rajawat *et al.*, 2009, Troen 2003).

1.1.3. Delay brain aging

Life expectancy is increasing as a result of advances in medical science and the availability of better healthcare services; however, the proportion of elderly persons in the general population is therefore rising. A major goal of aging research is to extend healthspan by identifying approaches for delaying or preventing age-related diseases. Many are the preventive measures of cognitive impairments and neurodegenerative processes that are associated with aging (Tripathi 2012). Brain healthy 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, have been primarily reported to delay the effects of normal brain aging and cognitive decline (Tripathi 2012). Recent investigation on the impact of caloric restriction (CR) on brain aging and neurodegenerative disorders, has shown many striking features (Tripathi 2012). Recently, a large group of monkeys, ranging in age from middle-aged adults to the quite elderly, were fed only 70 % of their free-feeding diet for about 15 years (Bendlin *et al.*, 2012). As a result of eating just 30 % fewer calories, the brains of the monkeys on the CR diet aged significantly more slowly. Although several brains regions showed benefits, those brains regions that evolved most recently, such as the frontal lobes, and therefore tend to be more vulnerable to the consequences of aging, showed the greatest beneficial response to CR (Bendlin *et al.*, 2012).

In addition, regular physical exercise has been shown to increase neurogenesis (McCarter 1995, van Praag *et al.*, 1999). Hence, management of these conditions through medical and life style interventions is likely to benefit in order to cope with these age related impairments. Associated to increasing neurogenesis, exercise improves spatial memory in rodents (Fordyce *et al.*, 1993, van Praag *et al.*, 1999, Anderson *et al.*, 2000) and it has been suggested that exercise-induced hippocampal neurogenesis contributes to the learning enhancement (van Praag *et al.*, 1999).

1.2. Caloric Restriction

Caloric restriction (CR) consists of a reduced food intake without malnutrition, retaining the essential nutrients, extending and ensuring cellular function (Koubova *et al.*, 2003, Dirks *et al.*, 2006), by reduction of food intake to 30–60 % below ad libitum intake levels in relation to typical feeding (Masoro 2006). It is the form of nutritional intervention, non-pharmacological, more widely discussed, in order to increase the average life expectancy of a wide variety of species, from yeast to mammals including humans (Masoro *et al.*, 2006, Roberts *et al.*, 2007). In fact, some studies show that CR is the most robust anti-aging intervention (Bergamini *et al.*, 2007, Fontana *et al.*, 2010).

Since the pioneer work of McCay in 1935, which showed that the reduction of energy intake without lack of essential nutrients is capable of increasing the longevity in rats (McCay *et al.*, 1935), several studies suggest that CR could extend the longevity and have beneficial effects on health of various organism models. This discovery opened a new door to several scientific studies designed to learn more about the biology of aging. Subsequent studies have concluded that long-term CR slows the process of aging and extends the maximum life expectancy in different animals including nematelmintes, fruit flies and rodents (Braeckman *et*

al., 2006, Roberts *et al.*, 2007, Burger *et al.*, 2010). In addition of living longer, these animals kept under CR, remained healthy and active longer, even after the death of the control group (Braeckman *et al.*, 2006, Roberts *et al.*, 2007, Burger *et al.*, 2010), demonstrating that CR reduces the morbidity of a host of diseases, including (but not limiting) autoimmune diseases, atherosclerosis, cardiomyopathies, cancer, diabetes, renal diseases, neurodegenerative diseases, and respiratory diseases (Imai 2009, Vaquero *et al.*, 2009). Then becomes necessary to discern the mechanisms underlying CR's beneficial effects.

1.2.1. Physiological effects of caloric restriction

CR has been recognized as the best characterized and most reproducible strategy for extending maximum survival, retarding physiological aging, and delaying the onset of age-related pathological situations (Speakman *et al.*, 2011, Vendelbo *et al.,* 2011). There are several common changes in CR rodent models, such as decreased body temperature, decreased insulin secretion, lower blood glucose levels, decreased growth hormone and IGF-1 (insulin-like growth factor 1) secretion (Koubova *et al.*, 2003). Concomitantly, CR is known to decrease growth, when applied to young animals (Masoro 2005), as well as fertility and reproduction-related hormones (Nalam *et al.*, 2008, Barzilai *et al.*, 2009). Overall, CR has been shown to improve longevity and resistance to stress in animal models, but, though some epidemiological data exists, its long term potential benefits in human, except for weight loss purposes, are yet unproven and contested (Smith *et al.*, 2010). Increasing evidences supports, the notion that CR reduces diseases-related mortality by staving off cardiovascular disease, malignancy, diabetes and neurodegeneration (Calabrese *et al.*, 2009).

Currently, there are also two active randomized non-human primate studies, to assess the benefits of CR on longevity in rhesus monkeys (*Macaca mulatta*) (Smith *et al.*, 2010). In these studies, CR effectively lowered incidence of neoplasia, cardiovascular disease and type 2 diabetes and lowered body weight in both studies (Mattison *et al.*, 2012). In humans is difficult to definitively answer whether or not CR prolongs life, because of the ethical and logistical limitations of research design. The majority of work has been conducted on healthy, middle age, non-obese (normal or overweight) men and women, with a significant amount of investigations utilizing data obtained from Comprehensive Assessment of Long Term Effects of Reducing Caloric Intake (CALERIE) program (Trepanowski *et al.*, 2011); this program investigates the adaptive responses of CR on free-living humans (Holloszy *et al.*, 2007). In addition to the CALERIE program, another investigation of importance, Biosphere 2, contains subjects aged from 27-67 years (Walford *et al.*, 2002). These subjects lived in enclosed about 3 years "ecological miniworld" for 2 years (Hollosky *et al.*, 2007). Food intake was subnormal due

to the inability to grow enough food for ad libitum consumption; hence, subjects followed a CR plan by default. Being these long-term beneficial effects eventually proven, the successful application of CR to humans for long periods of time would be unlikely to the majority of the population, due to the severity of the restriction required. This fact led to the interest in developing CR mimetics, which could provide the pro-longevity benefits without the actual restriction (Ingram *et al.*, 2006).

1.2.2. Mechanisms underlying 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. Here in we will summarize our current understanding, including recent findings that implicate specific enzymes and signaling pathways in the process.

1.2.2.1. Mitochondrial biogenesis

Recently, the effect of CR on mitochondrial biogenesis has been questioned, since the relationship between the decreases in metabolic rate in order to reduce oxidative stress is not well understood. Apparently, this mechanism may be contradictory, and several studies show that CR promotes the increase of the mitochondria number and activity (Speakman *et al.,* 2011). The peroxisome proliferator-activated receptor gamma coactivator-1alpha (PGC-1α) regulates genes involved in energy metabolism. This protein interacts with the peroxisome proliferator-activated receptor-gamma (PPAR-γ) allowing interaction of this protein with multiple transcription factors. The complex PGC-1α/PPAR-γ was widely described as a mediator of mitochondrial biogenesis (Song *et al.,* 2009, Manzanero *et al.,* 2011). Studies have concluded that CR does not reduce the metabolic rate, as previously thought, but actually increases it, and this increase is responsible for its buffering effect on oxidative stress.

1.2.2.2. Oxidative stress

ROS are formed under physiological conditions and in proportions controlled by cellular defense mechanisms. These chemical structures have an unpaired electron, making them unstable and with an enormous capacity to react to nonspecifically combine with numerous molecules, that comprise the cell structures and derivatives of each, leading to oxidation of proteins, lipids and DNA (Speakman *et al.,* 2011). These oxidative damages are strongly related to aging and the pathogenesis of several neurodegenerative diseases such as those mentioned above. And thus, reducing the metabolic rate following a CR system could decrease the

consumption of O_2, resulting in decreased formation of ROS and probably increased longevity (Heilbronn *et al.*, 2003). The mechanism by which CR decreases the formation of ROS is still unclear, but many are the inferences that one can already point out. In accordance with this, it is thought that CR reduces the energy flow and consequently lower levels of ROS, as well as, the rate of oxidative damage to vital tissues, thus promoting a reduction in energy metabolism in basal metabolic rate and body temperature central (Redman *et al.*, 2008). The brain is particularly susceptible to oxidative stress due to increased mitochondrial activity (Manzanero *et al.*, 2011). Furthermore, abundance of lipids in the CNS makes it a major target of this oxidative damage and plays an important role in numerous neurodegenerative diseases, as the damaged molecules tend to accumulate in long-lived, post-mitotic neurons, providing a connection between age and oxidative stress in the brain (Manzanero *et al.*, 2011, Speakman *et al.*, 2011).

1.2.2.3. Sirtuins

Sirtuins (SIRT), SIRT1 to SIRT7, are NAD^+ dependent deacetylase that appear to regulate activity of many proteins that are related to energy metabolism, cell survival and longevity (Michan *et al.,* 2007). Studies have shown that there is a correlation between the increased content of these enzymes in different tissues and nutritional status, since most of the cellular functions are depend on NAD^+ (Maalouf *et al.,* 2009, Thu *et al.,* 2010). One potential mechanism that might mediate the beneficial effects of CR on brain health is the activation of the nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylase SIRT1 (Bishop *et al.*, 2007, Lavu *et al.*, 2008).

1.2.2.4. Neurotrophic factors and neurogenesis

Recently, studies have shown that the levels of certain neurotrophic factors, especially brain-derived neurotrophic factor (BDNF), increases in areas of the hippocampus and cortex of rats kept under CR (Lee *et al.*, 2000). BDNF and other neurotrophic factors are known to protect neurons against excitotoxic, oxidative and metabolic insults in various experimental models of neurodegenerative diseases (Mattson *et al.*, 1997). During the last decade, several researchers have described the brains of adult mammals, proving the existence of populations of neural progenitor cells (NPC). This premise has come to reinforce the idea that neurogenesis does not occur only in brain development and that there is a continuous process throughout life (Kuhn *et al.*, 1996). In fact, new-born neurons are functionally integrated in the brain throughout the adult life, constituting an adaptive process to challenges imposed by physiological and/or environmental alterations, and not merely with restorative functions

(Lledo *et al.*, 2006). In the CNS, aging results in a precipitous decline in adult neural stem/progenitor cells and neurogenesis, with concomitant impairments in cognitive functions (van Praag *et al.*, 1999). However, it was shown that CR increases the number of neural cells newly formed in the dentate gyrus of the hippocampus of mice (Gomez-Pinilla 2008). In this study, additional data also indicated a higher production of BDNF, other beneficial effect of CR on the NPC. In addition, oxidative stress decreases BDNF levels and there by adversely affects synaptic plasticity (Gomez-Pinilla 2008). On the other hand, CR decreases oxidative stress, leading to increased regulation of BDNF levels in the hippocampus, which is a key element of neurogenesis in the hippocampus, resulting in increased synaptic plasticity, making the process of learning and memory (Park *et al.*, 2011). However, the functional consequences of CR on neurogenesis are yet to be discern.

1.2.2.5. Metabolic reprogramming

The inverse linear relationship between calorie intake and lifespan suggests that regulators of energy metabolism are important in the actions of CR. Studies in several tissue-specific show changes in energy metabolism with CR, suggested that the metabolic reprogramming plays a critical role in the mechanism of aging retardation and promotes health and longevity (Anderson *et al.*, 2010). Other studies found that CR involves a coordinated increase in the expression of genes that are involved in energy metabolism. These changes have been reported in several tissues such as skeletal muscle, white adipose tissue and heart. Furthermore these changes suggest an increase in mithochondrial biogenesis and that resting energy metabolism at the organ level may be increased under CR (Anderson et al., 2010). Moreover, these alterations are directly correlated with the manner in which energy is generated and how it is spent. A key metabolic change during CR is a shift from carbohydrate metabolism to fat metabolism (Cao *et al.*, 2001, Chen *et al.*, 2008). How this shift occurs is interesting because overall substrate oxidation must balance intake plus synthesis. Animals on CR have lower intake of all calories, including those from fat, this implies that to elevate their overall fatty acid oxidation, they must use some of the ingested energy to synthesize fat, which can then be mobilized (Duffy *et al.*, 1989, Masoro *et al.*, 1992).

1.2.2.6. Inflammation

Aging and several neurodegenerative diseases that are related to it are characterized by high levels of various inflammatory mediators (Qiu *et al.*, 2010). The activation of nuclear factor kappa B (NF-κB) is the central component of the inflammatory process (Maalouf *et al.*, 2009). The NF-κB is composed of several regulatory subunits (Perkins 2007), which mediate

neuroprotection and the process of apoptosis, depending on the function of the subunit (Kaltschmidt *et al.*, 2005). Thus, activation of this component, triggered by ROS for example, promotes increased transcription interleukin-1 beta (IL-1β), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), tumor necrosis factor-beta (TNF-β), pro-inflammatory enzymes such as cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) in several tissues, including brain (Valerio *et al.*, 2006). Studies indicate that CR suppresses the expression and activation of NF-κB, a SIRT1-dependent process blocking the synthesis of interleukins and TNF-α and TNF-β and suppressing the activity of COX-2 and iNOS both in animal models and humans, diminishing the state of systemic inflammation and chronic systemic diseases associated to non-infectious inflammation (Maalouf *et al.*, 2009, Manzanero *et al.*, 2011).

1.2.2.7. Autophagy

Further evidence demonstrates that CR as a dietary anti-aging intervention stimulates autophagy, a highly regulated intracellular process involved in the turnover of most cellular constituents and in the maintenance of cellular homeostasis in several species (Bergamini *et al.*, 2007, Hansen *et al.*, 2008, Blagosklonny 2010). Increasing evidence show that autophagy is involved in anti-aging mechanisms of CR (Donati 2006). 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 (Marino *et al.*, 2008). Autophagy deficiency contributes to different aspects of the aging phenotype and to the aggravation of detrimental age-related diseases (Cuervo 2008), such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) (Contestabile *et al.*, 2004). Thus, CR prevents the age-depend decline of autophagic proteolysis (Bergamini *et al.*, 2007).

1.2.2.8. Neuroendocrine alterations

The major neuroendocrine effect of CR is the increase of neuropeptide Y (NPY), in the arcuate nucleus of the hypothalamus (ARC) (Minor *et al.*, 2009). The hypothalamus has a key role in the control of body homeostasis, neuroendocrine outputs and food intake, being NPY the most potent hypothalamic orexigenic peptide (Wieland *et al.*, 2000). Aging is associated with reduced levels of NPY in the brain in general and in response to fasting (Gruenewald *et al.*, 1996). In addition, reduced NPY is associated with neurodegenerative diseases (Rose *et al.*, 2009, Decressac *et al.*, 2010, Decressac *et al.*, 2012) and the development of “anorexia of aging”, characterized by reduced food intake and body weight, responsible for age-associated under nutrition and physical deterioration (Matsumoto *et al.*, 2000, Morley *et al.*, 2001). On

the other hand, 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). These evidences suggest that NPY may play an important role in CR-induced lifespan, and could be a CR mimetic. On other hand, CR also increases the circulating levels of ghrelin, other orexigenic peptide that is produced by the stomach in response to fasting (Lutter *et al.*, 2008). During CR, ghrelin levels rise and recent studies show that the actions of ghrelin are elevated during negative energy balance, as ghrelin primarily functions to shift an organism from negative to neutral energy balance (Briggs *et al.*, 2011). In addition to promoting food intake after fasting (Salome *et al.*, 2009), ghrelin helps to maintain blood glucose levels during CR (Zhao *et al.,* 2010). On the other hand, diet-induced obesity suppresses many of the metabolic actions of ghrelin, including food intake and growth hormone secretion (Perreault *et al.,* 2004, Briggs *et al.,* 2010). These studies imply that metabolic status plays a fundamental role in the effectiveness and actions of ghrelin in the body, whereby negative energy balance enhances ghrelin action and diet-induced obesity attenuates ghrelin action. These evidences suggest that ghrelin may also play as a great potential CR mimetic. Leptin, a peptide hormone secreted mainly from adipocytes, was first identified as a molecule that regulates appetite and energy expenditure via the CNS. Dysfunctions on its signaling result in hyperphagia and obesity (Zhang *et al.*, 1994, Schwartz *et al.*, 2000). Although the primary role of leptin is as a long-term regulator of body fat via hypothalamic control of feeding, leptin also suppresses the gonadal, somatotropic, and the thyroid axes, thus reduced leptin may be a critical modulator of CR and longevity (Shimokawa *et al.*, 1999, Shimokawa *et al.*, 2001). Many studies have reported that CR reduces circulating levels of IGF-1 and insulin (and glucose) (Argentino *et al.*, 2005a, Argentino *et al.*, 2005b). The reductions in IGF-1 and insulin signaling that occur under CR have been suggested to be causally linked to the lifespan enhancing effects of CR. This was initially based on observations in *C. elegans* and *D. melanogaster* that mutants with defects in the intracellular IGF-1/insulin signaling pathway had increased lifespan (Berryman *et al.*, 2008, Kenyon 2011).

1.3. Autophagy

Cell growth and homeostasis are governed by tightly regulated biosynthetic and catabolic processes (He *et al.*, 2009, Yang *et al.*, 2013). Consequently, 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. There are two main pathways for cellular constituents' clearance in eukaryotic cells (Klionsky *et al.,* 2000,

Mizushima 2004): the ubiquitin-proteasome system (UPS), which is mostly responsible for the turnover of short-lived proteins (Hershko *et al.*, 1998) 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 of substrate proteins and cytosolic constituents are known (Mizushima 2004). The first one is endocytosis/phagocytosis, responsible for the degradation of extracellular materials and cell membrane proteins. The other three, which are characterized by the sequestration of cytosolic components and subsequent delivery to the lysosome, are intended for the turnover of intracellular constituents and are considered different types of autophagy. The term autophagy, coined from the Greek words of αυτς ('autos', self) and φαγειν ('phagein'), meaning 'eating', was first used in 1963 by Christian de Duve to establish a nomenclature for different cellular pathways and compartments in the endosomal-lysosomal pathway (Jaeger *et al.*, 2009, Klionsky 2008). Recently, increasing attention has been focused on the role of autophagy on metabolism of misfolded proteins and neuronal cell death, in neurodegeneration.

Autophagy is involved in the intracellular turnover of proteins and cell organelles and has an important role in regulating cell fate in response to stress (Shintani *et al.*, 2004, Levine 2005). Autophagy is a primordial and highly conserved intracellular process that occurs in all species and cell types studied thus far. Three main types of mammalian autophagy have been identified and implicated in CNS injury and disease: chaperone mediated-autophagy (CMA), microautophagy and macroautophagy (Mizushima 2004, Yang *et al.*, 2005). In CMA, target proteins, containing a specific consensus peptide sequence (KFERQ) are recognized by a chaperone complex, which delivers them to the lysosome, across the membrane, through a specific receptor – lysosomal-associated membrane protein 2 (LAMP-2A) (Li *et al.*, 2011). In microautophagy, the lysosomal membrane directly invaginates or exvaginates, in order to sequester the cytosolic component and buds into the lysosomal lumen for degradation (Kunz *et al.*, 2004). Finally, macroautophagy, the most well studied type of autophagy (Yu *et al.*, 2008), is characterized by the formation of a double membrane vesicle named autophagosome, which engulfs long-lived proteins and/or organelles, and, after fusion with a lysosome, degrades them.

Other specialized forms of autophagy exist, such as mitophagy, direct targeting of mitochondria to lysosomes (Kanki *et al.*, 2008); pexophagy, selective degradation of peroxisomes (Iwata *et al.*, 2006, Sakai *et al.*, 2006); xenophagy, degradation of intracellular bacteria and viruses (Levine 2005, Huang *et al.*, 2007); crinophagy, lysosomal fusion with re-directed exosomes (Sandberg *et al.*, 2007); microautophagy direct engulfment of cytosol by lysosomes (Marzella *et al.*, 1981, Ahlberg *et al.*, 1985); and piecemeal microautophagy of the

nucleus, partial sequestration and degradation of the nucleus (Kvam *et al.*, 2007), but most of them have only been observed in yeast or under special conditions. Briefly, the different types of autophagy mentioned differ in mechanism whereby substrates are delivered to lysosomes, their regulation and their selectivity.

1.3.1. Macroautophagy machinery

The macroautophagy process (hereafter referred to as autophagy) was firstly observed in mammalian cells, in the late 1950's, through electron microscopy (Clark 1957, Novikoff 1959). Although autophagy was first identified approximately 50 years ago, the molecular understanding of it only started in the past decade (Klionsky 2007), based on the discovery of autophagy-related genes (ATG), genes initially discovered in yeast followed by the identification of homologs in higher eukaryotes (Yang *et al.*, 2009). Among Atg proteins, one subset, including Atg1 to Atg10, Atg12 to Atg14 and Atg16 to Atg18, is essential for autophagosome formation in mammalians cells, as shown in Figure 1.2, and is referred to as the core molecular machinery (Xie *et al.*, 2007). 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), nucleation of the membrane (II), autophagosomal elongation (III), cargo selection (IV) and closure of autophagosomal vesicles and fusion to lysosomes (V). In this dissertation the main focus will be on mammalian autophagy.

Induction of autophagosome formation step can occur through mTOR (mammalian target of rapamycin)-dependent and independent pathways. The mTOR is an evolutionarily conserved serine/ threonine protein kinase complex that comprises the mTOR complex (mTORC) 1 (mTORC1) and mTORC2. The mTORC1 negatively regulates autophagy (Laplante *et al.*, 2009). This complex integrates upstream nutrient and growth factor-derived signals, to control cell growth, from several pathways, such as insulin, class I phosphatidylinositol-3-kinase (PI_3K) and protein kinase B (PKB) (also known as Akt) (Laplante *et al.*, 2009, Sengupta *et al.*, 2010). 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 PI_3K or the Atg6-Beclin-1, crucial for the nucleation step.

The nucleation step overall, begins with the formation of the phagophore, a small portion of membrane, near the endoplasmic reticulum (ER), which then elongates to engulf the cargo and culminates with the formation of a whole vesicle, the autophagosome (Beau *et al.*, 2011).

This process starts with the interaction between the unc-51 like kinase (ULK) complex (ULKs-Atg13-FIP200) and the class III PI_3K complex located at the isolation membrane (IM). The complex contains the class III PI_3K subunits, mVps34 and p150; along with the protein Beclin-1 working as an interacting partner (Volinia *et al.*, 1995, Kihara *et al.*, 2001). This kinase plays a crucial role in the phosphoinositide phosphatidylinositol-3-phosphate (PI(3)P) phosphorylation, a phospholipid required for autophagosome formation (Zeng *et al.*, 2006).

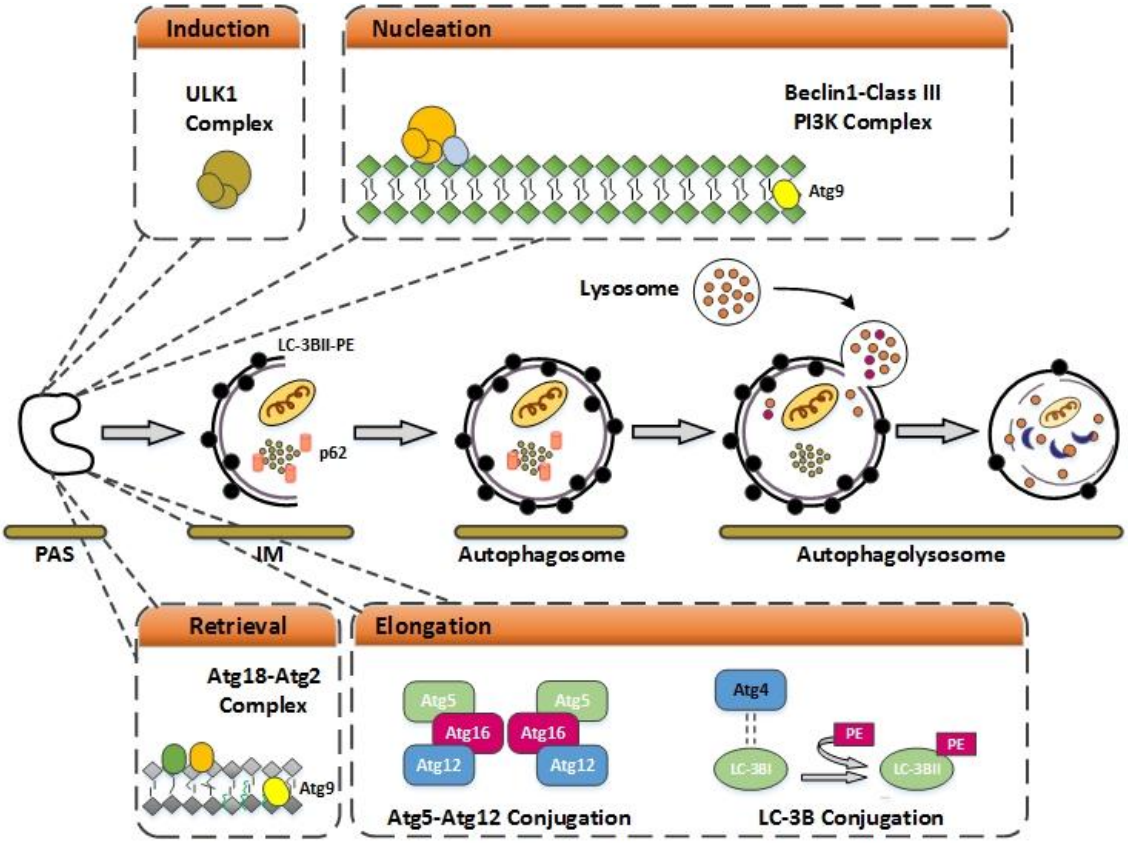

Figure 1.2 - The core molecular machinery of autophagy in mammalian cells.
Adapted from (Ciu *et al.*, 2013).

Autophagosomal elongation step is contingent on the coordinated actions of several core autophagy machinery proteins involved in two ubiquitin-like conjugation systems: the Atg12-Atg5 and the light chain-3B (LC-3B)-phosphatidylethanolamine(PE) system (Xie *et al.*, 2008, Yin *et al.*, 2008). In the Atg5-Atg12 system, Atg12 is activated by Atg7, an ubiquitin-activating enzyme (E1)-like protein, and conjugated to Atg5 via Atg10, an ubiquitin carrier protein (E2)-like protein (Mizushima *et al.*, 1998). Lastly, Atg12-Atg5 forms a multimer complex with Atg16L (forming Atg12-Atg5-Atg16L) (Kuma *et al.*, 2002, Yin *et al.*, 2008), which is localized to the outer portion of the autophagossomal membrane (Hanada *et al.*, 2007). Though crucial for pre-autophagosomal elongation, once a fully functional autophagosome forms, the Atg12-Atg5-Atg16L complex dissociates (Ravikumar *et al.*, 2010). In the LC-3B-PE system, LC-3B is

initially synthesized as a precursor (Pro LC-3B), as it possesses an additional arginine residue at the C terminus that is immediately cleaved by Atg4B, a cysteine protease, to become LC-3BI (Tanida *et al.*, 2004). LC-3BI is 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. Whereas the unconjugated form of LC-3B, known as LC-3BI resides in the cytosol, the conjugated form, LC-3BII, is localized to the autophagosomal membrane, thus making it a very effective as an important marker for autophagy (Shibata *et al.*, 2010).

The cargo selection step involves cargos recognized through interactions with specific receptor proteins. Further evidences suggest that the autophagy selectivity related to ubiquitinated substrates may be mediated by p62/sequestosome 1 (p62/SQSTM1) (Bjorkoy *et al.*, 2005, Pankiv*, et al.*, 2007), previously reported to interact with ubiquitinated proteins for UPS degradation (Seibenhener *et al.*, 2004, Babu *et al.*, 2005) and found to co-localize to ubiquitinated protein aggregates, LC-3B and lysosomes. Also, the formation of autophagosomes proved to be dependent of p62 even upon starvation (Bjorkoy *et al.*, 2005). The p62 is a signaling-adaptor protein which has a multidomain structure that allows it to interact with a myriad of other proteins, namely microtubule-associated LC-3B, which is recruited to the autophagosome membrane, and ubiquitin, among others, as well as itself, leading to oligomerization. The p62 binds to LC-3B, which in turn, integrates the autophagosome membrane and is ultimately degraded upon the autophagy late stages, leading to the concomitant degradation of p62 and proteins connected to it (Weidberg *et al.*, 2011). Also, apparently, p62 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 p62 delays the proteasomal degradation of poly-ubiquitinated aggregates (Korolchuk *et al.*, 2009, Moscat *et al.*, 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. A failure on the cargo recognition step can be responsible for the ineffectiveness of this pathway (Martinez-Vicente *et al.*, 2010).

The closure and maturation step involves the sealing of the membrane in order to form complete autophagosomes, which then fuse to endosomes and/or to lysosomes. This last step of autophagy involves the fusion between autophagosomes and lysosomes, to form autolysosomes, where the cargo is degraded (Tong *et al.*, 2010). This process is dynamic and mediated by the cytoskeleton, namely, the microtubules network, to which the

autophagosomes are associated. There, the fusion event requires the LAMP-2 and the small guanosine triphosphatase (GTPase) Rab7 (Jager *et al.*, 2004). Rab7 associates with autophagosomes and mediates the fusion event through LAMP-2, in a way resembling endosome maturation (Gutierrez *et al.*, 2004, Jager *et al.*, 2004). Posteriorly to the autolysosome formation, with the fusion between the lysosome and the outer membrane of the autophagosome, the inner membrane and the cargo, including bound LC-3BII, are degraded by several proteases, namely cathepsins B, D and L (Tanida *et al.*, 2005). The products of degradation are then released to the cytosol, potentially leading to the activation of mTOR and the disassembly of the autolysosome (Tong *et al.*, 2010).

Importantly, an increased number of autophagosomes may not necessarily correspond to an increased autophagic activity, but instead a blockage in the autophagy pathway downstream of autophagosome formation, such as in the maturation step (Cuervo 2004, Boland *et al.*, 2008, Mizushima *et al.*, 2010).

1.3.2. Signaling pathways regulating autophagy

Autophagy is known to be induced by a wide variety of stimuli, such as nutrient and growth factor depletion, hypoxia, drug and radiation treatment. For the same reason, tight regulation mechanisms exist to control its induction (Weidber *et al.*, 2011). The principal pathway, called of canonical one, culminates in the inhibition of mTOR, but other pathways exist, that 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.

1.3.2.1. 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 PI_3K−Akt−mTORC1. Upon insulin or IGF-1 binding to the insulin receptor, insulin receptor substrates (IRS) 1/2 (IRS1/2) are phosphorylated and recruited to the membrane, forming a docking scaffold for class I PI_3K (He *et al.,* 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 revert the action of PI_3K (Arico *et al.*, 2001). Generation of $PI(3,4,5)P_3$ increases membrane recruitment of Akt and its activator, phosphoinositide-dependent protein kinase 1 (PDK1), which phosphorylates the prior. The mTORC2 also plays a role at this point, contributing to the activation of Akt, though its upstream regulating mechanisms remain unknown (Dunlop *et al.,* 2009). After activation, Akt phosphorylates protein 2 from the tuberous sclerosis complex (TSC) 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 trisphosphate (GTP) into guanosine diphosphate (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 prevents autophagy (He *et al.,* 2009).

1.3.2.1. 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 *et al.,* 2010b). During intracellular low-glucose periods, mitochondria produce less adenosine triphosphate (ATP), leading to an increase of the AMP/ATP ratio (Hardie *et al.*, 2003). Upon this increase, liver kinase B-1 (LKB-1, also known as serine/threonine kinase 11) potentiates AMPK, which induces the formation of TSC1/2 complex and the consequent inhibition of mTORC1 through Rheb (Corradetti *et al.*, 2004). Plus, 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, one of the constituents of mTORC1. 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 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 (Hoyer-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 (REDD) 1 and 2 (REDD1 and REDD2), which induce the TSC1/2 complex (Dunlop *et al.,* 2009).

1.3.2.3. 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. Vps34, a Class III PI_3K with an important role in mediating the autophagic machinery induction downstream of ULK–Atg13–FIP200 complex, was the first protein to be linked to nutrient-sensing within the mTORC1 pathway (Dunlop *et al.,* 2009). But curiously, contrary to what would be expected, it was shown to respond to amino acid signaling, inducing mTOR kinase activity and inhibiting autophagy (Byfield *et al.*, 2005, Nobukuni *et al.*, 2007). This contradiction may be related to the existence of different PI_3K protein sub-populations or complexes in the cell, carrying out different functions, under different signals (He *et al.,* 2009). Otherwise, Vps34 activation by ULK and consequent production of PI_3P may also function as a negative feedback control, reactivating mTOR and restricting autophagy induction. The way through which Vps34 activates mTOR is not fully established yet, but it may presumably be dependent on the recruitment of FYVE and PX domain-containing proteins, by PI_3P (Nobukuni *et al.*, 2007). Though, the link between amino acids signaling and Vps34 has been attributed to calcium increase, which binds calmodulin and, in turn, activates Vps34 (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 Vps34 to activate mTORC1, inhibiting autophagy. Byfield and his collaborators have previously shown that the Vps34 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 (Hoyer-Hansen*, et al.*, 2007) and/or –independent (Grotemeier *et al.*, 2010), may overcome the Vps34 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 mitogen-activated protein kinase (MAPK) family protein. MAP4K2 overexpression was found to increase the phosphorylation of 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. Ras-related small GTPases Rag has been found to play an important role in amino

acid signaling, also positively regulating mTORC1 activation (Kim, *et al.,* 2009). But rather than modulating its kinase activity, when induced by amino acids, they appear to simply translocate mTOR to a perinuclear region (Sancak *et al.*, 2008). Yet, it is not known how this translocation increases mTORC1 activity (Kim *et al.,* 2009) and also, no direct connection has been made with autophagy induction.

1.3.2.4. The MAPK/ERK pathway

Other studies demonstrate that the Ras/MAPK 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 class I PI_3K activity and Raf-1/MAPK cascade activation (Furuta *et al.*, 2004). Although class I PI_3K signals through Akt, as previously described, Raf-1 (MAP3K) phosphorylates MEK 1/2 (MAP2K), which in turn phosphorylates extracellular signal-regulated kinases 1 and 2 (ERK 1/2). ERK 1/2 then phosphorylates TSC2, inhibiting its assembly with TSC1, thus allowing Rheb to activate mTORC1 (Furuta *et al.*, 2004, Ma *et al.*, 2005). A substrate of ERK, ribosomal s6 protein kinase α 1 (RSK1, or p90) has also been found to phosphorylate TSC2, contributing to the activation of mTORC1 (Roux *et al.*, 2004, Kwiatkowski *et al.,* 2005). Plus, both ERK 1/2 and RSK1 phosphorylate S6K1 (Steelman *et al.*, 2011), further indicating their pro-proliferative role in this pathway. Conversely, Ogier-Denis and collaborators have found that ERK 1/2 stimulates autophagy in the human colon cancer cell line HT-29 (Ogier-Denis *et al.*, 2000). They have shown that ERK 1/2 phosphorylates G-protein subunit α (Gα)-interacting protein (GAIP), thus leading to accelerated GTPase activity of inhibitory Gα-3 ($G\alpha_i3$). 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.

1.3.2.5. The cAMP-Epac-PLC-ε-IP_3 and Ca^{2+}-calpain-$G\alpha_s$ pathways

Williams and his collaborators suggested a role for inositol triphosphates (IP_3) in the regulation of autophagy, independently of mTORC1 (Williams *et al.*, 2008). These authors first observed that lithium-induced autophagy was dependent on 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 by cAMP (Epac) −phospholipase C-ε (PLC-ε) −IP_3 and

Ca^{2+}–calpains–stimulatory protein G subunit α ($G\alpha_s$) (Williams *et al.*, 2008, Sarkar *et al.*, 2009). Basically, an increase in cAMP caused by $G\alpha_s$-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 $G\alpha_s$, 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).

1.3.2.6. Ras/cAMP-dependent PKA pathway

Besides mTORC1, the Ras/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. Thus, in response to nutrient rich conditions, two redundant Ras GTPases, Ras1 and Ras2, are activated and subsequently stimulate adenylate cyclase to produce cAMP. Then cAMP is able to bind to the regulatory PKA subunit, Bcy1, allowing its dissociation from the PKA catalytic subunits (Portela *et al.,* 2006), and consequently the activation of PKA. When this activation occurs, the autophagy induced by mTOR inhibition is suppressed, indicating that PKA 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 and dissociated from the phagophore assembly site (PAS), whereas in contrast, 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 LC-3B (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, Fontana *et al.*, 2010, Chen *et al.*, 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.

1.3.3. Autophagy in the brain

It has been well established that autophagy regulates important biological functions, such as cell survival, cell death, cell metabolism, development, aging, infection and immunity. As mentioned above, autophagy is a basic cellular mechanism for recycling of nutrients and is for this very reason involved in many physiological processes in higher eukaryotes (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 *et al.*, 2010a). 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 such as neurons (Rami 2009).

The role of autophagy in neurons was until recently unknown, despite its known presence in neurons (Boellaard *et al.*, 1989, Tasso *et al.,* 1978). Recent findings boosted research on the role of autophagy in the CNS, specifically in neurodegenerative diseases. Accumulation of autophagic vacuoles (AV) was found in brains of animal models and patients with AD (Cataldo *et al.*, 1994, Cataldo *et al.*, 1996, Stadelmann *et al.*, 1999, Yang *et al.*, 2008), PD (Anglade *et al.,* 1997, Spencer *et al.,* 2009) and HD (Sapp *et al.*, 1997, Kegel *et al.*, 2000). This accumulation of AV in late stages of disease was initially incorrectly associated with autophagic cell death (Bursch 2000, Bursch *et al.*, 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 *et al.*, 2008, Kroemer *et al.*, 2009, Levine *et al.*, 2009). In fact, it is currently accepted that an accumulation of AV is not correlated with an excessive autophagy but an impaired autophagic process, such as deficient fusion with lysosomes and corresponding degradation (Cuervo 2004, Nixon *et al.,* 2005, Boland *et al.*, 2008, Mizushima *et al.*, 2010, Wong *et al.*, 2010a, Wong *et al.*, 2010b).

1.4. Neuropeptide Y

In 1982, Tatemoto isolated and sequenced neuropeptide Y (NPY) from extracts of porcine brain using a chemical assay for the C-terminal amide fragments (Tatemoto 1982, Tatemoto *et*

al., 1982) (Figure 1.3). NPY is one of the most abundant peptides found in the brain, although it is also present in the peripheral nervous system and in other peripheral tissues (Everitt *et al.*, 1984, Allen *et al.*, 1987, Zukowska *et al.*, 2003).

NPY is a 36 amino acid peptide exhibiting a carboxyl-terminal amidation. The presence of this α-amidated carboxyl terminus was the determinant key for the purification of the NPY-related peptides (Tatemoto *et al.*, 1978). In addition, the carboxyl-terminal of the peptide is responsible for its biological activity while the amino-terminal is involved in receptor affinity. The most important characteristic of NPY is the presence of certain amino acid residues, in specific tyrosine residues, to adopt a specific three-dimensionnal structure, named the pancreatic polypeptide fold (PP-fold) (Blundell *et al.*, 1981). NPY belongs to gastric peptide family that includes peptide YY (PYY) and pancreatic polypeptide (PP) (Tatemoto 1982, Schwartz *et al.*, 1990). NPY, PYY and PP share 70-50 % homology (Pedrazzini *et al.*, 2003). NPY is one of the most evolutionarily conserved neuropeptide while the PP varies considerably between species (Larhammar *et al.*, 1992).

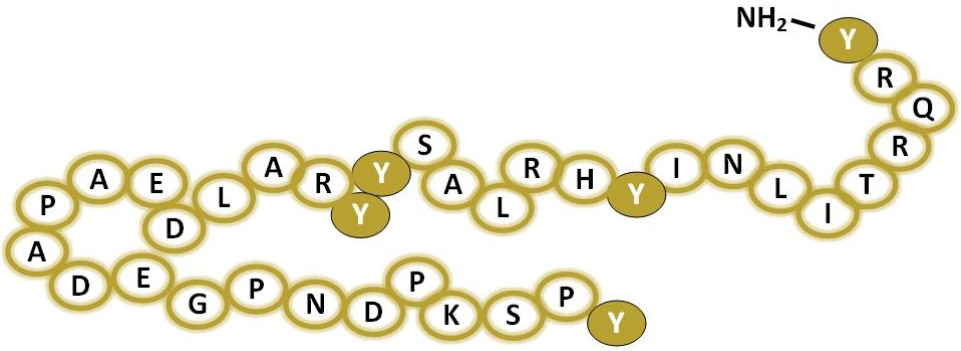

Figure 1.3 - Structure of human NPY. Human NPY has 36-amino acid peptides, with a carboxyl-terminal amidation. This α-amidated carboxyl terminus is essential during the purification of the NPY-related peptides. Adapted from (Schwartz *et al.,* 1990).

1.4.1. Synthesis of neuropeptide Y, metabolization and localization

Like all polypeptides and proteins, NPY is produced by cleavage of a peptide precursor, which is, in turn, synthesized in ribosomes and transported into the lumen of the ER and thus, to the Golgi complex (Beck-Sickinger *et al.*, 1995). The NPY gene is located on the human chromosome 7 at the locus 7p15.1, and is divided into 4 exons that are separated by 3 introns (Minth *et al.*, 1984). In mouse, the NPY gene is located in chromosome 6, locus 6 B3; 6 26.0 cM while in rat is localized in chromosome 4, locus 4q24 (Pruitt *et al.*, 2012). The translation of the messenger ribonucleic acid (mRNA) results in the synthesis of a pre-pro-neuropeptide Y (Pre-Pro-NPY), a 97 amino acid precursor (Minth *et al.*, 1984), and this process occurs in the secretory granules. Pre-Pro-NPY is further processed to pro-neuropeptide Y (Pro-NPY). The 28 amino acids signal peptide is cleaved by a signal peptidase enzyme that produces 69 residues

Pro-NPY. The Pro-NPY usually travels to the Golgi complex and further to the trans-Golgi. The Pro-NPY undergoes cleavage by proconverting enzymes, prohormone convertase (PC) 1 or 3 (PC1 or PC3) and/or PC2, releasing the C-Terminal Flanking Peptide of NPY (CPON), NPY peptide flanker (Mains *et al.*, 2006). NPY_{1-39} is further processed to NPY_{1-37} by carboxypeptidase E (CPE) enzyme and amidated by peptidylglycine α-amidating monoxygenase (PAM) to NPY_{1-36} or simply NPY that is the biologically active form (Figure 1.4) (Medeiros *et al.*, 1996, Silva *et al.*, 2002, Mains *et al.*, 2006). Although this peptide is already in its biologically active form, it can be further cleaved by certain peptidases, such as dipeptidyl-peptidase IV (DPP-IV, EC 3.4.14.5) or aminopeptidase P (AP-P, EC 3.4.11.9) (Pedrazzini *et al.*, 2003).

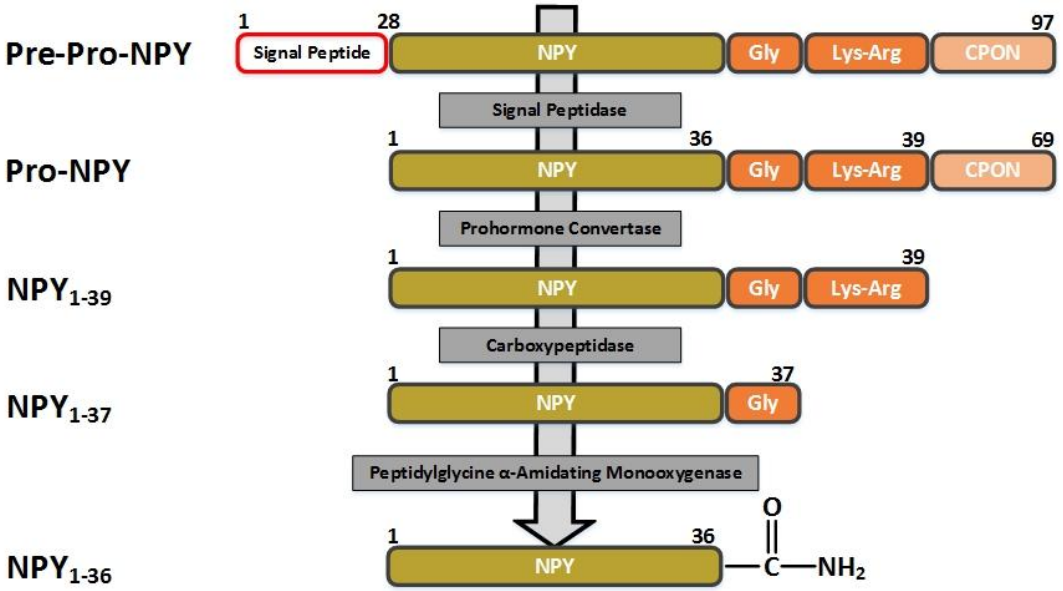

Figure 1.4 - Schematic biosynthesis of NPY. Adapted from (Silva *et al.*, 2002).

NPY metabolization by DPP-IV productive rise to C-terminal fragments (e.g. NPY_{2-36}), which have different receptor-activation/affinity profiles and, thus, different biological activities (Medeiros *et al.*, 1996). This exopeptidase belongs to the family of propyl-oligopeptidases and is a protease bounded to a plasma membrane that cleaves a peptide bond in the target peptide that have a proline amino acid in the penultimate position. Thus, DPP-IV acting on the NPY, cleaves the bond Ser-Pro of the terminal amine giving rise to NPY_{3-36}. This peptide loses affinity for Y_1 receptor and becomes agonist to Y_2 and Y_5 receptors (Mentlein *et al.*, 1993, Medeiros *et al.*, 1994, Medeiros *et al.*, 1996). 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 hydrolyze the peptide bond between the first and second amino acid in the amino terminus of the peptide. The second amino acid is the proline resulting in NPY_{2-36} (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 and the Tir^{20} Tir^{21}-Leu^{30}-Ile^{31} giving rise to biologically inactive peptides and NPY_{1-20} and NPY_{31-36} (Yaron *et al.*, 1968, Medeiros *et al.*, 1994, Medeiros *et al.*, 1996). NPY is one of the most abundant peptides expressed in several brain regions (for example hypothalamus, amygdala, hippocampus and cerebral cortex), as well as in the periphery (for example liver, heart, spleen, endothelial cells of blood vessels), showing both pre- and post-synaptic actions. However, the adrenal medulla is the primary source of circulating NPY.

Within the CNS, NPY is expressed both during development and adulthood. Concentrations of NPY have been determined in different brain regions of the rat brain during development using radioimmunoassay and chromatography. NPY is detected as early as embryonic day 14 in the diencephalon and the brainstem. The concentrations of NPY show a rapid post-natal rise in all regions examined (Allen *et al.*, 1984, Woodhams *et al.*, 1985), resulting in a widespread expression throughout the brain in adulthood. The distribution of

NPY in the human (Adrian *et al.*, 1983) and rodent (Allen *et al.*, 1983) CNS is well described. NPY is widely expressed within the brain but immunoreactive cell bodies and fibers are most prevalent in the cortical and hypothalamic regions (e.g. arcuate nucleus, dorsomedial nucleus, paraventricular nucleus), but with a particularly higher expression in the hypothalamus (Adrian *et al.*, 1983, Chan-Palay *et al.*, 1985). Within the hypothalamus, NPY is a potent orexigenic peptide playing a pivotal role in the physiological control of food intake and body weight (Chee *et al.,* 2008).

1.4.2. Regulation of neuropeptide Y expression

The NPY expression in the hypothalamus is regulated by multiple neural and peripheral signals including the food deprivation and hormones. An important factor that influences hypothalamic NPY expression is fasting. During food deprivation and food restriction in rodents, the expression of orexigenic NPY increases in the ARC (Beck *et al.*, 1990, Brady *et al.*, 1990, Bi *et al.*, 2003) with the consequent increase of NPY content in the paraventricular nucleus (PVN) (Sahu *et al.,* 1988, Beck *et al.*, 1990, Kalra *et al.*, 1993). Moreover, the NPY levels return to initial values within 6 to 24 hours after re-feeding (Beck *et al.*, 1990, Kalra *et al.*, 1993, Sanchez *et al.*, 2008). Additionally, NPY expression in the ARC is regulated by peripheral hormones such as insulin, leptin and ghrelin. 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, Schawartz *et al.*, 1992, Frederich *et al.*, 1995, Saladin *et al.,* 1995). Insulin receptors (IRS) and leptin receptors (LepR) are expressed in NPY neurons located in the ARC (Hakansson *et al.*, 1996, Mercer *et al.*, 1996,

Schawartz *et al.*, 1996a, Baskin *et al.*, 1999, Obici *et al.*, 2002) where these hormones act to suppress NPY expression and decrease food intake (Schawartz *et al.*, 1992, Sipols *et al.*, 1995, Schawartz *et al.*, 1996b). Ghrelin is an orexigenic peptide mainly synthesized by the stomach (Kojima *et al.*, 1999, Ariyasu *et al.*, 2001) and regulated by ingestion of nutrients such that, peripheral ghrelin levels rise before a meal and rapidly decrease after food intake (Ariyasu *et al.*, 2001, Cummings *et al.*, 2001, Tschop *et al.*, 2001b). Ghrelin receptors are present on NPY neurons in the ARC (Willesen *et al.*, 1999, Mondal *et al.*, 2005) where ghrelin acts to stimulate NPY production and increase food intake (Kamegai *et al.*, 2001, Guan *et al.*, 2010). Additionally, NPY neurons located in the ARC are activated by low glucose concentrations, in vivo and in vitro (Akabayashi *et al.*, 1993, Fioramonti *et al.*, 2007, Muroya *et al.*, 1999, Sergeyev *et al.*, 2000).

1.4.3. Neuropeptide Y receptors subtypes and signaling pathways

The existence of multiple NPY receptor subtypes was first proposed by Wahlestedt (Wahlestedt *et al.*, 1986). The NPY system has emerged as one of the most complex networks of related peptides and receptors, not only because it has a large number of physiological effects, but also because it includes an unusually broad repertoire of receptor subtypes called Y receptors. The family of NPY receptors is the same for all members of the NPY family (NPY, PP and PYY), 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 *et al.*, 2004).

1.4.3.1. Neuropeptide Y Y_1 receptor

The NPY Y_1 receptor was the first NPY receptor to be cloned (Eva *et al.*, 1990) and its gene is localized in the chromosome 4q(31,3-32) (Pedrazzini *et al.*, 2003). NPY binding activity to this receptor is largely impaired when enzymes, like DPP-IV, cleave the NPY N-terminal peptides (Silva *et al.*, 2002). However, when the C-terminal peptides are modified, NPY retains its full binding capacity to Y_1 receptor, suggesting that this neurotransmitter binds this receptor through its N-terminal region (Silva *et al.*, 2002). The Y_1 receptor has high affinity to NPY_{1-36} and PYY, but the affinity weakens as the N-terminal part of the peptide is cleaved by peptidases. The changes of NPY in the terminal carboxylic acid also result in similar assets (Silva *et al.*, 2002). The NPY Y_1 receptor is richly expressed in the CNS and in blood vessels. It induces vasoconstriction and proliferation in several types of cells. Smooth muscle cells (Zukowska-Grojec *et al.*, 1998), olfactory epithelium (Hansel *et al.*, 2001), progenitor cell of the hippocampus (Howell *et al.*, 2003, Howell *et al.*, 2005, Howell *et al.*, 2007), pancreatic cells

(Cho *et al.*, 2004), Müller cells (Milenkovic *et al.*, 2004) and cancer cells (Korner *et al.*, 2004) are some examples. The Y_1 receptor activation decreases anxiety and depression (Sajdyk *et al.*, 1999, Redrobe *et al.*, 2002), increases appetite (Corp *et al.*, 2001, Lecklin *et al.*, 2003) and alcoholic consumption (Kelley *et al.*, 2001, Thiele *et al.*, 2002). It also modulates pulpal inflammation (Rethnam *et al.*, 2010) and the antagonism of this receptor increases bone mass (Baldock *et al.*, 2007, Sousa *et al.,* 2012).

1.4.3.2. Neuropeptide Y Y_2 receptor

The Y_2 receptor was first cloned in 1995 (Rose *et al.*, 1995) and it is located in the chromosome 4q31, close to the Y_1 and Y_5 receptor locus (Pedrazzini *et al.*, 2003). The Y_2 receptor is characterized pharmacologically by its high affinity for NPY and PYY, but in contrast to the Y_1 receptor, the affinity to the Y_2 receptor does not suffer from further cleaving of the N-terminal ligand, NPY_{2-36}, NPY_{3-36}, NPY_{13-36} and PYY_{3-36} bind to the receptor (Michel *et al.*, 1998). The activation of Y_2 inhibits the neurotransmitter release, regulates appetite (Naveilhan *et al.,* 1999, Batterham *et al.,* 2002, Sainsbury *et al.,* 2002), is involved in neuronal excitability in epilepsy (El Bahh *et al.*, 2002, Herzog 2002, Vezzani *et al.*, 2004), in angiogenesis (Zukowska-Grojec *et al.*, 1998, Ekstrand 2003, Lee *et al.*, 2003) and presents a putative neuroprotective effect in Parkinson disease (Decressac *et al.*, 2012).

1.4.3.3. Neuropeptide Y Y_4 receptor

The cloning of the Y_4 receptor was first described in 1995 (Bard *et al.*, 1995) and it is located in the chromosome 10q 11-12 (Pedrazzini *et al.*, 2003). It exhibits a very high affinity for PP with relatively lower affinity for NPY, and it was reported to be internalized after agonist stimulation (Parker *et al.*, 2001), while another group reported that no internalization occurs for Y_4 receptor (Voisin *et al.*, 2000). Interestingly, it was described that this receptor forms a constitutive homodimers when expressed in mammalian cells (Berglund *et al.*, 2003) and that these dimers dissociates upon agonist stimulation. Human Y_4 receptor mRNA was found in prostate, colon, pancreas, small intestine, smooth muscle cells and brain, particularly in hypothalamus (Barrios *et al.*, 1999, Misra *et al.*, 2004). PP through this receptor is able to inhibit exocrine release from pancreas, induce the relaxation of biliary vesicle and stimulate the release of luteinizing hormone (Horvath *et al.*, 2001a, Andersen 2007).

1.4.3.4. Neuropeptide Y Y_5 receptor

The Y_5 receptor was cloned in 1996 and it is located in chromosome 4q32, in the same locus as Y_1 receptor, although their transcription is in opposite directions (Pedrazzini *et al.,*

2003). The Y_5 receptor is activated by NPY, PYY analogs, and fragments of peptides, such as NPY_{3-36} and PYY_{3-36} (Gerald *et al.,* 1996, Michel *et al.,* 1998). This receptor is localized centrally, in the hypothalamus, playing an essential role in appetite stimulation (Hwa *et al.*, 1999, Cabrele *et al.*, 2000, Lecklin *et al.*, 2003, Beck 2006) and peripherally, in human and murine adrenal glands (Cavadas *et al.*, 2001, Cavadas *et al.*, 2006).

1.4.3.5. Neuropeptide Y y_6 receptor

The y_6 receptor was initially cloned from mouse DNA and subsequently in other species including rabbit, monkey and human (Gregor *et al.,* 1996, Matsumoto *et al.,* 1996, Weinberg *et al.,* 1996). However, the importance of this receptor is not yet clear. This receptor is localized in the chromosome 5q31 (Pedrazzini *et al.,* 2003) and binding properties resemble that of Y_4, and PP shows higher affinity to y_6 than PYY and NPY (Jin *et al.,* 2000, Popovic *et al.,* 2001). Some reports show the presence of this receptor in some mammals, like mouse, rabbit dog, cow and primates, including humans, but absent in rats (Burkhoff *et al.,* 1998). The y_6 mRNA is located in hypothalamus, hippocampus, small intestine and adrenal glands of rabbits. It can also be found in heart, skeletal muscle and hypothalamus of humans (Gregor *et al.,* 1996, Matsumoto *et al.,* 1996, Weinberg *et al.,* 1996). However the pharmacological properties of y_6 are divergent; some authors defend that it is functional in mouse and rabbit and nonfunctional in human and other primates, as well as in guinea-pig and pig; as a result of frame shift mutations (Gregor *et al.,* 1996, Matsumoto *et al.*, 1996; Weinberg *et al.*, 1996, Mullins et al., 2000, Starback *et al.,* 2000) it becomes a pseudogene in some mammals (Bromee *et al.*, 2006). The NPY y_6 receptor is also present in chicken, amphibians and bony fishes and the y_6 gene appears to be functional in the shark, *Squalus acanthias* (Salaneck *et al.*, 2003).

1.4.3.6. Neuropeptide Y Y_7 receptor

The NPY Y_7 receptor was more recently discovered in non-mammalian jawed vertebrates and examples of some species that not have lost this receptor during evolution are chicken, fishes, like zebrafish *Danio rerio*, rainbow trout *Oncorhynchus mykiss*, as well as amphibians, like two species of frogs (*Xenopus tropicalis* and the marsh frog *Rana ridibunda*) (Fredriksson *et al.*, 2004, Bromee *et al.*, 2006, Larsson *et al.*, 2006, Larsson *et al.*, 2009). In opposition mammalian do not present this receptor (Larhammar *et al.*, 2004).

1.4.3.7. Neuropeptide Y Y_8 receptor

The NPY Y_8 receptor is present in fishes and frogs, like elephant shark, *Callorhinchus milii* and *Xenopus tropicalis*, respectively (Larsson *et al.*, 2009). The Y_8 gene has been lost in the

lineage leading to mammals. The Y_8 receptor is also missing in all amniote genomes; however is in duplicate (Y_8a and Y_8b) in the teleost fishes *Tetraodon nigroviridis* and *Takifugu rubripes*. In *T. rubripes*, Y_8 receptor is expressed in brain and in peripheral organs (Larsson *et al.*, 2008).

1.4.3.8. Molecular signaling pathways associated with neuropeptide Y receptors

Generally, NPY receptors use similar signal transduction pathways, acting via pertussis toxin-sensitive G-proteins, i.e., and members of the G_i and G_o family. Thus, inhibition of adenylyl cyclase upon NPY receptor activation is found in almost every tissue and cell type investigated (Michel 1991). However, the inhibition of adenylyl cyclase cannot probably explain all functional responses observed upon stimulation of NPY receptors (Michel *et al.,* 1998). Additional signaling responses that are restricted to certain cell types include modulation of the Ca^{2+} or K^+ channels conductance (Michel *et al.,* 1995, Xiong *et al.,* 1995). Moreover, there are also evidences suggesting that NPY may be associated to the activation of phospholipase A2 (Martin *et al.,* 1989), MAPK (Keffel *et al.,* 1999, Alvaro *et al.,* 2008a, Rosmaninho-Salgado *et al.,* 2009, Thiriet *et al.,* 2011), PKC (Rosmaninho-Salgado *et al.,* 2007, Chen *et al.,* 2008, Pons *et al.,* 2008, Rosmaninho-Salgado *et al.,* 2009), PI_3K (Zhou *et al.*, 2008), guanylyl cyclase (Rosmaninho-Salgado *et al.*, 2007), nitric oxide (NO) synthesis (Rosmaninho-Salgado *et al.*, 2009, Ferreira *et al.*, 2010), or even with the inhibition of PKA (Pons *et al.,* 2008, Rosmaninho-Salgado *et al.*, 2009).

1.4.4. Physiological functions of neuropeptide Y

NPY is the most potent endogenous orexigenic factor known to date (Minor *et al.*, 2009) and it is usually induced by hunger-signaling hormones, such as ghrelin and glucagon, and inhibited by satiety signals, such as insulin and leptin (Naslund *et al.*, 2007). Furthermore, ARC NPY neurons innervate a widespread area of the hypothalamus, regulating the feeding behavior, as well as growth and development. NPY has also been implicated in several other physiological functions, such as memory and learning, anxiety, circadian rhythm, locomotion and cardiovascular function (Thorsell *et al.*, 2002, Minor *et al.*, 2009). It has also been shown to modulate proliferation in several cell types (Álvaro *et al.*, 2008a, Jia *et al.*, 2010) and to promote neuroprotection against excitotoxicity-related apoptosis (Silva *et al.*, 2005, Álvaro *et al.*, 2008b). While it is predominant in the CNS, it is also expressed in the sympathetic peripheral nervous system (Pernow *et al.*, 1987) and in other tissues, such as the gut (Cox 2007), the spleen (Ericsson *et al.*, 1987), the adipose tissue (Yang *et al.*, 2008) and the adrenal medulla (Bernet *et al.*, 1998), which releases it into circulation. Overall physiological actions

include stimulation of fat storage and weight gain, along with hunger and decreasing body temperature, sex drive and locomotion (Minor *et al.*, 2009).

1.4.5. Neuropeptide Y in cerebral cortex

NPY is present in most cerebral cortical areas during fetal and postnatal development (Leroux 2002). Comparatively with other regions of brain, the highest concentrations of NPY are measured in the hypothalamus and limbic structures and to a lesser extent in the cerebral cortex, where it is synthesized by neurons in all lamina (Hendry *et al.*, 1984, Chrownwall *et al.*, 1985). A particular enrichment of NPY-immunoreactivity (NPY-ir) in cerebral cortex is present at the highest concentration in the cingulated and temporal cortices and at the lowest in the occipital lobe (Adrian *et al.*, 1983). NPY-labeled cells are also found in subcortical white matter, whereas this expression is negative in white matter areas away from the cortex (Adrian *et al.*, 1983).

Recent studies investigated the distribution of NPY receptors throughout different regions of the cerebral cortex (Parker *et al.*, 1999). These studies seem of particular relevance as multiple functions of NPY are almost certainly mediated by different receptor subtypes, as previously mentioned (Vezzani *et al.*, 1999, Furtinger *et al.*, 2001). In addition, these studies revealed that the rat frontal cortex is rich in Y_1 receptor subtype (Dumont *et al.*, 1993, Larhammar 1996) and both Y_1 receptors (Caberlotto *et al.*, 1997) and Y_2 receptors (Jacques *et al.*, 1997, Statnick *et al*., 1997) are present in the human frontal cortex. Interestingly, Y_2 rather than Y_1 receptors appear to predominate in the frontoparietal cortex of a developing rat (Leroux 2002), possibly suggesting a varying importance of the two-receptor subtypes in the immature and mature rat cortices.

The expression of NPY mRNA and its distribution of NPY-ir in axons and cortical neurons is affected in a variety of neuropathological processes, including depression, bipolar disorder, schizophrenia, schizoaffective disorder, and neurodegenerative disorders, such as AD, PD and HD (Beal *et al.*, 1986, Kowall *et al.*, 1988, Caberlotto *et al.*, 1999, Kuromitsu *et al.*, 2001, Moris *et al.*, 2009, Morales-Medina *et al.*, 2010). Within the cerebral cortex, NPY is involved in synaptic transmission (Bacci *et al.*, 2002), regulation of cerebral blood flow (Estrada *et al.*, 1998, Cauli *et al.*, 2004) and inhibition of neuronal excitability (Colmers *et al.*, 1994). Recently, NPY has been implicated in learning and memory, and the density of NPY-expressing cortical neurons and axons is reduced in depression, bipolar disorder, schizophrenia, and neurodegenerative diseases (Raghanti *et al.*, 2013).

1.5. Ghrelin

In 1999, Kojima identified ghrelin in the rat stomach (Kojima *et al.*, 1999). The name ghrelin originates from the word *ghre*, a word root in Proto-Indo-European languages for "growth", in reference to its ability to stimulate growth hormone (GH) release (Kojima 2008) (Figure 1.5). Ghrelin is an orexigenic peptide (Kojima *et al.*, 1999, Ariyasu *et al.*, 2001) and it is regulated by nutrients levels, since peripheral ghrelin levels rise before a meal and rapidly decrease after food intake (Ariyasu *et al.*, 2001, Cummings *et al.*, 2001, Tschop *et al.*, 2001b). Ghrelin is a 28-amino acid peptide (Kojima *et al.*, 1999) in which the third N-terminus amino acid, a serine residue (Ser^3), is post-translational modified with a 8-carbon acyl group (octanoylation). This post-translation modification that is essential for binding to respective receptor and so far unique to this peptide, as no other naturally occurring peptides have been shown to have this acyl group as a post-translation modification. This particular modification is capable of increasing the lipophilicity of the molecule (Rindi *et al.*, 2004).

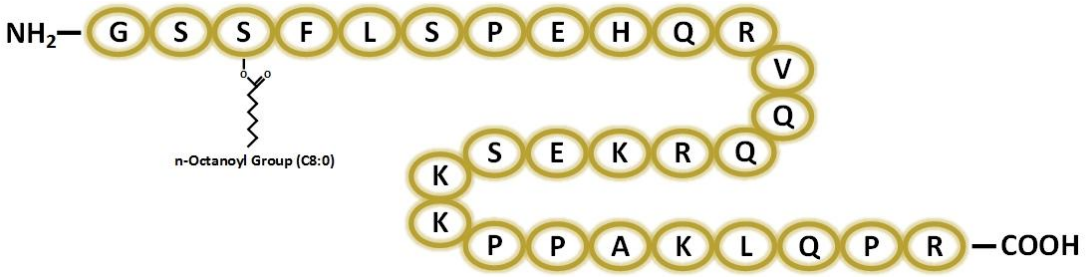

Figure 1.5 - Structure of human ghrelin. Human ghrelin has 28-amino acid peptides, in which Ser_3 is modified by a fatty acid, *n*-octanoic acid. This modification is essential for ghrelin's activity. Adapted from (Kojima *et al.,* 2005).

1.5.1. Ghrelin synthesis, metabolization and localization

The ghrelin gene is located on chromosome 3p at the locus 3p25-26 and contains four prepro-ghrelin coding exons 1–4 exons (Wajnrajch *et al.*, 2000) and one non-coding first exon, 20 bp exon 0, (Kanamoto *et al.*, 2004, Nakai *et al.*, 2004). The described human complementary deoxyribonucleic acid (cDNA) codes for a 117 amino acid long prepro-ghrelin (Kojima *et al.*, 1999), which contains a 23 amino acid signal peptide and 94 amino acid pro-ghrelin (with 28 amino acid corresponding to the mature ghrelin and a 66 amino acid tail – C-terminal polypeptide or C-ghrelin). Upon pre-pro-ghrelin processing, the 23 amino acid secretion-signal peptide is cleaved from the 117 amino acid precursor, resulting in the 94 amino acid pro-ghrelin peptide, amino acids 24–117. This pro-ghrelin peptide is then cleaved via the prohormone convertase 1/3 (PC1/3) and gives rise to the 28 amino acid ghrelin peptide, amino acids 24–51, and the 66 amino acid polypeptide C-ghrelin (Zhu *et al.*, 2006) (Figure 1.6).

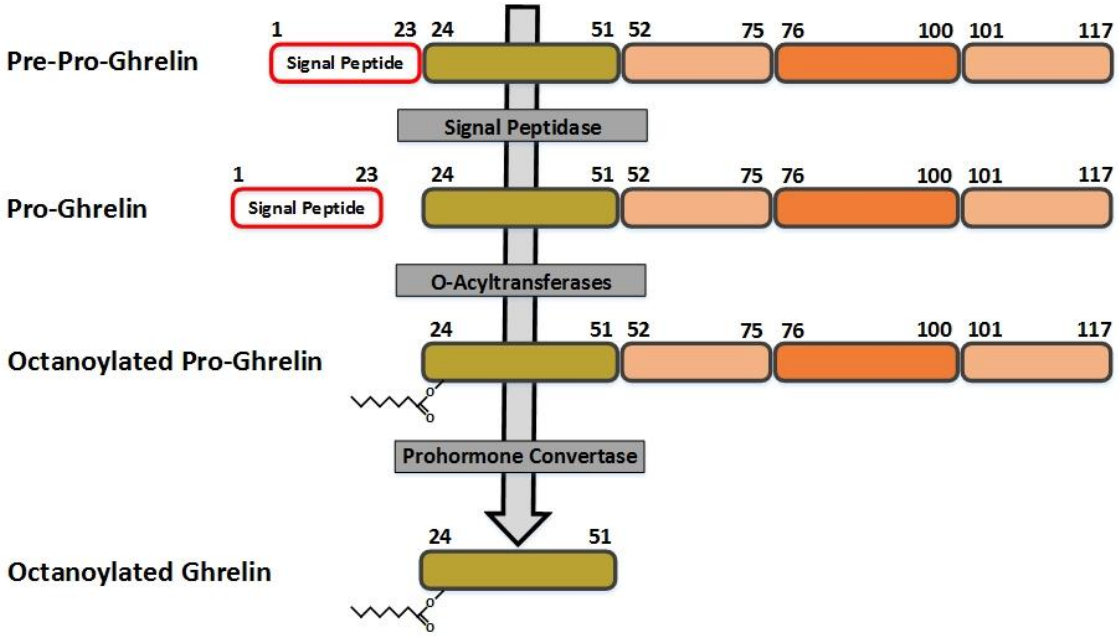

Figure 1.6 – Schematic biosynthesis of ghrelin. Adapted from (Garg 2007).

Ghrelin is predominantly produced in the stomach (Baskin *et al.*, 1999, Date *et al.*, 2000, Ariyasu *et al.*, 2001, Xu *et al.*, 2004) although small amounts also originates in the intestine (Ariyasu *et al.,* 2001), pancreas, pituitary gland, kidney, placent (Hosoda *et al.*, 2002), hypothalamus (Horvath *et al.*, 2001b), immune cells, lungs (Ariyasu *et al.*, 2001), testis, ovary (Korbonits *et al.,* 2004), heart, thyroid and neoplastic tissues (De Ambrogi *et al.*, 2003).

In the CNS, the main site of ghrelin expression, although at much lower levels than the stomach, is the hypothalamus (Ferrini *et al.*, 2009). This region is known to control appetite, suggesting therefore that the production of ghrelin in this area may be involved in the regulation of food intake. Moreover, it is also found in the hippocampus, suggesting thereby a possible role in regulating and development of memory (Kojima *et al.*, 2002, De Ambrogi *et al.*, 2003). Of note, ghrelin neurons are located not only in hypothalamus, but also in the cortex (sensorimotor area, cingular gyrus), and the fibers of ghrelin neurons in hypothalamus project directly to the dorsal vagal complex (Hou *et al.*, 2006).

1.5.2. Regulation of ghrelin release

The most important factor for the regulation of ghrelin secretion is feeding. Levels of endogenous ghrelin change according to acute, as well as chronic nutritional status. Fasting causes elevation of ghrelin levels. Plasma ghrelin levels increase nearly two-fold immediately before each meal, and fall to levels within 60–120 minutes after food intake (Tschop *et al.*, 2000, Tschop *et al.*, 2001a, Ariyasu *et al.*, 2001, Cummings *et al.*, 2001, Cummings *et al.*, 2002). It is still not clear what factors are involved in the regulation of ghrelin secretion upon fasting

and after food intake (postprandial) (Patterson *et al.*, 2011). The postprandial ghrelin suppression is proportional to the ingested calorie load (Callahan *et al.*, 2004). The increased ghrelin during fasting is a result of increased pulse frequency as well as pulse amplitude, which together with the synchronized lower leptin (orexigenic compound) pulses result in a powerful orexigenic effect (Bagnasco *et al.*, 2002). Fasting increases stomach ghrelin expression, but not pituitary or hypothalamic ghrelin expression (Torsello *et al.*, 2003). Additionally, prolonged fasting for 3 days did not change ghrelin levels significantly compared to the baseline state suggesting that the meal-related changes are rather decreased after food intake than increased due to fasting (Chan *et al.*, 2004). However, in disagreement with Chan and co-workers, it was found that caloric restriction in mice with a diet containing 60 % of normal calories for ten days resulted in a four-fold increase in circulating levels of ghrelin (Lutter *et al.*, 2008).

1.5.3. Ghrelin receptor

The ghrelin receptor or growth hormone secretagogue receptor (GHS-R) gene, similarly to the ghrelin gene, is located on chromosome 3, at position 3q26.2, and encodes for two transcripts, the transcript 1a which encodes a full-length receptor (GHS-R1a) and the transcript 1b which codifies for a shortened version (GHS-R1b) (McKee *et al.,* 1997). The 1a type encodes a typical 7 seven transmembrane domains (TMs) G-coupled protein receptor (GPCR) with binding and functional properties consistent with its role as the ghrelin receptor (Davenport *et al.,* 2005, Sempera *et al.,* 2005). This receptor is mainly expressed in somatotropic cells the anterior pituitary and hypothalamus (Xu *et al.,* 2004). It is also scattered in other parts of the brain, as well as the stomach, intestines, kidneys, pancreas, heart and aorta in humans and rodents. The wide distribution of GHS-R may explain the multifaceted functions of ghrelin and GHS-R1a (Ueno *et al.,* 2005). In addition, GHS-R1a is also expressed in the cerebral cortex, and parts of the midbrain, pons, medulla oblongata and hippocampus (Howard *et al.,* 1996, Guan *et al.,* 1997, Zigman *et al.,* 2006). Regarding isoform 1b GHS-R are not mentioned specific relations with the functions of ghrelin.

1.5.3.1. Molecular signaling pathways associated with ghrelin receptor

Ghrelin biding to GHS-R1a cause conformational changes of intracellular loops of the receptors, which expose binding sites to G proteins. The coupling of G proteins to GHS-R1a promotes guanosine diphosphate (GDP) release and guanosine triphosphate (GTP) binding to the G protein α subunit, thus activating G protein subunits to initiate intracellular signaling cascades by acting on various downstream effectors. The signaling transduction mechanisms

underlying ghrelin function involve the regulation of ionic currents and protein phosphorylation-based intracellular signaling (Gao *et al.,* 2007, Muccioli *et al.,* 2007). Once bound to ghrelin, activated GHS-R1a normally binds the Gαq/11 subunit of a G-protein, which leads to activation of phosphatidylinositol-specific phospholipase C (PI-PLC) to generate inositol 1,4,5-triphosphate (IP_3) and diacylglicerol (DAG) from phosphatidylinositol 4,5-diphosphate (PIP_2) (Howard *et al.,* 1996, Smith *et al.,* 1997). The intracellular free Ca^{2+} concentration increases because of the rapid, though transient, release of Ca^{2+} from IP_3-responsive cytoplasmatic storage pools in the ER. Increased Ca^{2+} and DAG levels activate PKC, which, in turn, inhibits the K^+ channels, causing depolarization (Camiña 2006). This depolarization causes the opening of voltage-dependent L-type Ca^{2+} channels, causing more sustained accumulation of intracellular Ca^{2+}. Together with the blockade of K^+ channels, the intracellular rise in free Ca^{2+} exacerbates the cellular depolarization. Ghrelin also activates MAPK and PI_3K cascades in different cellular systems to promote proliferation (Kim *et al.,* 2004a, 2004b, Mazzocchi *et al.,* 2004). Moreover, ghrelin induces an increase in intracellular Ca^{2+} concentration through N-type Ca^{2+} channels influx in isolated NPY producing hypothalamic neurons (Kohno *et al.,* 2003). Because N-type Ca^{2+} channels are modulated by cyclic adenosine monophosphate (cAMP)-dependent PKA activation, this suggests that GHS-R1a is coupled to Gs in NPY-producing neurons in the hypothalamus (Kohno *et al.,* 2003). Also in the hypothalamus, it was found that ghrelin enhances the activity of 5'-AMP-activated protein kinase (AMPK) (Andersson *et al.,* 2004). AMPK activity is strongly implicated in energy homeostasis and is downregulated by leptin administration in the hypothalamus (Carling 2005). However, the molecular mechanism by which ghrelin regulates AMPK is still unknown. Finally, ghrelin can also inhibit vascular inflammation through the activation of the calmodulin-dependent kinase kinase (CaMKK), AMPK and endothelial nitric oxide synthase (eNOS). Altogether, these observations suggest that GHS-R1a activation is coupled to several signaling pathways, and subsequent signaling effectors, in particular PKA, PKC, PI_3K, and MAPK.

1.5.4. Physiological functions of ghrelin

Ghrelin is currently the only described orexigenic hormone from the periphery, which acts centrally to modulate the energy homeostasis (Kojima *et al.*, 2010, Sato *et al.*, 2012). The important role of ghrelin in the regulation of appetite and satiety is well established, and the orexigenic effects of peripheral or central ghrelin administration are widely documented in rodents (Wren *et al.*, 2001b, Finger *et al.*, 2011), as well as in (lean and obese) humans (Wren *et al.*, 2001a, Druce *et al.*, 2005). In other hand, recent studies demonstrating that ghrelin is also involved in neuroprotection (Lago *et al.*, 2005, Ferrini *et al.*, 2009, Bayliss *et al.*, 2013),

protection achieved by the activation of several mechanisms, such as inhibition of ROS generation, stabilization of mitochondrial transmembrane potential, increase of the Bcl-2/Bax ratio, prevention of cytochrome c release, and inhibition of caspase 3 activation (Chung *et al.*, 2007, Ferrini *et al.*, 2009). Ghrelin may also acts in memory and learning processes (Nakazao *et al.*, 2001, Miao *et al.,* 2007, Ferrini *et al.*, 2009, Moon *et al.*, 2011).

1.6. Main Objectives

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. CR delays the aging and improves resistance to disease in yeast to primates. These beneficial effects in mammals include the prevention of age-associated cognitive impairment and neurodegeneration. Autophagy is known to mediate lifespan extension induced by CR. Although CR induces hypothalamic NPY expression, a potent neuroprotective agent in several brain areas, it was not known whether NPY could play a role in autophagy regulation in the brain. Recently, our group found that NPY not only induces autophagy in hypothalamic neurons but also mediates nutrient deprivation induced autophagy in these cells, suggesting that NPY may mediate CR's effects on autophagy. However, its potential role in lifespan extension is not known. Even though NPY produces vast physiological effects, mostly consistent with the ones observed after CR, the effect of NPY in autophagy in cortical neurons has not been studied yet. On the other hand, CR is known to increase the levels of ghrelin, an orexigenic peptide secreted by the stomach. Since ghrelin shares some of the physiological effects induced by CR it has been suggested as a metabolic regulator signal during CR. Therefore we hypothesize that ghrelin, similarly to NPY, could be involved in nutrient deprivation-induced autophagy in cortical neurons.

The aims of this study are: 1) to evaluate the effect of CR on autophagy in rat cortical neurons; 2) to evaluate the potential involvement of NPY and NPY receptors on CR-induced autophagy and the signaling pathways underlying this process; 3) to evaluate the effect of NPY on the regulation of autophagy in cortical neurons and to investigate by which NPY receptors subtypes and signaling pathways are involved in NPY-induced autophagy; 4) to evaluate the potential involvement of ghrelin and ghrelin receptor on CR-induced autophagy; 5) to evaluate the effect of ghrelin on autophagy in cortical neurons.

Elucidation of the molecular mechanisms whereby nutritional/metabolic cues impinge on neuronal survival and health may be an avenue to new pharmacological strategies, that exploit nutrient-sensitive protective circuitries to prevent the catastrophic impact of aging and

dysmetabolism on the brain. Understanding how CR, NPY and ghrelin may modulate autophagy-related proteins and autophagic flux in cortical neurons, should provide a basis for more comprehensive approaches, in the future, aiming at a better and deeper perception of the mechanisms regulating autophagy, in the brain.

Chapter II

Materials and Methods

2.1. Materials and reagents

Neurobasal medium, fetal bovine serum (FBS), penicillin-streptomycin, B27 supplement, L-glutamine, trypsin, Hoechst 33342, Alexa-Fluor 488-conjugated goat anti-rabbit immunoglobulin G (IgG) and Alexa-Fluor 594-conjugated-goat anti-mouse IgG secondary antibodies from Molecular Probes were obtained from Life Technologies (from Invitrogen) (Carlsbad, CA, USA). Dulbecco's Modified Eagle Medium (DMEM), DNAse I, poly-D-lysine, chloroquine, rapamycin, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phenylmethylsulfonyl fluoride (PMSF), mouse monoclonal anti-microtubule-associated protein 2 (MAP2) and anti-β-tubulin I antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). Neuropeptide Y (NPY) was obtained from Phoenix Europe GmbH (Karlsruhe, Germany). NPY receptors antagonists BIBP3226 (NPY receptor Y_1 antagonist), BIIE0246 (NPY receptor Y_2 antagonist) and L-152,804 (NPY receptor Y_5 antagonist) and ghrelin receptor antagonist [D-Lys^3]-GHRP-6 were purchased from Tocris Bioscience (Bristol, UK). Ghrelin and NPY receptors agonists Leu^{31}Pro^{34} (Y_1agonist), NPY_{13-36} (Y_2 agonist), r-PP (Y_4 agonist) and NPY_{19-23}(Gly^1, Ser^3, Gln^4, Thr^6, Ala^{31}, Aib^{32}, Gln^{34})PP (Y_5 agonist) were purchased from Bachem (Bubendorf, Switzerland). Ghrelin was obtained from Bachem (Bubendorf, Switzerland). The bicinchoninic acid (BCA) protein quantification assay kit was purchased from Pierce/Thermo Fisher Scientific (Rockford, IL, USA). The Complete Mini protease inhibitor cocktail tablets were purchased to Roche Diagnostics (Basel, Switzerland). Electrophoresis and Western Blotting systems as well as sodium dodecyl sulphate (SDS), 30 % acrylamide/Bis-acrylamide and precision plus protein dual color standards, used for molecular weight marking, were obtained from Bio-Rad (Hercules, CA, USA). Mouse monoclonal anti-glial fibrillary acidic protein (GFAP) and anti-NeuN antibodies as well as polyvinylidene fluoride (PVDF) membranes and rabbit polyclonal antibody against GHS-R1a were purchased from Millipore (Billerica, MA, USA). Rabbit polyclonal antibodies against LC-3B, p62/SQSTM1, mTOR and phosphorylated-mTOR (Ser2448) were obtained from Cell Signaling Technology (Beverly, MA, USA). Alkaline phosphatase-linked goat anti-rabbit and anti-mouse IgG and enhanced chemifluorescence (ECF) reagent was purchased from GE Healthcare (Buckinghamshire, UK). Fluorescence mounting medium was purchased from Dako (Glostrup, Denmark). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Merck KGaA (Darmstadt, Germany).

2.2. Animals

Female Wistar rats were purchased from Charles River Laboratories (Wilmington, MA, USA). All experimental procedures were performed in accordance to the guidelines of the

European Community for the use of animals in laboratory (2010/63/EU) and the Portuguese law for the care and use of experimental animals (DL n.° 129/92 and norm 1005/92). In addition, animals were 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. Center for Neurosciences and Cellular Biology animal experimentation board approved the utilization of animals for this project (reference PTDC/SAU-FCF/099082/2008).

2.3. Cellular models

2.3.1. Primary rat cortical neurons culture

The experiments were performed on primary cultures of rat cortical neurons, as previously described (Sciarretta *et al.*, 2010), with minor modifications. Briefly, embryonic cortical tissue was isolated from rat embryos with 18-19 days (E18-19) of gestation. The pregnant females were sacrificed by cervical dislocation and subjected to cesarean section in order to remove the uterine horns containing the embryos. The brains were removed from the skull and transferred to a petri dish containing phosphate buffered saline (PBS) solution supplemented with 5.5 mM glucose, 100 $U.mL^{-1}$ penicillin and 100 $\mu g.mL^{-1}$ streptomycin (dissection buffer). Cortices were dissected and meninges thoroughly removed. Cortical tissue was placed in a conical tube with dissection solution and centrifuged at 59 g, for 4 minutes. The supernatant was discarded and cortices were ressuspended in a 0.25 % trypsin solution and then incubated in a water-bath (37 °C) for 15 min. After digestion, 50 $\mu L.mL^{-1}$ DNAse I (5 $mg.mL^{-1}$) were added to the tube with tissue/trypsin. After centrifugation (59 g, for 1 min, room temperature), the supernatant was taken off immediately and the pellet was covered with 3 mL of FBS. The cortical tissue was gently triturated by using a glass Pasteur pipette and the dissociated cortical tissue was let stand undisturbed for 3 min to allow for the cell debris to settle down. The supernatant, containing the cell suspension, was then centrifuged at 59 g, for 4 min. The supernatant was discarded and cells were ressuspended in 5 mL of neurobasal medium, supplemented with 500 µM L-glutamine, 2 % B27 suplement, 100 $U.mL^{-1}$ penicillin and 100 $\mu g.mL^{-1}$ streptomycin. Cell density was determined after tripan-blue staining in a hemocytometer. The cells were plated at a density of 132,000 $cells.cm^{-2}$ on 24-well cell culture plates (cell viability assay), 12-well cell culture plates with 16 mm coverslips (immunocytochemistry) or 60 mm cell culture dishes (protein cell lysates), precoated with poly-D-lysine. The cells were maintained at 37 °C in a humidified incubator with 5 % CO_2/air for

8 days. The cell culture medium was replaced every fourth day by aspirating half of the medium from each well and replacing it with fresh medium.

2.4. Cell treatments

2.4.1. Caloric restriction mimetic condition

To investigate the effect of caloric restriction on autophagy in cortical neurons, primary rat cortical neuronal cultures were subjected to nutrient deprivation to mimic a caloric restriction condition. Cortical neurons were exposed to DMEM low glucose medium (1 $g.L^{-1}$ glucose, 100 $U.mL^{-1}$ penicillin and 100 $\mu g.mL^{-1}$ streptomycin, without B27 supplementation), for 0.5, 1, 3, 6 and 24 hours. After determining the best nutrient deprivation condition (higher cell viability and autophagy induction), a 6 h exposure to DMEM low glucose was chosen for further experiments. In order to confirm that an efficient autophagic flux occured under nutrient deprivation conditions, chloroquine, a lysossomal protein degradation inhibitor, was added to the cell culture medium 30 min prior to nutrient deprivation treatment, to a final concentration of 100 µM. To determine if NPY plays a role in nutrient deprivation-induced autophagy in cortical neurons, NPY receptors selective antagonists were used: Y_1 antagonist (BIBP3226), Y_2 antagonist (BIIE024) and Y_5 antagonist (L-152,804). Each receptor antagonist was added to the cell culture medium to a final concentration of 1 µM, 30 min before nutrient deprivation treatment. To further determine the molecular pathways involved in nutrient deprivation-induced autophagy, cortical neurons were exposed to PI_3K inhibitor (LY294002), ERK 1/2 inhibitor (U0126) or PKA inhibitor (H89). Each protein kinase inhibitor was added individually to the cell culture medium to a final concentration of 1 µM, 30 min before nutrient deprivation treatment. In order to determine if ghrelin plays a role in nutrient deprivation-induced autophagy in cortical neurons, ghrelin receptor selective antagonist ([D-Lys^3]-GHRP-6) was used. [D-Lys^3]-GHRP-6 was added to the cell culture medium to a final concentration of 100 µM, 30 min before ghrelin treatment. Rapamycin (100 nM, 1 h) was used as a positive control of autophagy induction.

2.4.2. Neuropeptide Y

To study the role of NPY in autophagy induction in cortical neurons, primary cell cultures of rat cortical neurons were exposed to 100 nM NPY, for 0.5, 1, 3, 6 and 24 h. In order to confirm that NPY treatment increases the autophagic flux in cortical neurons, cells were treated with chloroquine (100 µM), 30 min prior to NPY treatment. To assess which NPY receptor subtype modulates the NPY effect on autophagy induction, different NPY receptor

selective antagonists were used: Y_1 antagonist (BIBP3226), Y_2 antagonist (BIIE0246) and Y_5 antagonist (L-152,804). Each receptor antagonist was added individually to the cell culture medium to a final concentration of 1 µM, 30 min before NPY treatment. To confirm which NPY receptor subtypes were involved in NPY-induced autophagy in cortical neurons, different NPY receptor selective agonists were used: Y_1 agonist ($Leu^{31}Pro^{34}$), Y_2 agonist (NPY_{13-36}), Y_4 agonist (r-PP) and Y_5 agonist ($NPY_{19-23}(Gly^1, Ser^3, Gln^4, Thr^6, Ala^{31}, Aib^{32}, Gln^{34})PP$). Each agonist was added individually to the cell culture medium to a final concentration of 100 nM for 6 h. To further determine the molecular pathways involved in NPY-induced autophagy, cortical neurons were incubated with PI_3K inhibitor (LY294002), ERK 1/2 inhibitor (U0126) or PKA inhibitor (H89). Each inhibitor was added individually to the cell culture medium to a final concentration of 1 µM, 30 min before NPY treatment. Rapamycin (100 nM, 1 h) was used as a positive control of autophagy induction.

2.4.3. Ghrelin

To investigate the effect of ghrelin on autophagy induction in cortical neurons, primary cell cultures of rat cortical neurons were exposed to 10 nM ghrelin for 6 and 24 h. In order to confirm that ghrelin increases the autophagic flux in cortical neurons, chloroquine (100 µM) was added to the cell culture medium 30 min prior to ghrelin treatment. To assess if ghrelin receptor GHS-R1a modulates the ghrelin effect on autophagy induction, the ghrelin receptor antagonist ([D-Lys^3]-GHRP-6) was used. [D-Lys^3]-GHRP-6 was added to the cell culture medium to a final concentration of 100 µM, 30 min before ghrelin treatment. Rapamycin (100 nM, 1h) was used as a positive control of autophagy induction.

2.5. Cell viability assay

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. This colorimetric assay allows cell viability measurement, since the yellow MTT tetrazole crystals are reduced to purple formazan crystals by mitochondrial reductases of live cells. MTT was added to the cell culture medium to a final concentration of 0.5 $mg.mL^{-1}$ and cells were incubated during 1.5 h, at 37 °C in a humidified incubator with 5 % CO_2/air. The medium was removed and the formazan crystals were dissolved in 0.04 M HCl/isopropanol. The volume used of 0.04 M HCl/isopropanol solution depended on the amount of precipitate (MTT reduction extension) and was the same for each well (300 to 600 µL) in each independent experiment. Two hundred microliters from each well were transferred

to a 96-well plate for absorbance reading in a spectrophotometer (SLT spectra II), at 570 nm, using a reference filter at 620 nm. All experiments were carried out in triplicate.

2.6. Gene expression analysis

2.6.1. Purification and quantification of total RNA

The total ribonucleic acid (RNA) was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Briefly, cells were disrupted with buffer RLT and homogenized by pipetting up and down to disrupt cell clumps. The lysate was then placed in a QIAshredder homogenizer spin column and centrifuged for 2 min at 14,100 g. Afterwards, 70 % ethanol was added to the homogenized lysate which was then transferred to an RNeasy spin column and centrifuged for 15 seconds at 8,000 g. Total RNA was adsorbed to a silica matrix, washed with the recommended buffers and eluted with 30 µL of RNase-free water by centrifugation for 1 min at 8,000 g. The total RNA was quantified by optical density (OD) measurements using a ND-1000 Nanodrop Spectrophotometer (Thermo Scientific), and the purity was evaluated by measuring the ratio of OD at 260 and 280 nm. RNA samples were treated with RNase-free DNAse (Qiagen) to eliminate any contamination with genomic DNA. RNA samples were kept at −80 °C until use.

2.6.2. Reverse transcription

Reverse transcription into cDNA was carried out using the iScript Select cDNA Synthesis Kit (Bio-Rad) following the manufacturer's instructions. Briefly, 1 µg of total RNA from each sample was reverse transcribed into cDNA in a 20 µL reaction containing 1x reaction buffer, 1x random primers, and 50 units of reverse transcriptase. Reverse transcription reactions were performed in a thermocycler at 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min, and 4 °C for 5 min. cDNA samples were then stored at -20 °C until use.

2.6.2. Polymerase chain-reaction

PCR was performed using specific primer pairs for rat NPY and NPY receptors. The primers were (forward and reverse and product size: Y_1, 5'-AACCTCTCCTTCTCAGACTTGC-3', 5'-CACAGTGTTGAAGATGGTAAGG-3' (616 bp); Y_2, 5'-CTCCAAGCAAATCAGCTTCC-3', 5'-GTTTTGTGCCTTCGCTGATGG-3' (318 bp); Y_4, 5'-AACCTACTCATTGCCAACCTG-3', 5'-ATGTAGCAGACCAGGATGAAG-3' (476 bp); Y_5, 5'-GTGTTCCCGAGGTGCTTCTA-3', 5'-ATTCCGAGCAGCAGCTGTAT-3' (248 bp) (Eurofins MWG Synthesis GmbH, Ebersberg, Germany). The primers for the reference rat gene (rat HPRT, NM-012583) was pre-designed

and validated by QIAGEN (QuantiTect Primers, Qiagen). As positive controls, wild type rat hypothalamic cDNA and rat retina cDNA were used. Negative controls were performed without RNA sample, which was substituted by water. All PCR reactions were performed in a 25 µL reaction containing 1x PCR Buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 U of DFS-(DNA Free Sensitive) Taq polymerase (Bioron), 250 nM of each gene specific primer and 1 µL of template cDNA. The reactions were performed in the following sequence of steps: 95 °C for 10 min, followed by 35 cycles at 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were separated by electrophoresis on 2 % agarose gels.

2.6.3. Quantitative real-time polymerase chain reaction

Quantitative real-time PCR was performed in an iQ_5 thermocycler (Bio-Rad) using 96-well microtiter plates and the QuantiTect SYBR Green PCR Master Mix (Qiagen). The primers for the target rat gene (NPY, NM-012614), (Ghrelin, NM-021669) and the reference gene (rat HPRT, NM-012583) were pre-designed and validated by QIAGEN (QuantiTect Primers, Qiagen). A master mix was prepared for each primer set, containing the appropriate volume of 2× QuantiTect SYBR Green PCR Master Mix and 10× QuantiTect Primer (both from QIAGEN). For each reaction, 18 µL of master mix were added to 2 µL of template cDNA. All reactions were performed in duplicate (two cDNA reactions per RNA sample) at a final volume of 20 µL per well. Negative controls were performed without RNA sample, which was substituted by water. The reactions were performed according to the manufacturer's recommendations: 95 °C for 15 min, followed by 40 cycles at 94 °C for 15 sec, 55 °C for 30 sec and 72 °C for 30 sec. The melting curve protocol started immediately after amplification. qRT-PCR products were run by electrophoresis on a 2% agarose gel containing GreenSafe, a DNA stain. The amplification efficiency for each gene and the threshold values for threshold cycle determination (Ct) were determined automatically by the iQ_5 Optical System Software (Bio-Rad). Relative mRNA quantification was performed using the ΔCt method for genes with the same amplification efficiency.

2.7. Protein expression analysis

2.7.1. 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 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; 0.5 % (w/v) deoxycholate 0.1 % (w/v) sodium dodecyl sulphate (SDS); 200 µM phenylmethylsulfonylfluoride (PMSF); 1 mM dithiothreitol (DTT); 1 mM sodium orthovanadate (ORTO); 10 mM sodium fluoride (NaF)], supplemented with complete mini protease inhibitor cocktail tablet. 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 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 $mg.mL^{-1}$) 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 % SDS (w/v), 0.6 M DTT, 0.012 % bromophenol blue (w/v)), boiled for 5 min at 95 °C and stored at -20 °C until use.

2.7.2. Western blotting

Western Blotting technique was used in order to immunodetect the expression of autophagy-related proteins. Equal amounts of protein were loaded per lane and proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), using 8 %-12 % gels. The electrophoresis was run on a Tris-Bicine buffer (25 mM Tris; 25 mM Bicine; 1 % SDS (w/v); 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 PVDF membranes, in CAPS transfer buffer (10 mM CAPS, pH 11.0; 10% (v/v) methanol), at a 750 mA constant current, for 2.5 h, at 4 °C. Afterwards, the membranes were blocked for 1 h at room temperature (RT), in 5 % (w/v) non-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: rabbit polyclonal anti-LC-3B, anti-p62/SQSTM1, anti-phosphomTOR (Ser2448) and anti-mTOR. After the incubation, the membranes were washed with TBS-T and incubated with an alkaline phosphatase-linked secondary antibody, specific to rabbit IgG, in a 1:10,000 dilution in TBS-T with 5 % (w/v) BSA, for 1 h, at RT. The membranes were then washed in TBS-T and protein immunoreactive bands were visualized by chemifluorescence using the ECF substrate. Fluorescence was detected on Versa Doc Imaging System (Bio-Rad). The optical density of the bands was quantified with the Quantity One Software (Bio-Rad). For protein loading control, the membranes were reprobed overnight with a mouse monoclonal anti β-tubulin I immunoreactivity (Sigma, T7816), in a

1:1,000 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, in a 1:10,000 dilution in TBS-T with 5 % (w/v) non-fat milk, for 1 h, RT.

2.7.3. Immunocytochemistry

After the treatments, cells were washed twice with pre-warmed PBS (pH 7.4) and then fixed with ice-cold 4 % paraformaldehyde/PBS for 15 min. Cells were permeabilized with 0.25 % (v/v) TX-100/PBS for 10 min, washed in PBS, and blocked for 1 h in 10 % goat serum/PBS. The cells were incubated with primary antibodies overnight at 4 °C. After incubation, cells were washed in PBS and incubated for 1 h at room temperature with the respective secondary antibodies. The nuclei were stained with Hoechst 33342 (2 $\mu g.mL^{-1}$) during secondary antibody incubation. The coverslips were washed in PBS and mounted on glass slides with Dako Fluorescence Mounting Medium. The primary antibodies used were: rabbit anti-LC-3B (1:400), mouse anti-MAP2 (1:500), mouse anti-NeuN (1:400), rabbit anti-TUJ1 (1:500) and mouse anti-GFAP (1:500). 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. Cells were analyzed on a Zeiss Axiovert fluorescence microscope (Carl Zeiss, Oberkochen, Germany). The procedure was performed for three independent culture preparations.

2.8. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Data were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's post test, or Student's unpaired t test with two-tailed p value, as indicated in figure legends. A value of $p<0.05$ was considered significant. Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used for all statistical analysis.

Chapter III

Results

3.1. Characterization of primary rat cortical neurons cultures

In order to characterize the primary rat cortical neuronal cultures, cells were maintained in culture for 8 days in neurobasal medium. The expression of neuron specific markers, such as β–III tubulin (TUJ1; axons plus dendrites), MAP2 (dendrites) or NeuN (neuronal nuclei) and the glial specific marker GFAP were evaluated. Overall, these cultures show >90% neuronal population stained with β-III tubulin, MAP2 or NeuN, with an extensive neurite outgrowth as expected of healthy neurons (Figure 3.1A and B). Given that these rat cortical neuronal cultures are high density cell cultures with a high neurite extension complexicity, we decided to use the neuronal marker MAP2 in the further experiments described in this study. The culture also presents a minimum number of astrocytes labeled with GFAP in Figure 3.1C.

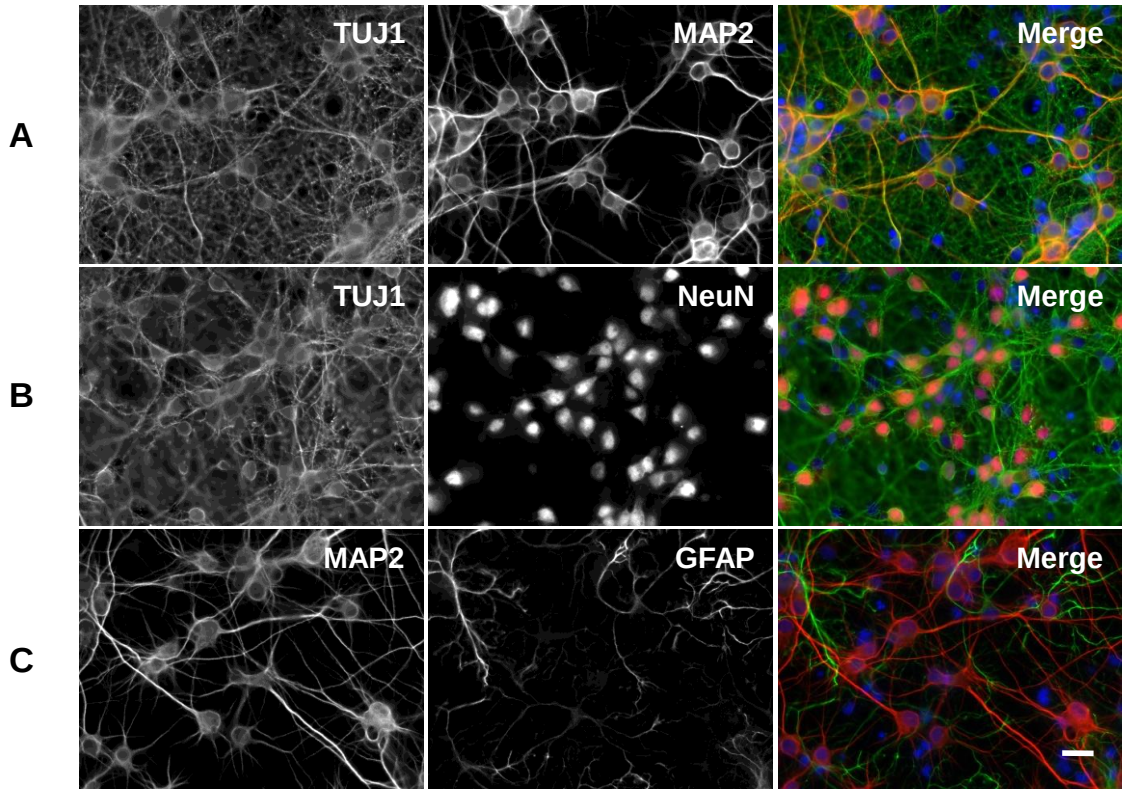

Figure 3.1 – Characterization of primary rat cortical neurons cultures. Primary rat cortical neuronal cultures were maintained in culture for 8 days, and were immunolabeled for (A) TUJ1 (green, β–III tubulin) and MAP2 (red, neurons), (B) TUJ1 (green, β–III tubulin) and NeuN (red, neuronal nuclei) and (C) MAP2 (red, neurons) and GFAP (green, glial cells). Nuclei were stained with Hoechst 33342 (blue). Characterization of primary rat cortical neurons cultures was assessed by immunocytochemistry assay, as described in Materials and Methods (see Chapter II). Figures are representative of 3 independents experiments. Scale bar, 20 µM.

3.2. Caloric restriction mimetic condition

3.2.1. Nutrient deprivation induces autophagy in cortical neurons

In order to assess the effect of nutrient deprivation on the viability of primary rat cortical neuronal cultures, cells were exposed to DMEM low glucose medium (1 $g.L^{-1}$ glucose, 100 $U.mL^{-1}$ penicillin and 100 $\mu g.mL^{-1}$ streptomycin, without B27 supplementation), to mimic a

caloric restriction condition for 6 and 24 h. Cell viability was assessed by the MTT reduction assay. As shown in Figure 3.2, nutrient deprivation did not significantly change cell viability after a 6 and 24 h treatments.

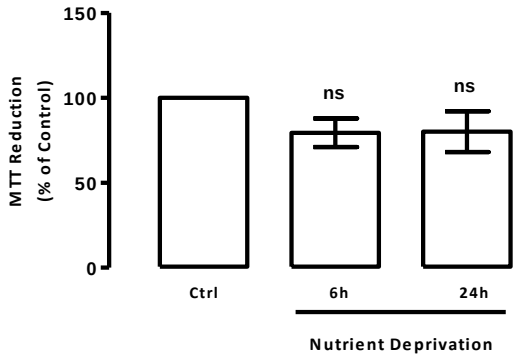

Figure 3.2 - Effects of nutrient deprivation does not change rat cortical neurons viability. Primary rat cortical neuronal cultures were incubated with nutrient deprivation mimetic medium (DMEM low glucose medium) for 6 h or 24 h. Untreated cells were used as control (Ctrl). Cell viability was assessed by the MTT reduction assay, as described in Materials and Methods (see Chapter II). The results represent the mean ± SEM of 3 independents experiments and are expressed as percentage of control. Student's t test. ns, not statistically different.

To further evaluate the autophagy-induction potential of nutrient deprivation, primary rat cortical neuronal cultures were exposed to DMEM low glucose medium for 30 min, 1, 3 or 6 h. Rapamycin (100 nM, 1 h), a known inductor of autophagy, was used as positive control. As shown in Figure 3.3A, nutrient deprivation induced an increase of the autophagic flux in cortical neurons, since the ratio of LC-3BII/β-tubulin increased in a time-dependent manner upon nutrient deprivation treatment (30 min: 112.4±7.5%, 1 h: 137.4±16.9%, 3 h: 157.0±16.3%, 6 h: 176.5±11.8% of control). Rapamycin induced an increase in LC-3BII/β-tubulin ratio (131.6±2.8% of control; Figure 3.3A). The effect of nutrient deprivation on the protein levels of p62/SQSTM1, a protein involved in regulation of autophagosome formation was also evaluated. Concomitant with the increase in LC-3BII/β-tubulin ratio, the protein content of p62/SQSTM1 significantly decreased after 6 h (83.6±5.8% of control) of nutrient deprivation exposure, as shown in Figure 3.3B. Rapamycin incubation also led to a decrease in p62/SQSTM1 protein (82.4±5.0% of control).

In order to confirm that nutrient deprivation induced an efficient autophagic flux, the LC-3BII/β-tubulin ratio and p62/SQSTM1 protein content in the presence of chloroquine, which inhibits lysosomal acidification and blocks the activity of lysosomal hydrolases, i.e., inhibiting lysosomal degradation (Barth *et al.,* 2010). Since LC-3BII and p62/SQSTM1 are degraded at the final stages of autophagy, chloroquine would impair this degradation, thus leading to an increased LC-3BII/β-tubulin ratio and accumulation of p62/SQSTM1. Primary rat cortical

neurons were pre-treated with chloroquine (100 μM) 30 min prior to nutrient deprivation treatment for 6 h. As shown in Figure 3.3C, cells under nutrient deprivation had an increased LC-3BII/β-tubulin ratio (159.9±9.1% of control) compared to untreated cells. Moreover, chloroquine induced a further increase (338.3±24.0% of control) when cells were incubated with nutrient deprivation medium. Chloroquine also increased p62/SQSTM1 levels in cells exposed to nutrient deprivation (132.9±10.9% of control) compared to cells under nutrient deprivation (79.0±4.1% of control), as shown in Figure 3.3D. These results indicate that autophagic flux is enhanced by nutrient deprivation in cortical neurons.

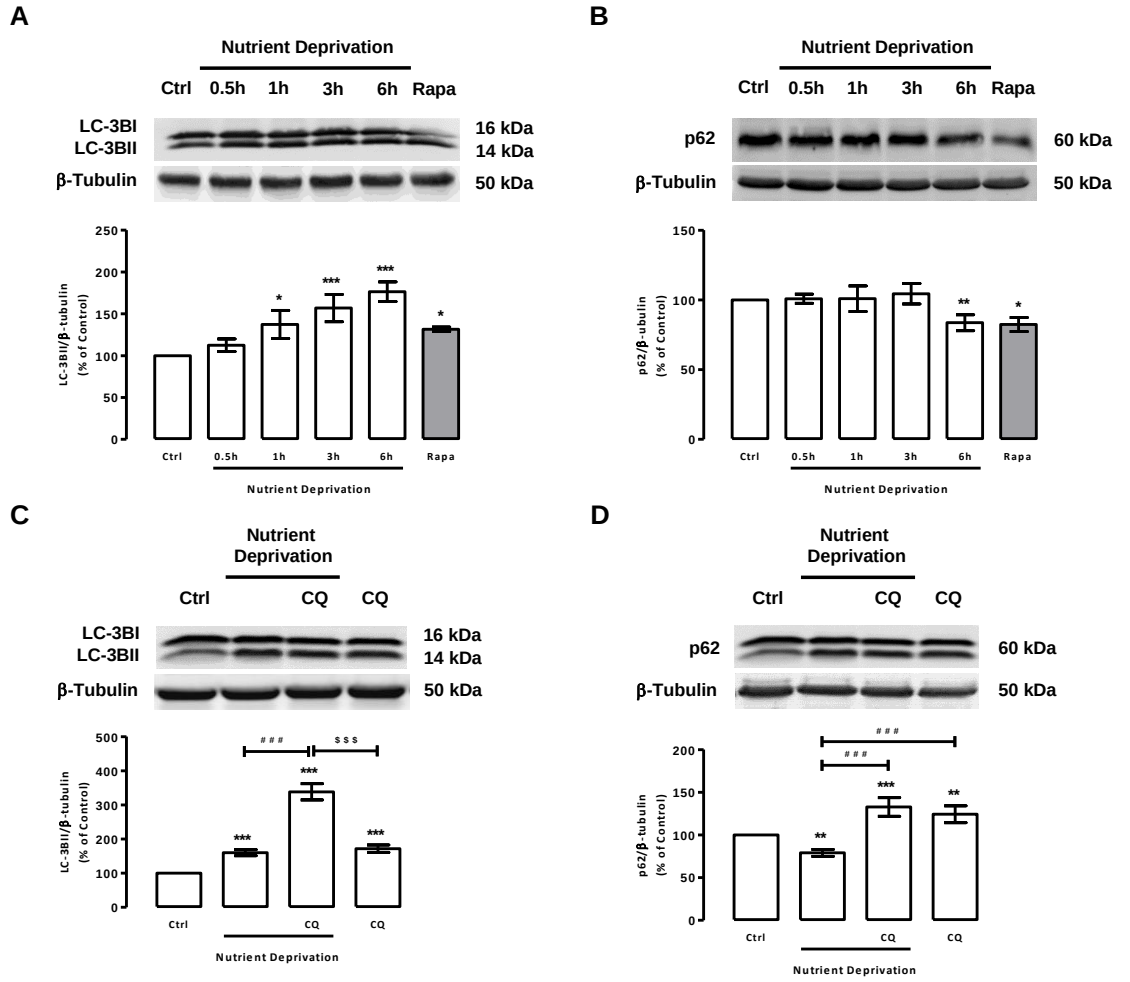

Figure 3.3 – Nutrient deprivation induces autophagy in rat cortical neurons. (A and B) Primary rat cortical neuronal cultures were incubated with nutrient deprivation mimetic medium (DMEM low glucose medium) for 30 min, 1 h, 3 h or 6 h. Untreated cells were used as control (Ctrl), and cells treated with rapamycin (Rapa, 100 nM) for 1 h were used as positive control of autophagy induction. (C and D) Primary rat cortical neuronal cultures were incubated with chloroquine (CQ, 100 μM), a lysosomal degradation inhibitor, 30 min before nutrient deprivation treatment for 6 h. Untreated cells were used as control (Ctrl). Whole cell extracts were assayed for LC-3BII (A and C), p62/SQSTM1 (B and D) and β-tubulin (loading control) immunoreactivity through Western blotting analysis, as described in Materials and Methods (see Chapter II). Representative Western blots for each protein are presented above each respective graph. The results represent the mean ± SEM of, at least, 3 independents experiments, and are expressed as percentage of control. *p<0.05, **p<0.01 and ***p<0.001, significantly different compared to control; $^{\#\#\#}$p<0.001, significantly different from nutrient deprivation 6 h; $^{\$\$\$}$p<0.001, significantly different from chloroquine-treated cells, as determined by ANOVA, followed by Bonferroni's post test.

As already referred, autophagy is characterized by the formation of intracellular vesicles named autophagosomes, which present LC-3B, until later stages. Therefore, formation of intracellular small LC-3B puncta or aggregates is indicative of autophagy induction. In order to analyze if nutrient deprivation induces LC-3B puncta formation in primary rat cortical neurons, cells were exposed to DMEM low glucose medium for 6 h. As shown in Figure 3.4, LC-3B localization within the cells is mainly perinuclear and with a punctuate distribution. While untreated cells revealed a more diffuse LC-3B distribution, with few puncta, in nutrient deprivation treated cells, a clear increase in LC-3B puncta number can be observed.

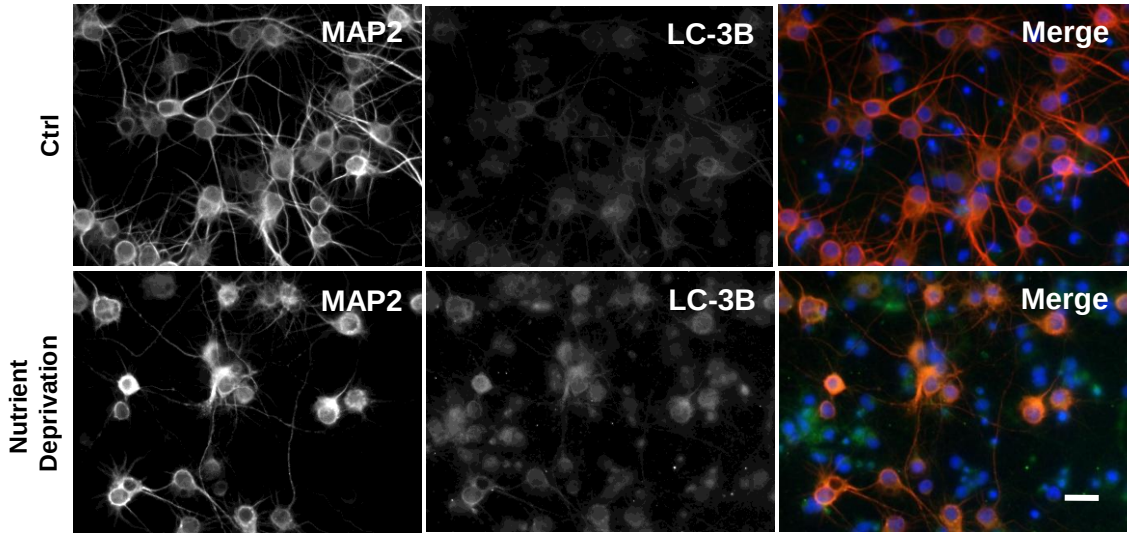

Figure 3.4 – Nutrient deprivation induces LC-3B puncta formation in rat cortical neurons. Primary rat cortical neuronal cultures were exposed to nutrient deprivation mimic medium (DMEM low glucose medium) for 6 h. Untreated cells were used as control (Ctrl). Cells were immunolabeled for LC-3B (green) and MAP2 (red, neurons). Nuclei were stained with Hoechst 33342 (blue). LC-3B puncta formation was assessed by immunocytochemistry assay, as described in Materials and Methods (see Chapter II). Figures are representative of 3 independents experiments. Scale bar, 20 μM.

3.2.2. Nutrient deprivation increases NPY levels in cortical neurons

Caloric restriction anti-aging effects are intimately tied to increased autophagy and alterations in the neuroendocrine system, such as the increase of NPY in the hypothalamus (Minor *et al.*, 2009). Preliminary data obtained by our group show that nutrient deprivation increases NPY expression in primary cultures of hypothalamic neurons. In addition, NPY not only induces autophagy, but also mediates nutrient deprivation-induced autophagy in hypothalamic neurons, supporting the hypothesis that NPY may mediate nutrient deprivation-induced autophagy. In line with these data and given that NPY may be expressed by cortical neurons, we hypothesized that NPY may also mediate nutrient deprivation effects on cortical neurons.

Firstly, to evaluate the presence of NPY and NPY receptors in cortical neurons, RT-PCR analysis was performed. To assess whether nutrient deprivation induce an increase in the expression levels of NPY in cortical neurons, cells were exposed to nutrient deprivation medium (DMEM low glucose medium) for 6 h, and total RNA was isolated and the transcript levels of NPY were analyzed by qRT-PCR. As shown in Figure 3.5A, nutrient deprivation increased NPY mRNA levels in primary rat cortical neurons (1.31±0.1 fold increase over control). As showed in Figure 3.5B, NPY Y_1, Y_2, Y_4 and Y_5 receptors mRNA are expressed by cortical neurons in culture.

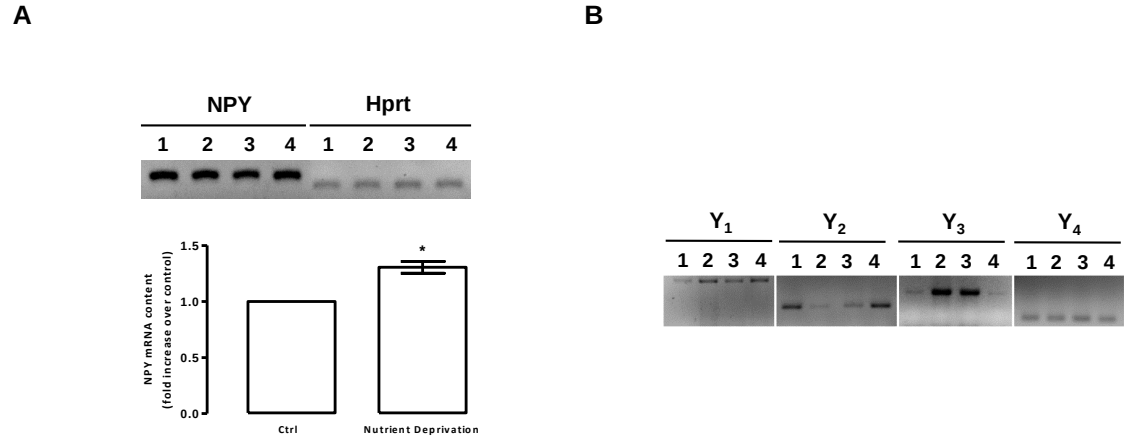

Figure 3.5 – Nutrient deprivation increases NPY mRNA levels in rat cortical neurons. (A) Primary rat cortical neuronal cultures were exposed to nutrient deprivation mimic medium (DMEM low glucose medium) for 6 h. Untreated cells were used as control (Ctrl). Total RNA was isolated, the transcript levels of NPY were analyzed by qRT-PCR and the resulting products were visualized by agarose gel electrophoresis, as described in Materials and Methods (see Chapter II). RT-PCR products (base pair (bp): NPY, 150bp and Hprt, 110bp, used an endogenous control. A representative image of 3 independent cell cultures is presented above the graph. The results represent the mean ± SEM of at 5 independents experiments and are expressed as the relative amount compared to control. *p<0.05, significantly different compared to control, as determined by Student's t test. (B) The presence of NPY receptors in rat cortical neurons was assessed by PCR, as described in Materials and Methods (see Chapter II). PCR products (base pair (bp): NPY Y_1, 616bp, NPY Y_2, 318bp, NPY Y_4, 476bp and NPY Y_5, 248bp. Legend: 1: primary cortical neuronal culture#1; 2: primary cortical neuronal culture#2; 3: rat cortex; 4: rat hypothalamus.

3.2.3. NPY Y_1, Y_2 or Y_5 receptors mediate autophagy induced by nutrient derivation

In order to assess the role of NPY in nutrient deprivation-induced autophagy, primary rat cortical neurons were incubated with different NPY receptors selective antagonists, NPY Y_1 antagonist (BIBP3226, 1 μM), NPY Y_2 antagonist (BIIE0246, 1 μM) and NPY Y_5 antagonist (L-152,804, 1 μM), 30 min prior the treatment with DMEM low glucose medium. After 6 h, LC-3BII/β-tubulin ratio and p62/SQSTM1 levels were evaluated by Western blotting. As shown in Figure 3.6A, the increase in LC-3BII/β-tubulin ratio induced by nutrient deprivation (162.5±10.0% of control) was significantly decreased in the presence of NPY receptor selective antagonists (Y_1: 109.0±7.3%, Y_2: 124.2±8.0% and Y_5: 121.7±11.6% of control). Associated to the increase of LC-3BII/β–tubulin ratio, nutrient deprivation induced a decrease in p62/SQSTM1

levels (79.1±3.7% of control), which was inhibited by NPY Y_1, Y_2 and Y_5 receptor selective antagonists (Y_1: 98.6±11.0%, Y_2: 97.3±9.9% and Y_5: 104.1±8.6% of control; Figure 3.6B). These results suggest that nutrient deprivation-induced autophagy in cortical neurons is mediated by NPY Y_1, Y_2 and Y_5 receptors activation.

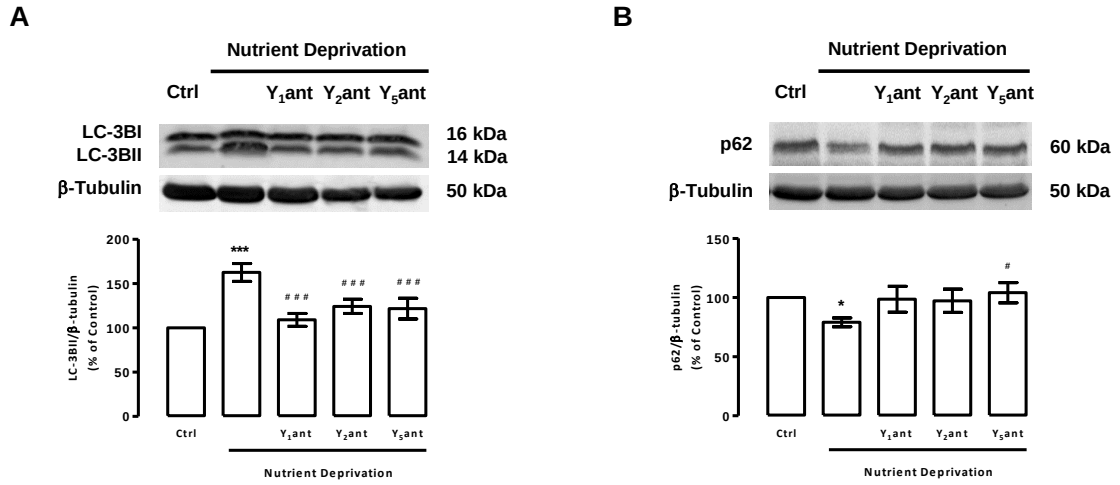

Figure 3.6 – NPY Y_1, Y_2 and Y_5 receptors mediate the effect of nutrient deprivation on autophagy induction. Primary rat cortical neuronal cultures were incubated with NPY Y_1 receptor antagonist BIBP3226 (Y_1ant, 1 μM), NPY Y_2 receptor antagonist BIIE0246 (Y_2ant, 1 μM) or NPY Y_5 receptor antagonist L152,800 (Y_5ant, 1 μM), 30 min before nutrient deprivation treatment for 6 h. Untreated cells were used as control (Ctrl). Whole cell extracts were assayed for LC-3BII (A), p62/SQSTM1 (B) and β-tubulin (loading control) immunoreactivity through Western blotting analysis, as described in Materials and Methods (see Chapter II). Representative Western blots for each protein are presented above each respective graph. The results represent the mean ± SEM of, at least, 3 independents experiments, and are expressed as percentage of control. *p<0.05, ***p<0.001, significantly different compared to control; $^{\#}$p<0.05 and $^{\#\#\#}$p<0.001, significantly different from nutrient deprivation 6 h, as determined by ANOVA, followed by Bonferroni's post test.

3.2.4. Molecular pathways involved in nutrient deprivation-induced autophagy

One of the molecular switches for autophagy induction is the inhibition of mTORC1 complex. The mTORC1 activity can be assessed by the analysis of mTOR phosphorylation at Ser2448, which is its active form. In order to evaluate if mTORC1 was being inhibited upon nutrient deprivation, primary rat cortical neurons were treated with DMEM low glucose medium for 30 min and 6 h and whole cell extracts were evaluated for phospho-mTOR (Ser2448) by Western blotting. As shown in Figure 3.7A, 30 min and 6 h of nutrient deprivation induced a phospho-mTOR decrease (78.7±3.5% and 60.2±6.3% of control, respectively). This result suggests that nutrient deprivation induces autophagy through the inhibition of mTORC1. However, several other pathways are known to regulate autophagy in mammalian cells. To further determine the molecular pathways involved in nutrient deprivation-induced autophagy, primary rat cortical neurons were incubated with PI_3K inhibitor (LY294002, 1 μM), ERK 1/2 inhibitor (U0126, 1μM) or PKA inhibitor (H89, 1 μM), in the presence or absence of

DMEM low glucose medium. After 6 h, LC-3BII/β-tubulin ratio and p62/SQSTM1 levels were evaluated by Western blotting. The increase of LC-3BII/β-tubulin ratio and the decrease of p62/SQSTM1 protein content induced by nutrient deprivation treatment were inhibited by all protein kinase inhibitors tested. As shown in Figure 3.7B, C and D, LC-3BII/β-tubulin ratio was increased in cells under nutrient deprivation (PI_3K: 169.4±9.6%, ERK: 183.2±9.4% and PKA: 167.5±14.3% of control) and this increase was inhibited in the presence of respective protein kinase inhibitor (PI_3K_i: 130.7±11.5%, ERK_i: 127.3±9.0% and PKA_i: 114.3±3.5% of control). As shown in Figure 3.7E, F and G, the decrease in p62/SQSTM1 levels induced by nutrient deprivation (PI_3K: 80.0±6.4%, ERK: 79.4±9.0% and PKA: 82.6±7.1% of control) was inhibited in the presence of respective protein kinase inhibitor (PI_3K_i: 96.8±3.9%, ERK_i: 88.5±5.1% and PKA_i: 88.7±2.0% of control). These results suggest that nutrient deprivation induces autophagy in rat cortical neurons through PI_3K, ERK 1/2 and PKA signaling pathways.

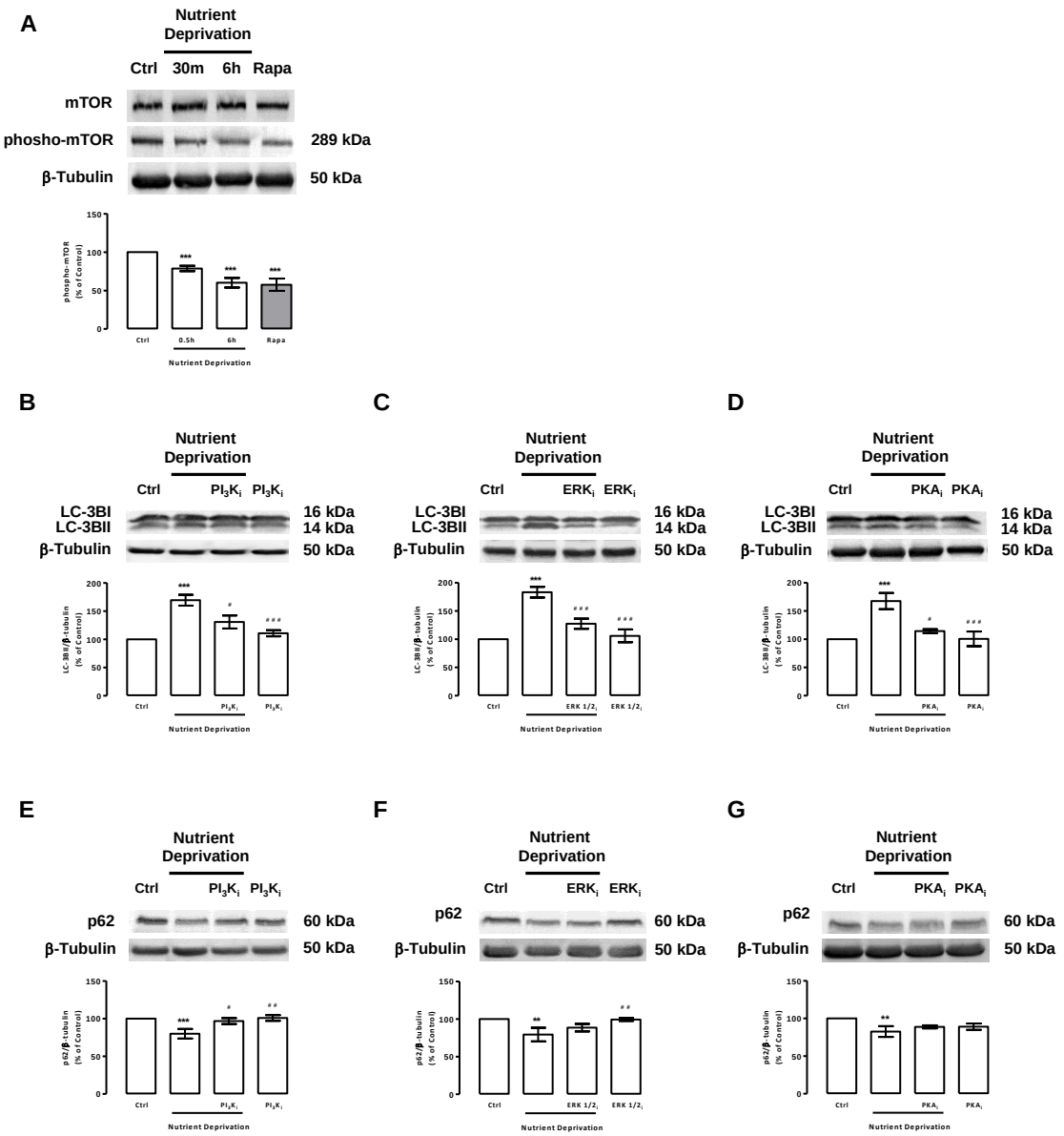

Figure 3.7 – Molecular pathways involved in nutrient deprivation induced autophagy in cortical neurons. (A) Nutrient deprivation induces autophagy via mTOR-dependent pathway. Primary rat cortical neuronal cultures were exposed to nutrient deprivation for 30 min or 6 h. Untreated cells were used as control (Ctrl). (B to G) The effect of nutrient deprivation on autophagy induction is mediated by PI_3K, ERK 1/2 and PKA. Primary rat cortical neuronal cultures were treated with PI_3K inhibitor (LY294002 (PI_3K_i), 1 μM), ERK 1/2 inhibitor (U0126 (ERK_i), 1 μM)) or PKA inhibitor (H89 (PKA_i), 1 μM), 30 min before nutrient deprivation treatment. Untreated cells were used as control (Ctrl). Whole cell extracts were assayed for phospho-mTOR (Ser2448) (A), LC-3BII (B, C and D), p62/SQSTM1 (E, F and G) and β-tubulin (loading control) immunoreactivity by Western blotting analysis, as described in Materials and Methods (see Chapter II). Representative Western blots for each protein are presented above each respective graph. The results represent the mean ± SEM of, at least, 3 independents experiments, and are expressed as percentage of control. **p<0.01 and ***p<0.001, significantly different compared to control; $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001, significantly different from nutrient deprivation 6 h, as determined by ANOVA, followed by Bonferroni's post test.

3.3. Neuropeptide Y

3.3.1. NPY induces autophagy in cortical neurons through NPY Y_1, Y_2 or Y_5 receptors activation

Since NPY mediates nutrient deprivation-induced autophagy, we hypothesize that NPY *per se* may induce autophagy in cortical neurons. To evaluate this hypothesis we first investigated the effect of NPY on viability of primary rat cortical neuronal cultures by the MTT reduction assay. Cells were exposed to NPY (100 nM), for 6 and 24 h. As shown in Figure 3.8, NPY had no effect on cortical neurons viability (6 h: 94.3±4.1% and 24 h: 107.2±1.5% of control).

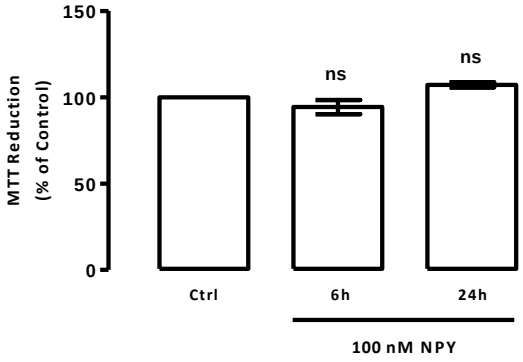

Figure 3.8 – NPY does not change rat cortical neurons viability. Primary rat cortical neuronal cultures were incubated with NPY (100 nM) for 6 or 24 h. Untreated cells were used as control (Ctrl). Cell viability was assessed by the MTT reduction assay, as described in Materials and Methods (see Chapter II). The results represent the mean ± SEM of 3 independents experiments, and are expressed as percentage of control. Student's t test. ns, not statistically different.

To evaluate the autophagy-induction potential of NPY, primary rat cortical neurons cultures were exposed to NPY (100 nM) for 30 min, 1 h, 3 h, 6 h or 24 h. Rapamycin (100 nM, 1 h), a known inductor of autophagy, was used as positive control of autophagy induction. As shown in Figure 3.9A, NPY induced an increase of the autophagic flux in cortical neurons since the ratio of LC-3BII/β-tubulin increased significantly upon 1 h (119.8±4.5% of control) and 3 h (114.9±2.7% of control) of treatment, but more markedly after 6h of NPY treatment (130.8±5.8% of control). However, the amount of LC-3BII/β-tubulin ratio decreased to basal levels after longer periods of incubation (101.8±5.6% of control), suggesting that is being degraded by the lysosome. Rapamycin induced an increase in LC-3BII/β-tubulin ratio (128.4±3.8% of control; Figure 3.9A). Concomitant with the increase of LC-3BII/β-tubulin ratio, the protein content of p62/SQTM1 was significantly decreased after 6 h (79.6±3.6% of control) of NPY treatment, as shown in Figure 3.9B. Rapamycin incubation also led to a decrease in p62/SQSTM1 protein (78.6±11.7% of control). In order to confirm that an efficient autophagic flux was occurring in NPY-treated cells, primary rat cortical neurons were treated with chloroquine (100 μM) 30 min prior the addition of NPY (100 nM) for 6 h. As observed

previously, cells treated with NPY had an increased LC-3BII/β-tubulin ratio (129.8±4.6% of control, Figure 3.9C) compared to untreated cells. However, in the presence of chloroquine, this increase was higher (277.7±28.2% of control) than in cells treated with NPY alone. Chloroquine treatment also increased p62/SQSTM1 levels in cells treated with NPY (111.9±1.6% of control) compared to untreated cells, as shown in Figure 3.9D. These results indicate that autophagic degradation is occurring and that NPY enhances autophagy in cortical neurons.

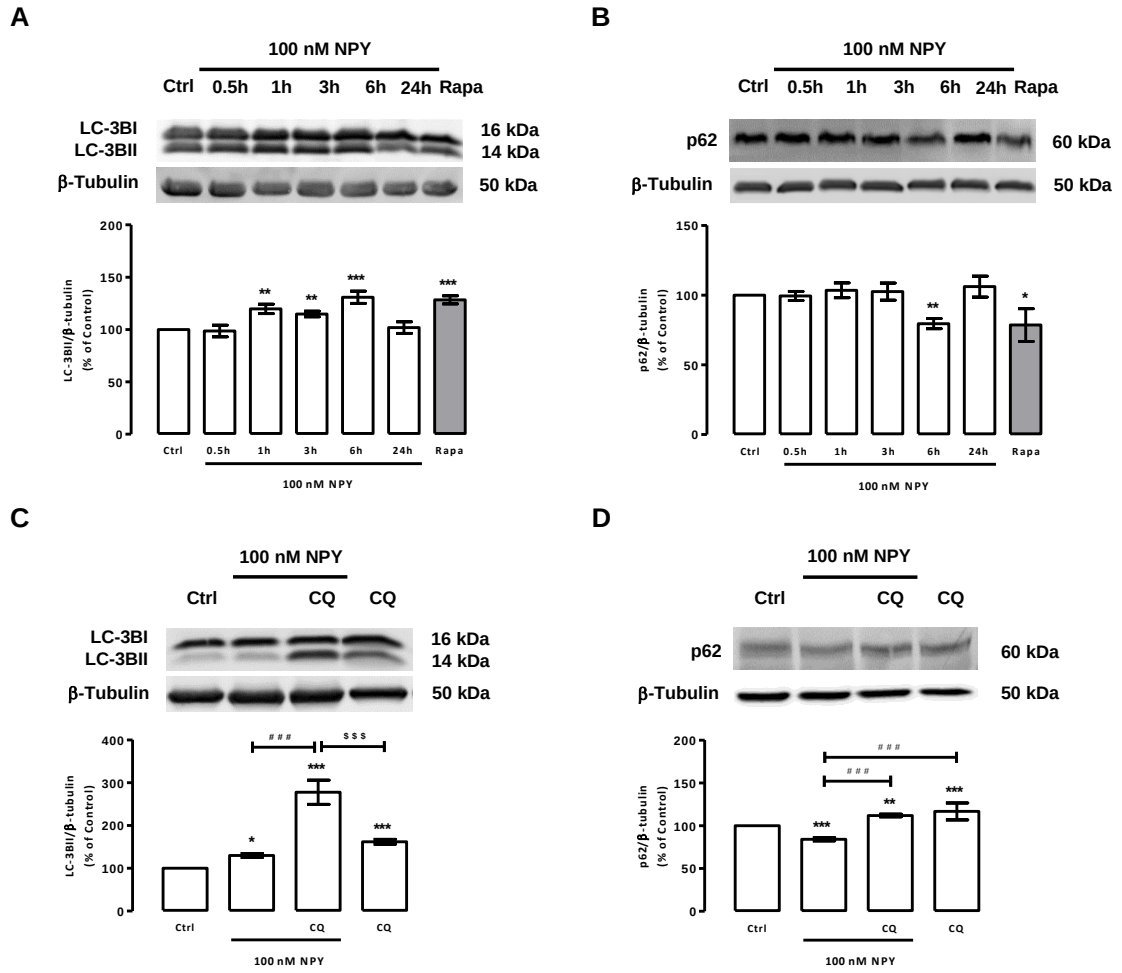

Figure 3.9 – NPY induces autophagy in rat cortical neurons. (A and B) Primary rat cortical neuronal cultures were incubated with NPY (100 nM) for 30 min, 1 h, 3 h, 6 h or 24 h. Untreated cells were used as control (Ctrl), and cells treated with rapamycin (Rapa, 100 nM) for 1 h were used as positive control of autophagy. (C and D) Primary rat cortical neuronal cultures were incubated with chloroquine (CQ, 100 μM), a lysosomal degradation inhibitor, 30 min before NPY (100 nM) treatment for 6 h. Untreated cells were used as control (Ctrl). Whole cell extracts were assayed for LC-3BII (A and C), p62/SQSTM1 (B and D) and β-tubulin (loading control) immunoreactivity through Western blotting analysis, as described in Materials and Methods (see Chapter II). Representative Western blots for each protein are presented above each respective graph. The results represent the mean ± SEM of, at least, 3 independents experiments, and are expressed as percentage of control. *p<0.05, **p<0.01 and ***p<0.001, significantly different compared to control; $^{###}$p<0.001, significantly different from NPY treatment for 6 h; $^{\$\$\$}$p<0.001, significantly different from chloroquine-treated cells, as determined by ANOVA, followed by Bonferroni's post test.

LC-3B immunoreactivity in primary rat cortical neurons was also evaluated by immunocytochemistry, when cells were exposed to 100 nM NPY for 6 h. As shown in Figure 3.10, LC-3B localization within the cells is mainly perinuclear and with a punctuate distribution. While untreated cells revealed a more diffuse LC-3B distribution, with few puncta, in nutrient deprivation condition, an increase in LC-3B puncta number was observed.

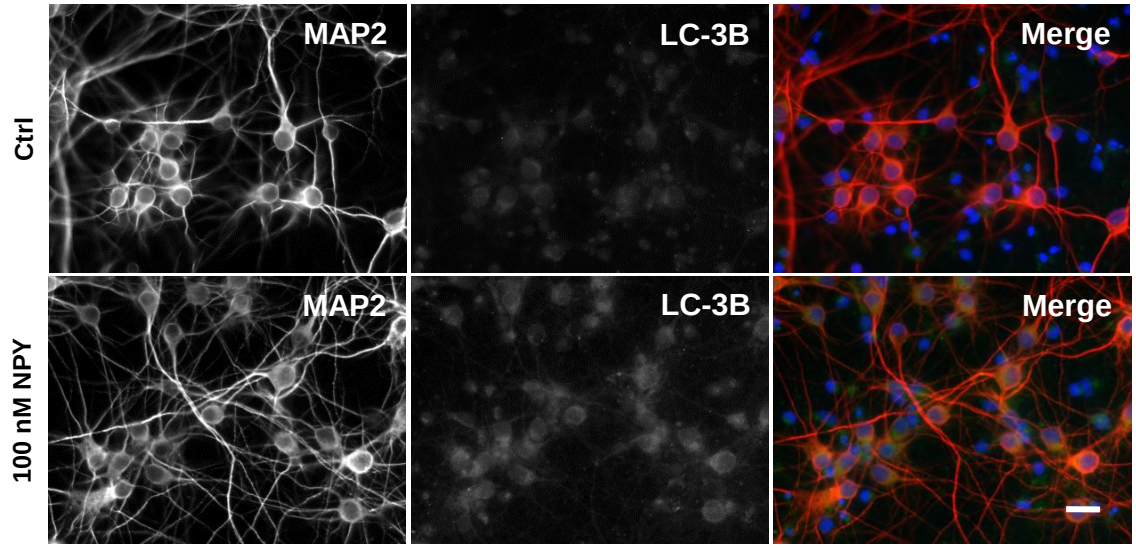

Figure 3.10 – NPY induces LC-3B puncta accumulation in rat cortical neurons. Primary rat cortical neuronal cultures were exposed to NPY (100 nM) for 6 h. Untreated cells were used as control (Ctrl). Cells were immunolabeled for LC-3B (green) and MAP2 (red, neurons). Nuclei were stained with Hoechst 33342 (blue). LC-3B puncta formation was assessed by immunocytochemistry assay, as described in Materials and Methods (see Chapter II). Figures are representative of 3 independents experiments. Scale bar, 20 µM.

In order to evaluate which NPY receptor(s) subtype(s) are involved in NPY signaling, primary rat cortical neurons were incubated with 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 prior to treatment with NPY (100 nM). After 6 h, LC-3BII/β-tubulin ratio and p62/SQSTM1 levels were evaluated by Western blotting. As shown in Figure 3.11A, the increase in LC-3BII/β-tubulin ratio induced by NPY (151.9±5.7% of control) was significantly prevented in the presence of NPY receptor selective antagonists (Y_1: 104.9±7.9%, Y_2: 112.4±12.9% and Y_5: 112.2±12.3% of control). Along with the increase of LC-3BII/β–tubulin ratio, NPY induced a decrease in p62/SQSTM1 levels (77.5±3.8% of control), which was inhibited by NPY Y_1, Y_2 and Y_5 receptor selective antagonists (Y_1: 110.8±7.4%, Y_2: 106.1±10.7% and Y_5: 110.8±7.0% of control), as shown in Figure 3.11B. These results suggest that NPY-induced autophagy is mediated by NPY Y_1, Y_2 and Y_5 receptors activation.

In order to confirm which NPY receptor subtypes were involved in NPY-induced autophagy in cortical neurons, different NPY receptor selective agonists were used: Y_1 agonist ($Leu^{31}Pro^{34}$) (Y_1 ago, 100 nM), Y_2 agonist (NPY_{13-36}) (Y_2 ago, 100 nM), Y_4 agonist (r-PP) (Y_4 ago, 100 nM) and Y_5 agonist ($NPY_{19-23}(Gly^1, Ser^3, Gln^4, Thr^6, Ala^{31}, Aib^{32}, Gln^{34})PP$) ($Y_5$ ago, 100 nM).

Cells were exposed to each NPY receptor agonist for 6 h. As shown in Figure 3.11C, similar to the effect of NPY (137.5±4.2% of control), all NPY receptors agonists tested increased the LC-3BII/β-tubulin ratio (Y_1: 168.8±27.5%, Y_2: 136.2±0.4%, Y_4: 141.3±7.9% and Y_5:163.0±5.1% of control). Associated to the increase of LC-3BII/β-tubulin ratio, NPY induced a decrease in p62 levels (78.1±4.3% of control), and in presence of NPY Y_1, Y_2, Y_4 and Y_5 receptor selective agonists decrease in the protein content was also observed (Y_1: 78.1±4.3%, Y_2: 70.9±8.9%, Y_4: 91.7.3±11.8% and Y_5: 69.2±5.3% of control), as shown Figure 3.11D. These results suggest that NPY-induced autophagy is mediated by NPY Y_1, Y_2 and Y_5 receptors activation.

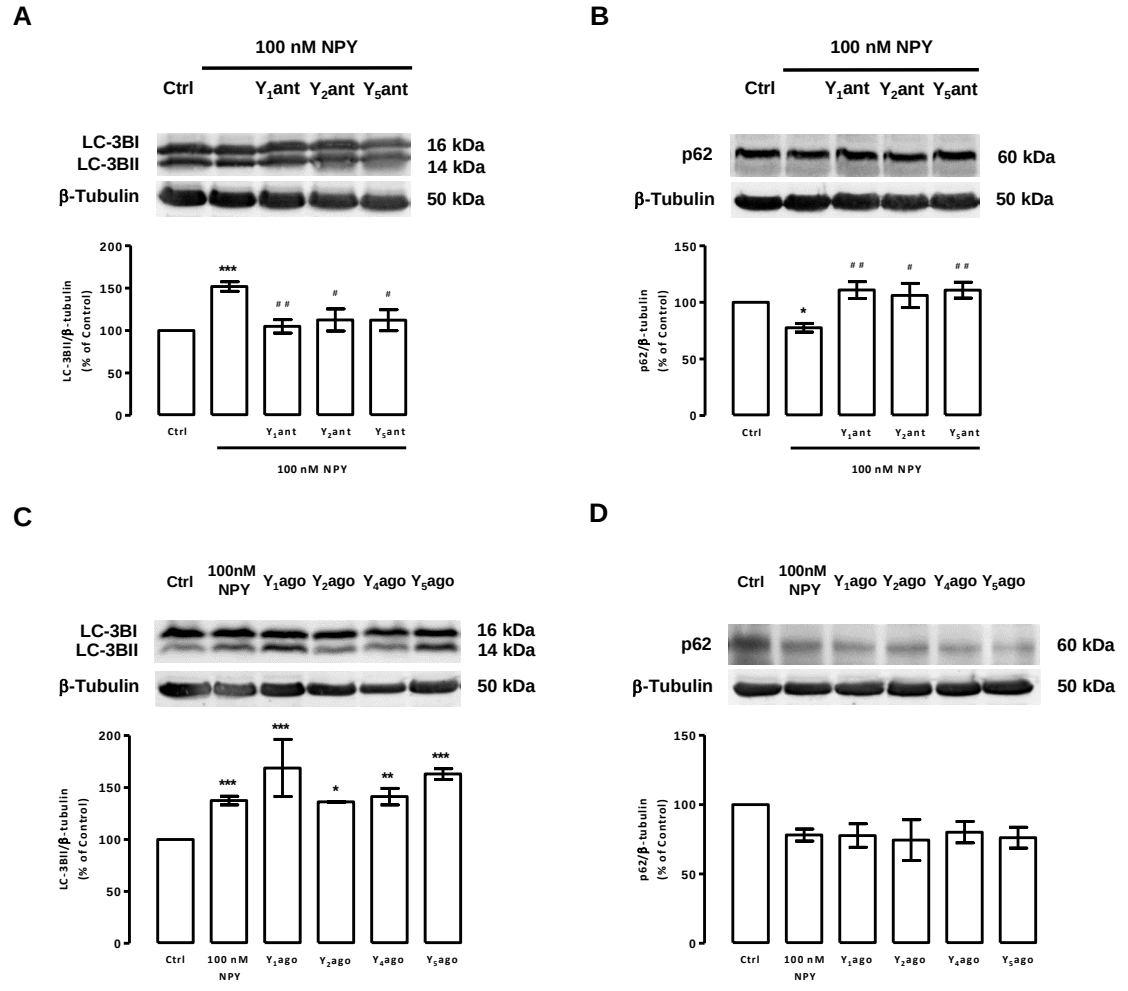

Figure 3.11 – NPY stimulates autophagy through NPY Y_1, Y_2 and Y_5 receptors activation. (A and B) Primary rat cortical neuronal cultures were incubated with Y_1 receptor antagonist BIBP3226 (Y_1ant, 1 μM), Y_2 receptor antagonist BIIE0246 (Y_2ant, 1 μM) or Y_5 receptor antagonist L152,800 (Y_5ant, 1 μM), 30 min before NPY (100 nM) treatment for 6 h. Untreated cells were used as control (Ctrl). (C and D) Primary rat cortical neuronal cultures were treated with Y_1 receptor agonist receptor $Leu^{31}Pro^{34}$ (Y_1ago, 100 nM), Y_2 receptor agonist NPY_{13-36} (Y_2ago, 100 nM), Y_4 receptor agonist r-PP (Y_4ago, 100 nM) or Y_5 receptor agonist NPY_{19-23} (Gly^1,Ser^3,Gln^4,Thr^6, Ala^{31},Aib^{32},Gln^{34}) (Y_5ago, 100 nM), for 6 h. Untreated cells were used as control (Ctrl). Whole cell extracts were assayed for LC-3BII (A and C), p62/SQSTM1 (B and D) and β-tubulin (loading control) immunoreactivity through Western blotting analysis, as described in Materials and Methods (see Chapter II). Representative Western blots for each protein are presented above each respective graph. The results represent the mean ± SEM of, at least, 3 independents experiments, and are expressed as percentage of control. *p<0.05, **p<0.01 and ***p<0.001, significantly different compared to control; $^{\#}$p<0.05, $^{\#\#}$p<0.01 significantly different from NPY treatment for 6 h, as determined by ANOVA, followed by Bonferroni's post test.

3.3.2. Molecular pathways involved in NPY-induced autophagy

The inhibition of mTOR is one of the major regulators of autophagy induction (He *et al.,* 2009, Jung *et al.,* 2010). In order to evaluate if mTORC1 was being inhibited upon NPY treatment, cells were treated with NPY (100 nM) for 30 min and 6 h and whole cell extracts were assayed for phospho-mTOR (Ser2884) by Western blotting. As shown in Figure 3.12A, NPY treatment led to a significant decrease in phospho-mTOR (Ser2448) levels (89.4±3.5% and 78.4±3.3% of control, respectively) in rat cortical neurons. This result suggests that NPY induces autophagy through through the inhibition of mTORC1. However, several other pathways are known to regulate autophagy in mammalian cells. To further determine the molecular pathways involved in NPY-induced autophagy, primary rat cortical neurons were incubated with PI_3K inhibitor (LY294002 (PI_3K_i), 1 μM), ERK 1/2 inhibitor (U0126 (ERK_i), 1 μM) or PKA inhibitor (H89 (PKA_i), 1 μM), in the presence or absence of 100 nM NPY. After 6 h, LC-3BII/β-tubulin ratio and p62/SQSTM1 levels were evaluated by Western blotting. The increase of LC-3BII/β-tubulin ratio and the decrease of p62 protein content, induced by NPY treatment, were inhibited by all kinase inhibitors tested. As shown in Figure 3.12B, C and D, NPY increased LC-3BII/β-tubulin ratio (PI_3K: 134.4±6.2%, ERK: 138.4±6.1% and PKA: 137.1±5.7% of control) which was decreased in the presence of respective protein kinase inhibitor (PI_3K_i: 105.5±8.4%, ERK_i: 124.9±14.4% and PKA_i: 118.7±13.7% of control). As shown in Figure 3.12E, F and G, cells treated with NPY showed a decreased in p62/SQSTM1 protein content (PI_3K: 86.5±2.4%, ERK: 85.2±3.3% and PKA: 83.9±3.6% of control) which was inhibited in the presence of respective protein kinase inhibitor (PI_3K_i: 102.2±2.6%, ERK_i: 103.9±6.8% and PKA_i: 110.1±3.1% of control). These results suggest that NPY induces autophagy in cortical neurons through PI_3K, ERK 1/2 and PKA signaling pathways.

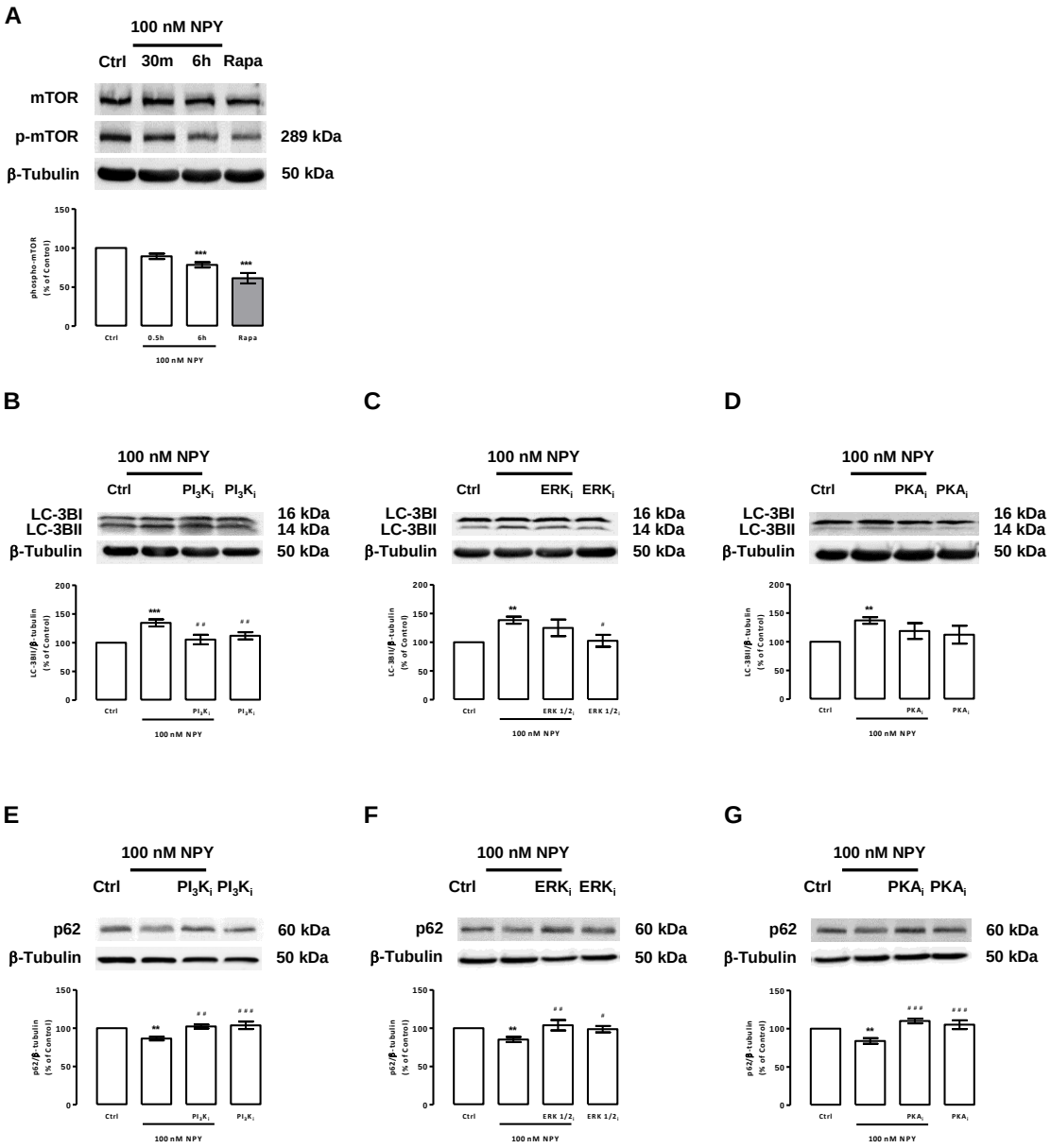

Figure 3.12 – Molecular pathways involved in NPY induced autophagy in cortical neurons. (A) NPY activates autophagy through a mTOR-dependent pathway. Primary rat cortical neuronal cultures were exposed to NPY (100 nM) for 30 min or 6 h. Untreated cells were used as control (Ctrl). (B to G) The effect of NPY on autophagy induction is mediated by PI_3K, ERK 1/2 and PKA. Primary rat cortical neuronal cultures were treated with PI_3K inhibitor (LY294002 (PI_3K_i), 1 µM), ERK 1/2 inhibitor (U0126 (ERK_i), 1 µM)) or PKA inhibitor (H89 (PKA_i), 1 µM) 30 min before NPY (100 nM) treatment. Untreated cells were used as control (Ctrl). Whole cell extracts were assayed for phospho-mTOR (Ser2448) (A), LC-3BII (B, C and D), p62/SQSTM1 (E, F and G) and β-tubulin (loading control) immunoreactivity by Western blotting analysis, as described in Materials and Methods (see Chapter II). Representative Western blots for each protein are presented above each respective graph. The results represent the mean ± SEM of, at least, 3 independents experiments, and are expressed as percentage of control. **p<0.01 and ***p<0.001, significantly different compared to control; $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.01 significantly different from NPY treatment for 6 h, as determined by ANOVA, followed by Bonferroni's post test.

3.4. Ghrelin

3.4.1. Nutrient deprivation-induced autophagy is mediated by ghrelin receptor activation

CR is known to increase the levels of ghrelin, an orexigenic peptide secreted by the stomach known to be involved in food intake and body weight regulation (Kojima *et al.*, 1999, Ariyasu *et al.*, 2001). Since ghrelin shares some of the physiological effects induced by CR it has been suggested as a metabolic regulator signal during CR. Therefore we hypothesize that ghrelin, similarly to NPY, could be involved in nutrient deprivation-induced autophagy in rat cortical neurons. Ghrelin effects are mediated by the activation of the ghrelin receptor, GHS-R1a (McKee *et al.*, 1997). Although is known that ghrelin receptor is ubiquitously distributed in the brain, included in the cortex, to confirm the presence of GHSR-1a receptor in primary rat cortical neuronal cultures, GHS-R1a immunoreactivity was evaluated by Western blotting. In Figure 3.13A we show that rat cortical neurons in culture express GHS-R1a receptor, and therefore, are able to respond to ghrelin stimulation.

To assess whether ghrelin plays a role in nutrient deprivation-induced autophagy in cortical neurons, cells were exposed to the ghrelin receptor antagonist ([D-Lys^3]-GHRP-6, 100 μM) 30 min prior to the incubation of nutrient deprivation medium. After 6 h, LC-3BII/β-tubulin ratio and p62/SQSTM1 levels were evaluated in whole cell lysates by Western blotting. As shown in Figure 3.13B, preliminary data (n=2-3) show that the increase in LC-3BII/β -tubulin ratio induced by nutrient deprivation (178.2±9.3% of control) was partially decreased in the presence of GHS-R1a antagonist (159.6±15.1% of control). In addition, GHS-R1a antagonist (86.7±5.6% of control; Figure 3.13C) partially inhibited the decreased in p62/SQSTM1 protein levels upon nutrient deprivation treatment (78.4±6.4% of control; Figure 3.13C). Of note, the receptor antagonist itself also seems to alter the LC-3BII/β-tubulin ratio and p62/SQSTM1 protein content in these cells. These effects may be related with the intrinsic constitutive activity of GHS-R1a receptor (Damian *et al.,* 2012). These preliminary results suggest that GHS-R1a may mediate nutrient deprivation-induced autophagy in cortical neurons.

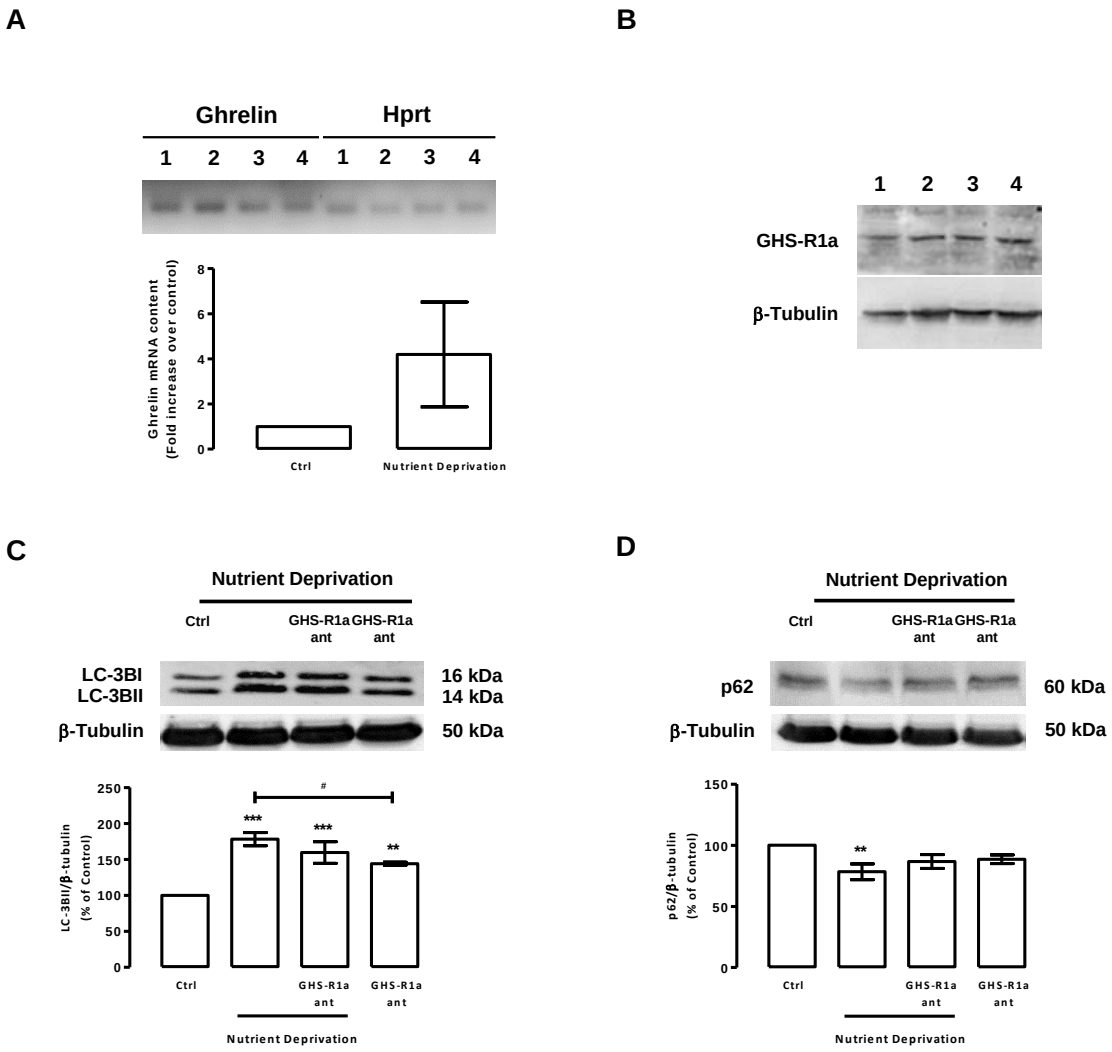

Figure 3.13 – The effect of GHS-R1a receptor antagonist on nutrient deprivation-induced autophagy. (A) Total RNA was isolated from primary cortical neuronal cultures, the transcript levels of ghrelin were analyzed by qRT-PCR and the resulting producs were visualized by agarose gel electrophoresis as described in Materials and Methods (see Chapter II). RT-PCR products (base pair (bp): Ghrelin, 120bp and Hprt, 110bp, used an endogenous control. A representative image of 3 independent cell cultures is presented above the graph. (B) Lysates from primary rat cortical neurons and from adult rat cortex were assayed for GHS-R1a and β-tubulin (loading control) immunoreactivity by Western blotting analysis, as described in Materials and Methods (see Chapter II). (C and D) Primary rat cortical neuronal cultures were treated with GHS-R1a receptor antagonist [D-Lys^3]-GHRP-6 (GHS-R1a ant, 100 μM) 30 min before nutrient deprivation treatment for 6 h. Untreated cells were used as control (Ctrl). Whole cell extracts were assayed for LC-3BII (C), p62/SQSTM1 (D) and β-tubulin (loading control) immunoreactivity through Western blotting analysis, as described in Materials and Methods (see Chapter II). The results represent the mean ± SEM of, at least, 3 independents experiments, and are expressed as percentage of control. **p<0.01 and ***p<0.001, significantly different compared to control; $^{\#}$p<0.001, significantly different from nutrient deprivation 6 h, as determined by ANOVA, followed Bonferroni's post test. Legend: 1: primary cortical neuronal culture#1; 2: primary cortical neuronal culture#2; 3: rat cortex; 4: rat hypothalamus.

3.4.2. Ghrelin induces autophagy in cortical neurons

Since GHS-R1a seems to play a role in nutrient deprivation-induced autophagy, we evaluated the effect of ghrelin on autophagy in cortical neurons. First, the effect of ghrelin on primary rat cortical neuronal viability was evaluated by the MTT reduction assay. Cells were

exposed to 10 nM ghrelin for 6 and 24 h. As shown in Figure 3.14, ghrelin did not affect the viability of cortical neurons (6 h: 96.3±4.1% and 24 h: 104.0±0.6% of control).

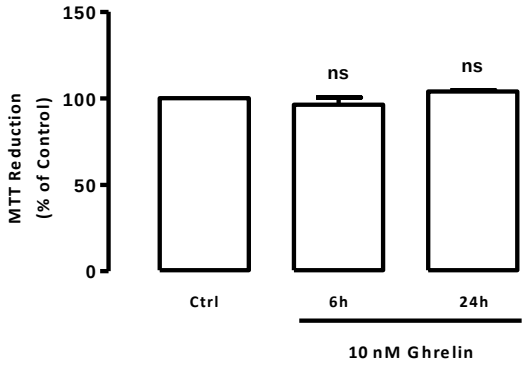

Figure 3.14 – Ghrelin does not change rat cortical neurons viability. Primary rat cortical neuronal cultures were incubated with ghrelin (10 nM) for 6 h or 24 h. Untreated cells were used as control (Ctrl). Cell viability was assessed by the MTT reduction assay, as described in Materials and Methods (see Chapter II). The results represent the mean ± SEM of 3 independents experiments, and are expressed as percentage of control. Student's t test. ns, not statistically different.

In order to evaluate the autophagy-induction potential of ghrelin, primary rat cortical neuronal cultures were exposed with 10 nM ghrelin for 6 and 24 h. Rapamycin (100 nM, 1 h), a known inductor of autophagy, was used as positive control. As shown in Figure 3.15A, ghrelin induced an increase of the autophagic flux in cortical neurons, since the ratio of LC-3BII/β–tubulin is increased after 6h (125.5±4.5%of control) of treatment; however, the amount of LC-3BII/β–tubulin ratio decreased to basal levels after longer-time exposures (92.9±3.2% of control), suggesting that LC-3BII is being degraded by the lysosome. Rapamycin induced an increase in LC-3BII/β-tubulin ratio (127.9±4.3% of control; Figure 3.15A). Parallel to the increase in LC-3BII/β–tubulin ratio, the protein content of p62/SQSTM1 was decreased after 6 h (76.4±4.4% of control) of ghrelin treatment. In order to confirm that ghrelin induces autophagic flux, cortical neurons were pre-treated for 6 h with chloroquine (100 uM), 30 min before ghrelin treatment. As shown in Figure 3.15C, cells incubated with ghrelin showed a higher LC-3BII/β-tubulin ratio (125.2±4.5% of control) compared to untreated cells. However, in the presence of chloroquine, this increase was higher (147.4±4.6% of control) than in cells treated with ghrelin alone. Chloroquine treatment also increased p62/SQSTM1 protein levels 137.1±10.1% of control) compared to untreated cells, as shown in Figure 3.15D. These results indicating that indeed ghrelin increases autophagic flux in cortical neurons.

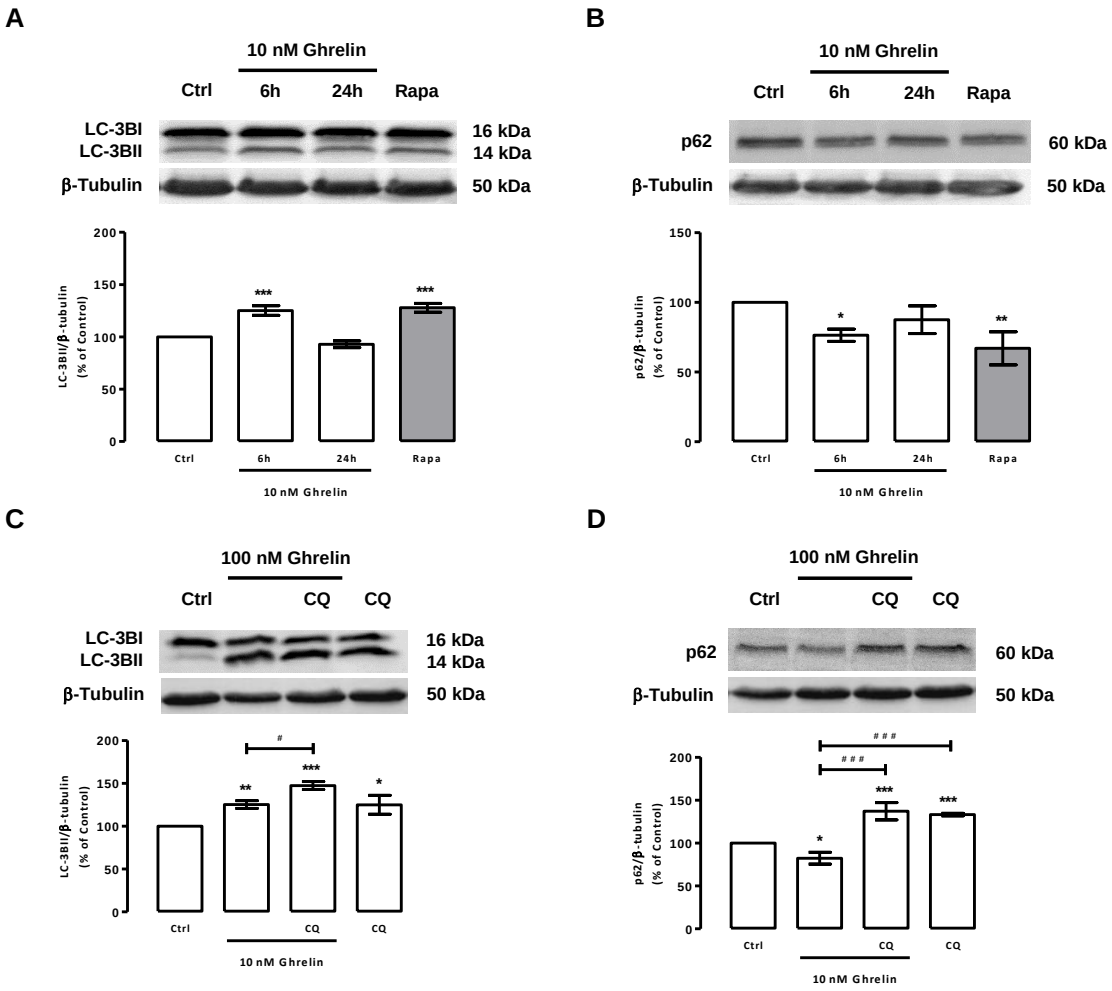

Figure 3.15 – Ghrelin induces autophagy in cortical neurons. (A and B) Primary rat cortical neuronal cultures were incubated with ghrelin (10 nM) for 6 h or 24 h. Untreated cells were used as control (Ctrl), and cells incubated with rapamycin (Rapa, 100 nM) for 1 h were used as positive control of autophagy. (C and D) Primary rat cortical neuronal cultures were incubated with chloroquine (CQ, 100 μM), a lysosomal degradation inhibitor, 30 min before ghrelin (10 nM) treatment for 6 h. Untreated cells were used as control (Ctrl). Whole cell extracts were assayed for LC-3BII (A and C), p62/SQSTM1 (B and D), and β-tubulin (loading control) immunoreactivity through Western blotting analysis, as described in Materials and Methods (see Chapter II). Representative Western blots for each protein are presented above each respective graph. The results represent the mean ± SEM of, at least, 3 independents experiments, and are expressed as percentage of control. *p<0.05, **p<0.01 and ***p<0.001, significantly different compared to control; $^{\#}$p<0.05 and $^{\#\#\#}$p<0.001, significantly different from ghrelin treatment for 6 h, as determined by ANOVA, followed by Bonferroni's post test.

LC-3B immunoreactivity in primary rat cortical neurons was also evaluated by immunocytochemistry. Cells were exposed to 10 nM ghrelin for 6 h. As shown in Figure 3.16, LC-3B localization within the cells is mainly perinuclear and with a punctuate distribution. While untreated cells revealed a more diffuse LC-3B distribution, with few puncta, in nutrient deprivation treated cells, a clear increase in LC-3B puncta number was observed.

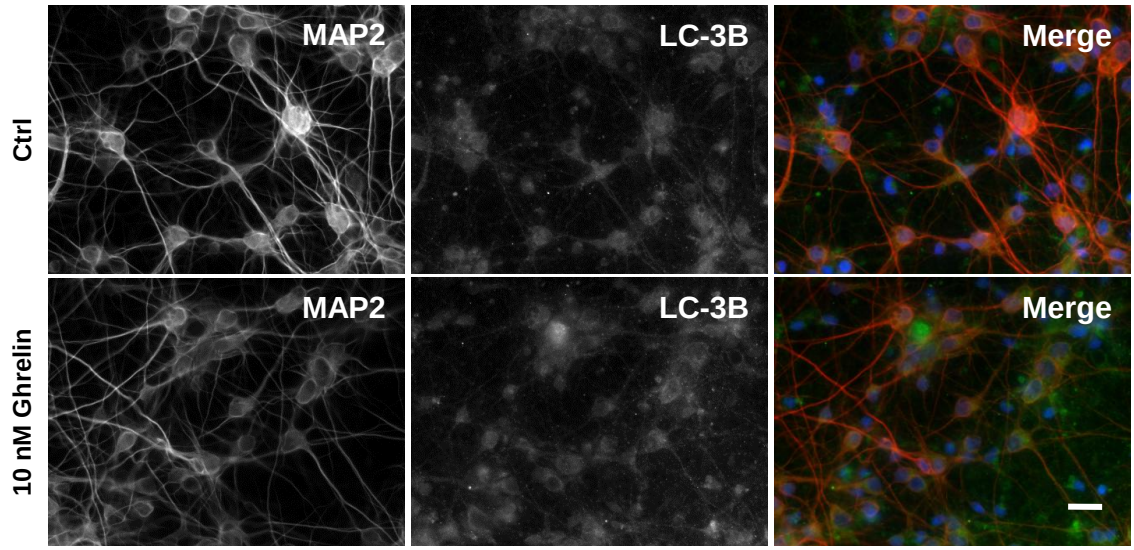

Figure 3.16 – Ghrelin induces LC-3B puncta accumulation in rat cortical neurons. Primary rat cortical neuronal cultures were exposed to ghrelin (10 nM) for 6 h. Untreated cells were used as control (Ctrl). Cells were immunolabeled for LC-3B (green) and MAP2 (red, neurons). Nuclei were stained with Hoechst 33342 (blue). LC-3B puncta formation was assessed by immunocytochemistry assay, as described in Materials and Methods (see Chapter II). Figures are representative of 3 independents experiments. Scale bar, 20 μM.

3.4.3. Ghrelin-induced autophagy is mediated by GHS-R1a receptor activation

Since GHS-R1a mediates ghrelin effects on cells, we next evaluated whether ghrelin receptor GHS-R1a modulates the ghrelin effect on autophagy induction. Cells were exposed to the ghrelin receptor antagonist (100 μM, [D-Lys^3]-GHRP-6) 30 min prior to ghrelin (10 nM) treatment. After 6 h, LC-3BII/β-tubulin ratio and p62/SQSTM1 levels were evaluated by Western blotting. As shown in Figure 3.17A, the increase in LC-3BII/β-tubulin ratio upon ghrelin treatment (128.7±4.2% of control) decreased in the presence of GHS-R1a antagonist (108.0±7.2% of control). Concomitant with the increase of LC-3BII/tubulin ratio, ghrelin-induced decrease in p62/SQSTM1 levels (76.4±4.4% of control), which was inhibited by GHSR-1a antagonist (113.0±10.8% of control), as shown in Figure 3.17B. However, when the cells are only exposed to the antagonist, the profile is similar to that with ghrelin exposure only (LC-3BII/β-tubulin: 144.2±2.0% and p62/SQSTM1: 88.6±3.5% of control). Nevertheless, these preliminary results suggest that ghrelin increases autophagy in rat cortical neurons and this is mediated by GHS-R1a receptor activation. In order to evaluate if mTORC1 complex was involved upon ghrelin treatment, primary rat cortical neurons were treated with 10 nM ghrelin for 30 min and 6 h and whole cell extracts were evaluated for phospho-mTOR (Ser2448) by Western blotting. As shown in Figure 3.17C, ghrelin led to a decrease in phospho-mTOR after 30 min and 6 h of incubation (96.9±7.5% and 80.8±3.3% of control, respectively). This result suggests that nutrient deprivation induces autophagy through the inhibition of mTORC1.

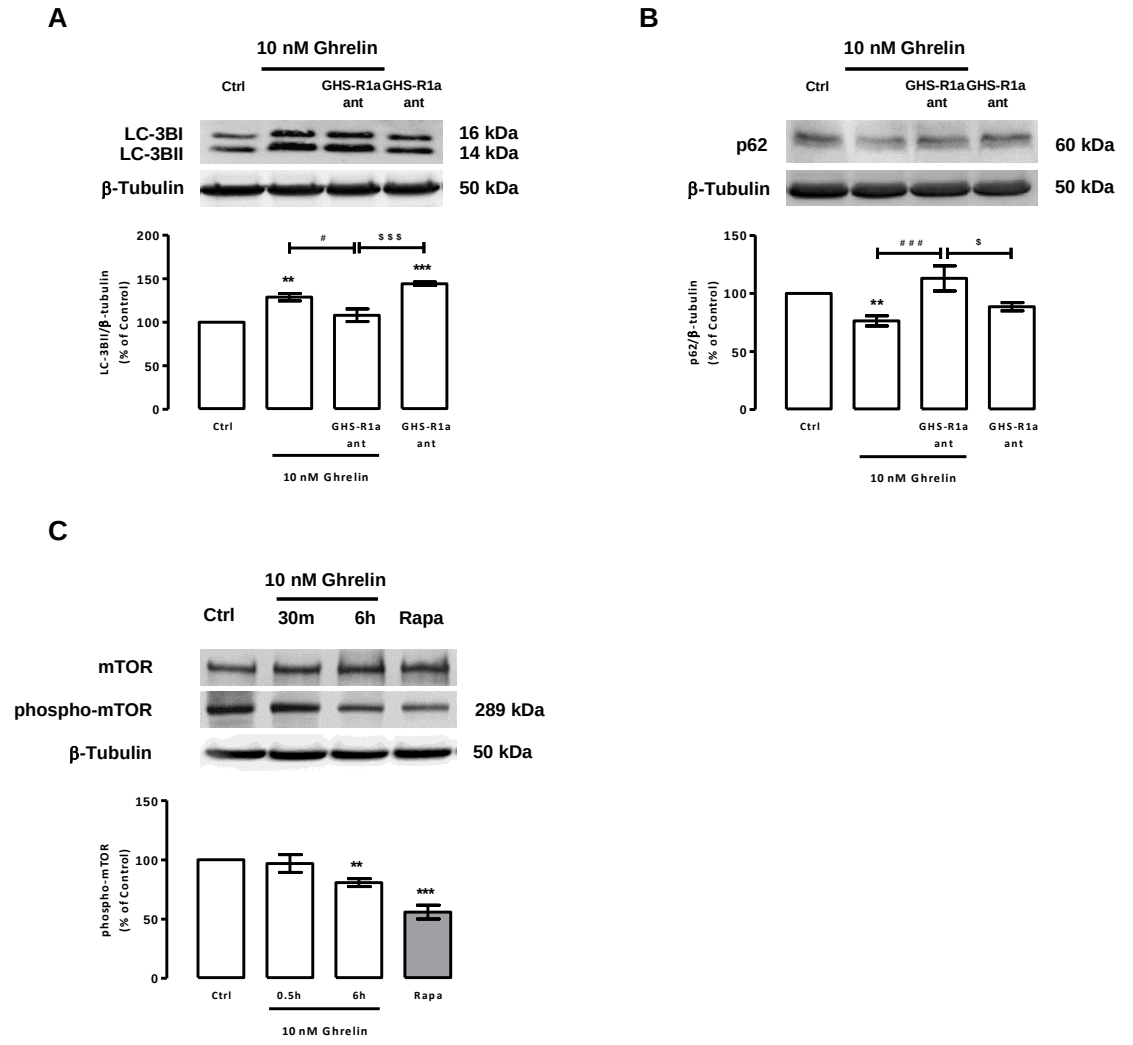

Figure 3.17 – The effect of GHS-R1a receptor antagonist on ghrelin-induced autophagy. Primary rat cortical neuronal cultures were treated with GHS-R1a receptor antagonist [D-Lys^3]-GHRP-6 (GHSR-1a ant, 100 μM), 30 min before ghrelin (10 nM) treatment for 6 h (A and B), or exposed to ghrelin alone for 6 or 24h (C). Untreated cells were used as control (Ctrl). Whole cell extracts were assayed for LC-3BII (A), p62/SQSTM1 (B), phosphor-mTOR (C) and β-tubulin (loading control) immunoreactivity through Western blotting analysis, as described in Materials and Methods (see Chapter II). Representative Western blots for each protein are presented above each respective graph. The results represent the mean ± SEM of 2 to 3 independents experiments, and are expressed as percentage of control. **p<0.01 and ***p<0.001, significantly different compared to control; $^{\#}$p<0.05 and $^{\#\#\#}$p<0.001, significantly different from ghrelin 6 h; $^{\$}$p<0.05 and $^{\$\$\$}$p<0.001 significantly different from GHS-R1a receptor antagonist treatment, as determined by ANOVA, followed Bonferroni's post test.

Chapter IV

Discussion

Aging and longevity are determined by multifactorial and complex processes whose molecular basis remains incompletely understood (Kirkwood 2005). The brain is the most affected organ by the aging process, exerting effects on the molecules, cells, vasculature, gross morphology, and cognition. In addition, several studies suggest that age-related reductions in brain weight were due, in part, to a decline in neuron number in all cortical layers (Brody 1955, Duan *et al.*, 2003). The aging process is also related with alterations in the neuroendocrine control of energy homeostasis that could lead to obesity or diabetes (Smith *et al.*, 2005, Carrascosa *et al.*, 2009), metabolic diseases that worsen quality of life and shorten lifespan.

Aging is driven by the accumulation of damaged macromolecules and organelles which compromises cellular function (Kirkwood 2005). The lifelong accumulation of abnormal cellular constituents leads to a decreased ability of cells, and ultimately of the organism, to survive. The continuous removal of worn-out components and replacement with newly synthesized ones ensures cellular homeostasis and delays the aging process. In addition, there is evidence that neurons are especially susceptible to proteasomal and autophagic dysfunction, possibly because they are long-lived cells with considerable specialized membrane and protein turnover. Autophagy is the major degradative process of eukaryotic cells (Levine *et al.*, 2008, Mizushima *et al.*, 2008) and is well characterized as a response to cellular stress with the dual effect of debris removal and provision of energy through recycling of valuable cellular resources. This process is required for the bulk clearance of damaged macromolecules, the turnover of long-lived proteins, and for the removal of superfluous or dysfunctional organelles within cells. Growing evidence indicates that basal autophagic activity decreases with age, thus contributing to the aging phenotype and to the aggravation of age related diseases (Cuervo 2008).

Actually, there are several interventions that try to delay the aging process and increase longevity, however there are no interventions or gene manipulations that stop or reverse the aging process. When considering all the possible strategies to slow down aging and prolong maximal lifespan, caloric restriction (CR) is the most robust anti-aging intervention known to increase maximal lifespan and healthspan from yeast to mammals (Bergamini *et al.*, 2007, Fontana *et al.*, 2010). In fact, CR was shown to slow aging in Rhesus monkeys by delaying the onset of age-associated pathologies, including cancer and cardiovascular diseases (Colman *et al.*, 2009). CR anti-aging effects are intimately tied to increased autophagy (Donati 2006) and alterations in the neuroendocrine system, particularly the increase of NPY in the hypothalamus (Minor *et al.*, 2009) and the increase in the circulating levels of ghrelin (Lutter *et al.*, 2008), a orexigenic peptide produced by the stomach in response to fasting conditions, which

stimulates hypothalamic neurons to increase food intake via NPY release (Nakazato *et al.,* 2001, Shintani *et al.,* 2001).

Aging is associated with reduced levels of NPY in several cerebral areas, such as hypothalamus, hippocampus and cortex (Higuchi *et al.*, 1988, Gruenewald *et al.*, 1994, Vela *et al.*, 2003). Also a decline in NPY plasma levels in humans correlated with increasing age (Chiodera *et al.*, 2000). In addition, reduced NPY is associated with neurodegenerative diseases (Decressac *et al.*, 2012) and the development of "anorexia in aging", characterized by reduced food intake and body weight, responsible for age-associated under nutrition and physical deterioration (Matsumoto *et al.*, 2000, Morley *et al.*, 2001). On the other hand, the increase in NPY can lead to several physiological modifications similar to those induced by CR. Central administration of NPY has been shown to induced hyperphagia (Stanley *et al.*, 1986, Beck *et al.*, 1992), lower blood glucose levels (Ahlborg *et al.*, 1994, Marks *et al.*, 1997, Bischoff *et al.*, 1998) and reduce core body temperature (Billington *et al.*, 1991, Kotz *et al.*, 2000). In humans, increased NPY levels may also be correlated with lifespan benefits, since long-lived female centenarians have higher NPY plasma levels compared to younger women (Baranowska *et al.*, 2006). Given the difficulty to implement and sustain a CR regimen in humans, there is an increasing interest on the identification of new agents that can mimic the beneficial effects of CR. It is not known whether NPY is a necessary precursor for the functional benefits associated with CR. Considering NPY's unique long term response to CR compared to other neuropeptides and the plethora of similar physiological actions (Bi *et al.*, 2003), NPY is a promising CR mimetic candidate.

Similarly to NPY, the circulating levels of ghrelin also decrease with increasing age (Rigamonti *et al.*, 2002), but exogenous restoration of ghrelin improves a variety of age-related immune, appetite, and insulin derangements. Additionally, ghrelin has been shown to be affected in the short-term by changes in feeding and in the long-term by changes in body weight (Tschop *et al.*, 2000, Wren *et al.*, 2000, Wren *et al.*, 2001b, Finger *et al.,* 2011a, Finger *et al.,* 2011b, Rolland *et al.,* 2011). These properties are important as CR modifies both. Like CR, ghrelin is known to increase food intake, inhibit insulin release and reduce blood glucose levels (Wierup *et al.*, 2004), to reduce the reproductive organ function (Fernandez-Fernandez, *et al.*, 2004), and maintenance of cognition (Carlini *et al.*, 2008). The effectiveness of ghrelin in these roles may be impaired as ghrelin levels decrease with age, perhaps contributing to other age-related conditions like insulin resistance and diabetes (Wierup *et al.*, 2004), reduced fertility (Fernandez-Fernandez *et al.*, 2004), and decreased performance on cognitive and memory tasks with advancing age (Carlini *et al.*, 2008). The significant overlap between CR-

and ghrelin-induced physiological processes suggest that ghrelin may play a role in the beneficial effects of CR on health and lifespan. However, its role in autophagy and consequently, lifespan extension, remains unkown.

In this study, we show for the first time that nutrient deprivation induces autophagy in rat cortical neurons by increasing LC-3BII/β-tubulin ratio and decreasing p62/SQSTM1 protein levels. In addition, nutrient deprivation induced the formation of numerous LC-3B punctuate structures or aggregates, indicative of increased formation of autophagosomes in the cytoplasm. An increase in LC-3BII/β-tubulin ratio or LC-3B aggregates are not sufficient to guarantee an increase on the autophagic flux upon nutrient deprivation treatment. LC-3BII can be accumulated due to enhanced autophagosome formation or inhibition of autophagic degradation, perphaps 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 LC-3BII/β-tubulin ratio and LC-3B punctate is due to an inhibited autophagosome degradation rather than autophagosome formation, we measured endogenous autophagic flux by the difference in the LC-3BII/ β-tubulin ratio and p62/SQSTM1 protein levels in the presence or absence of chloroquine, an inhibitor of autophagic degradation (Barth *et al.*, 2010). Chloroquine increased LC-3BII/β-tubulin ratio and p62/SQSTM1 protein levels in both nutrient deprivation-treated and non-treated cells, but the increase in nutrient deprivation-treated cells was much larger than in untreated cells. These results suggest that nutrient deprivation induces autophagy and accelerates autophagic flux in cortical neurons.

CR anti-aging effects are intimately linked to the increase of autophagy and alterations in the neuroendocrine system, such as the increase of NPY in the hypothalamus (Minor *et al.*, 2009). Preliminary data obtained by our group shows that nutrient deprivation increases NPY expression in primary cultures of hypothalamic neurons. In addition, NPY not only induces autophagy, but also mediates nutrient deprivation-induced autophagy in hypothalamic neurons, supporting the hypothesis that NPY may mediate nutrient deprivation-induced autophagy. In line with these preliminary data and knowing that NPY may be expressed by cortical neurons is reasonable to hypothesize that NPY may mediate nutrient deprivation effects on cortical neurons. In fact, this hypothesis was confirmed by evaluating the presence of NPY and its receptors in these primary cultures of rat cortical neurons. NPY exerts its biological functions through G-protein-coupled receptors (NPY Y_1, Y_2, Y_4, Y_5) (Gehlert 2004), all of which have been reported to be present in the cortex (Parker *et al.*, 1999). However, the NPY Y_1 and Y_2 receptors are the most common (Leroux 2002). The presence of NPY and NPY

receptors in cortical neurons was demonstrated and confirmed by mRNA expression. The mRNA for NPY and NPY Y_1, Y_2, Y_4 and Y_5 were detected in cortical neurons in culture. In the present study, we observed that NPY Y_1, Y_2 and Y_5 receptor antagonists decreased the effect of nutrient deprivation on autophagy induction, suggesting that nutrient deprivation-induced autophagy is dependent on NPY Y_1, Y_2 and Y_5 receptor activation in cortical neurons. Though, no receptor seems to take a major role in mediating this effect, since all three antagonists equally reduced LC-3BII/β-tubulin ratio and increased p62/SQSTM1 protein levels. This leads to the hypothesis that all the three tested receptors may play redundant roles in signaling autophagy induction, supported by the fact that all three trigger basically the same molecular pathways, through $G\alpha_{i/o}$ protein activation. Furthermore, these three NPY receptor subtypes are considered to be the most active in the regulation of feeding circuitries and energy balance in mammals (Duhault *et al.*, 2000, Lecklin *et al.*, 2002, Henry *et al.*, 2005).

The canonical pathway for autophagy induction is usually dependent on mTORC1 inhibition (Jung *et al.*, 2010). Activation of mTOR kinase activity, in response to replete nutrient conditions and insulin/growth factor signaling suppresses autophagy, while inhibition of mTOR by starvation induces autophagy. In this study, we observed that nutrient deprivation decreases mTOR activity, the core kinase of mTORC1. On the other hand, several other pathways are known to regulate autophagy in mammalian cells. PI_3K inhibitors, including 3-methyladenine, wortmannin, and LY294002 have been well established as autophagy inhibitors (Petiot *et al.*, 2000, Blommaart *et al.*, 2009). These inhibitors inhibit class I as well as class III PI_3K. While class III PI_3K is essential for autophagosome formation and therefore, autophagy induction, class I PI_3K 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.*, 2009, Cagnol *et al.*, 2010). Several studies involving pharmacological inhibition of ERK demonstrated that it mediates starvation- and TNF-α-induced autophagy (Cheng *et al.*, 2008, Ogier-Denis *et al.,* 2000). Consistent with these reports, we showed that nutrient deprivation induced autophagy in cortical neurons via the ERK pathway, as showed by a decline in LC-3BII/β-tubulin ratio by ERK inhibitor. In addition to PI_3K and ERK, we also revealed that nutrient deprivation enhances autophagic flux in cortical neurons via PKA pathway. However, it is described that PKA negatively regulates autophagy either by directly phosphorylating LC-3B (Cherra *et al.*, 2010) or by activating TORC1, which inhibits autophagy (Mavrakis 2006). In this study, we also observed that PI_3K, ERK 1/2 and PKA inhibitors

decreased autophagic flux induced by nutrient deprivation. These results suggest that nutrient deprivation induces autophagy in cortical neurons through mTOR, PI_3K, ERK 1/2 and PKA signaling pathways.

Since nutrient deprivation-induced autophagy is mediated by NPY receptors activation and an increase in NPY expression, we evaluated the hypothesis that NPY also induces autophagy in rat cortical neurons. In this study, we showed for the first time that NPY increases LC-3BII/β-tubulin ratio and decreases p62/SQSTM1 protein levels, enhancing this way the autophagic flux in cortical neurons. Furthermore, it was observed the formation of LC-3B punctuate structures or dots in NPY-treated cells, which indicates an increased formation of autophagosomes in cortical neurons. To confirm if NPY-induced indeed autophagy and the lysosomal degradation in the late stages was taking place, the cortical neurons were treated with chloroquine prior to NPY incubation. The results obtained clearly show that NPY is indeed inducing autophagy, ultimately leading to LC-3BII degradation. This supports that NPY induces autophagy in a time-dependent manner and that the induced autophagy is dynamic and complete. NPY exerts its biological functions through G-protein-coupled receptors (NPY Y_1, Y_2, Y_4, Y_5) (Gehlert 2004), all of which were shown to be present in cortical neurons. In the present study we observed that NPY Y_1, Y_2 and Y_5 receptor antagonists decreased the effect of NPY on autophagy induction, suggesting that NPY-induced autophagy is dependent on NPY Y_1, Y_2 and Y_5 receptor activation in cortical neurons. Though, no receptor seems to take a major role in mediating this effect, since all three antagonists equally reduced LC-3BII/β-tubulin ratio and increased p62/SQSTM1 protein levels. To better understand the involvement of each NPY receptor in autophagy regulation, specific agonists of NPY Y_1, Y_2, Y_4 and Y_5 were used. Similar to NPY, all four agonists increased LC-3B/β-tubulin ratio and decreased p62/SQSTM1 protein content. The experiments demonstrate that NPY regulates cortical neuronal autophagy through the activation of these four receptors. Like nutrient deprivation, NPY decreased the activity of mTOR, which is a signal for autophagy induction. On the other hand, we also observed that PI_3K, ERK and PKA inhibitors decreased the autophagic flux induced by NPY. These results suggest that NPY induces autophagy in cortical neurons through PI_3K, ERK 1/2 and PKA signaling pathways. 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. The similarity between the effects of nutrient deprivation and NPY on cortical neuronal autophagy supports the hypothesis that NPY may be considered as a CR mimetic.

Finally, we explore the potential of ghrelin, other putative CR mimetic, to regulate cortical neuronal autophagy. In the CNS, the main site of ghrelin expression, although at much lower levels than the stomach, is the hypothalamus. However, it has also been found in the hippocampus (Kojima *et al.*, 2002, De Ambrogi *et al.*, 2003) and cortex (Hou *et al.*, 2006). On the other hand, ghrelin receptor is ubiquitously distributed in the brain, being found in the hypothalamus, hippocampus and cortex (Howard *et al.*, 1996, Guan *et al.*, 1997, Zigman *et al.*, 2006). To confirm that cortical neurons in culture express ghrelin and ghrelin receptor, we performed qPCR and Western blotting analysis to evaluate the expression of ghrelin and the immunoreactivity of GHS-R1a in these cells. We observed that cortical neurons in culture express ghrelin and GHS-R1a receptor, and therefore, are able to respond to ghrelin. Then, to investigate the involvement of ghrelin in nutrient deprivation-induced autophagy, via GHS-R1a activation, we used a specific antagonist for this receptor: [D-Lys^3]-GHRP-6. We observed that in the presence of [D-Lys^3]-GHRP-6, the effects of nutrient deprivation cortical neuronal autophagy are partially diminished, suggesting that this receptor may play a role in autophagy regulation. We then evaluated whether ghrelin *per se*, like NPY, could regulate autophagy in cortical neurons. We observed that, ghrelin induces autophagy in cortical neurons, since LC-3BII/β-tubulin ratio increase and p62/SQSTM1 decrease, beyond ghrelin decreased the activity of mTOR, which is a signal for autophagy induction, like NPY. In addition, chloroquine increased LC-3BII/β-tubulin ratio and p62/SQSTM1 protein levels in ghrelin-treated suggesting that ghrelin enhances the autophagic flux in cortical neurons. We also observed that this effect is mediated by the activation of GHS-R1a receptor. However, it is important to refer that these effects may be related with the intrinsic constitutive activity of GHS-R1a receptor (Damian *et al.*, 2012). Altogether, these preliminary results suggest that ghrelin not only increases autophagy in rat cortical neurons but also mediates nutrient deprivation-induced autophagy. These evidences, even preliminary, in addition to the fact that ghrelin shares some of the beneficial effects of CR support the potential role of ghrelin as a CR mimetic.

Since cortical autophagy decreases with age, modulation of NPY and ghrelin levels may acts as a protective mechanism against impaired cortical dysfunction associated with age. Furthermore, a better understanding of the role of NPY and ghrelin in the regulation of cortical autophagy and on other beneficial mechanisms, induced by CR, will provide new putative therapeutic strategies to extend longevity and ameliorate age-related deteriorations in combination with CR. In addition, since is difficult to implement CR and it is known that ghrelin regulates the expression of NPY, which has been shown to induce obesity, it would be important to understand how ghrelin can be modulated in order to regulate NPY expression,

introducing ghrelin as a new putative therapeutic strategies to delay the aging process and promote healthy lifespan.

Chapter V

Conclusions

The results presented in this thesis allowed the drawing of the following main conclusions:

Although the knowledge surrounding autophagy and its effects has considerably grown in the last decade, there is still much to clarify and understand, as its relation with caloric restriction effects in the brain. This study provided new evidences regarding the autophagy regulation in caloric restriction, having NPY and ghrelin as key mediators.

The results showed an autophagy induction in cortical neurons by CR and NPY, in a time-dependent manner and that this induction appears to be equally mediated by NPY Y_1, Y_2 and Y_5 receptors. Likewise, and in both conditions, it was additionally demonstrated that CR and NPY induce autophagy through the canonical (mTORC1) and non-canonical signaling pathways.

We observed for the first time that ghrelin, similarly to NPY, contributes to the autophagy induction mediated by nutrient deprivation.

Other new main finding that NPY and ghrelin, both considered caloric restriction mimetics, enhance autophagy in cortical neurons *per se*.

Further studies, should provide additional data, in order to better understand how NPY and ghrelin may be acting in the brain and, eventually, how they may contribute to the enhancement of cellular resistance to aging, through autophagy.

Chapter VI

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