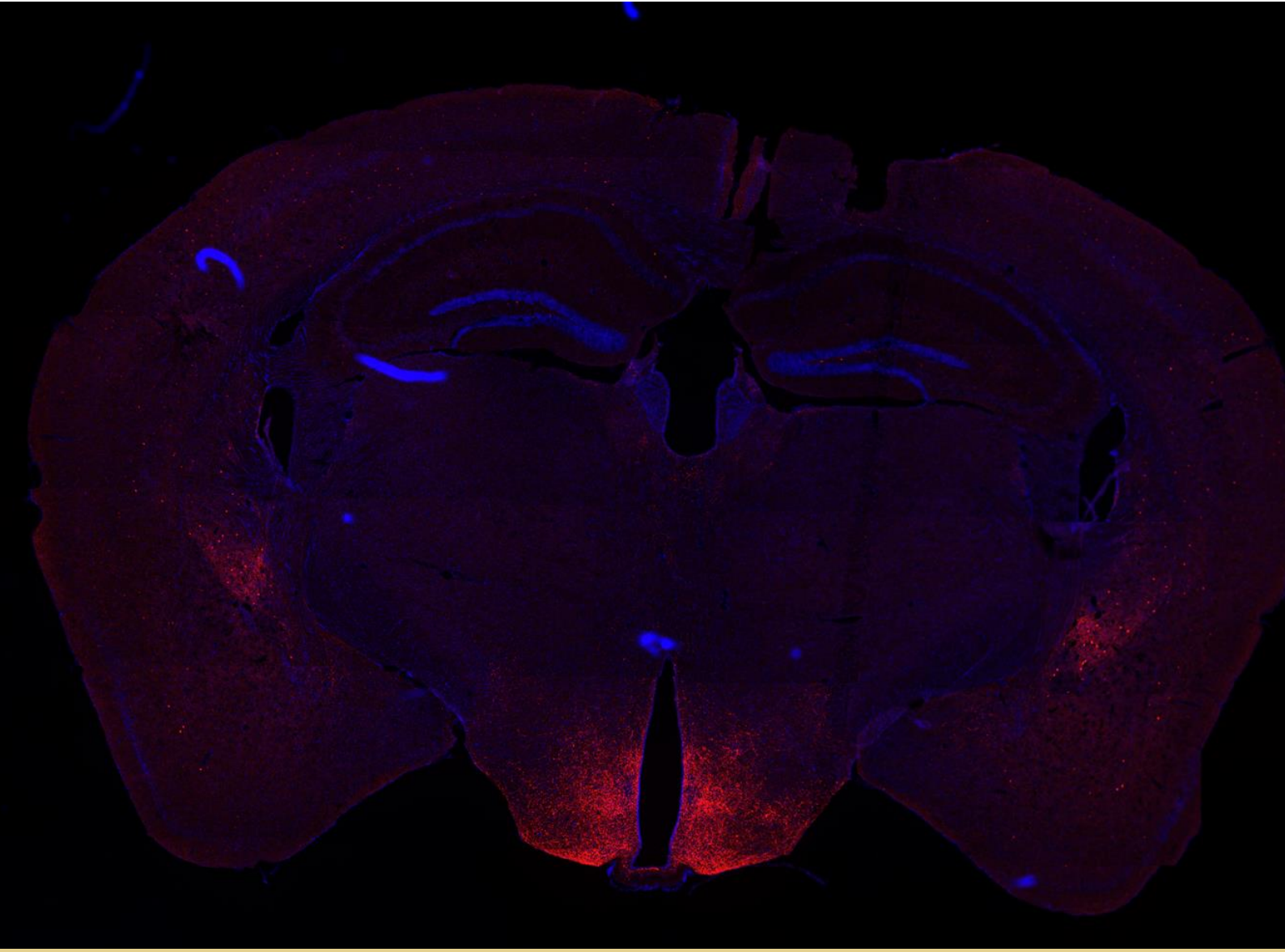

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Neuropeptide Y: a novel strategy to delay aging

Dissertação para obtenção de grau Mestre em Biotecnologia Farmacêutica, sob a orientação científica da Professora Doutora Cláudia Cavadas e da Doutora Célia Aveleira, apresentada à Faculdade de Farmácia da Universidade de Coimbra

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Dissertação apresentada à Faculdade de Farmácia da Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia Farmacêutica, realizada sob a orientação científica da Professora Doutora Cláudia Cavadas (Faculdade de Farmácia e Centro de Neurociências e Biologia Celular da Universidade de Coimbra) e da Doutora Célia Aveleira (Centro de Neurociências e Biologia Celular da Universidade de Coimbra).

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Cover Note: Microscopy image of mouse brain coronal section stained for neuropeptide Y (red) and nuclei (blue).

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List of abbreviations

AAV	Adenoassociated virus
AgRP	Agouti related peptide
AMPK	AMP-dependent protein kinase
ARC	Arcuate nucleus of hypothalamus
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CART	Cocaine amphetamine-regulated-transcript
CAAX	Four amino acid sequence in the carboxyl terminus of a protein
cDNA	Complementary DNA
CNS	Central nervous system
CPON	C-Terminal flanking peptide of NPY
CR	Caloric restriction
DDT	Dithiothreitol
DHA	Dorsal hypothalamic area
DMN	Dorsomedial nucleus
DNA	Deoxyribonucleic acid
DPP-IV	Dipeptidyl-peptidase-IV
DR	Dietary restriction
Dyrk1a	Dual specificity tyrosine-phosphorylation-regulated kinase 1a
ECF	Enhanced chemifluorescence
EDTA	Ethylenediamine tetraacetic acid
FOXO	Forkhead box O
GFAP	Glial fibrillary acidic protein
GH	Growth hormone
GPCR	G-coupled protein receptor
GS	Goat serum
HCl	Hydrogen chloride
HE	Hematoxylin-Eosin
HGPS	Hutchinson Gilford Progeria Syndrome
HSCs	Hematopoietic stem cells

Iba1	Ionized calcium-binding adapter molecule
IGF-1	Insulin like growth factor-1
IgG	Immunoglobulin G
IIS	Insulin and IGF-1 signaling pathway
IL-1β	Interleukin-1 beta
KCl	Potassium chloride
KH_2PO_4	Monopotassium phosphate
LC-3B	Light chain-3 B
LHA	Lateral hypothalamic area
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MAP2	Microtubule-associated protein 2
ME	Median eminence
mRNA	Messenger ribonucleic acid
MTOR	Mechanistic target of rampamycin
mTORC	mTOR complex
NaCl	Sodium chloride
NaF	Sodium fluoride
Na_2PO_4	Disodium phosphate
Na_3VO_4	Sodium orthovanadate
NeuN	Neuronal nucleic protein
NF-κB	Nuclear factor kappa B
NPY	Neuropeptide Y
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PFA	Paraformaldehyde
pIκBα	Phospho-IκBα
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C

PMSF	Phenylmethylsulfonyl fluoride
POA	Preoptic area
POMC	Pro-opiomelanocortin
PP	Pancreatic polypeptide
PP-fold	Pancreatic polypeptide fold
Pp53	Phospho-p53
Pre-Pro-NPY	Pre-pro-neuropeptide Y
Pro-NPY	Pro-neuropeptide Y
PVDF	Polyvinylidene fluoride
PVN	Paraventricular nucleus
PYY	Peptide YY
p62/SQSTM1	p62/Sequestosome1
rAAV	recombinant adeno-associated viral vectors
RIPA	Radio-immunoprecipitation assay
ROS	Reactive oxygen species
RT	Room temperature
SAM	Senescence-accelerated mice
SAMP	Senescence-prone strains
SAMR	Senescence-resistant strains
SCN	Suprachiasmatic nucleus
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Sirt1	Sirtuin 1
SON	Supraoptic nucleus
TBS	Tris-buffered saline
TBS-T	Tris-Buffered Saline and Tween 20
TNF-α	Tumor necrosis factor alpha
VMN	Ventromedial hypothalamic nucleus

Abstract

Aging is characterized by the gradual and overall loss of various physiological functions, leading to the end of lifespan. Because average human life expectancy has increased, but also the prevalence of chronic diseases and cognitive decline, aging research is now focused in finding strategies that increase both lifespan and healthspan.

The hypothalamus is a brain region crucial for the neuroendocrine interaction between the central nervous system and the periphery, controlling several survival functions such as development, growth, energy balance, sleep, reproduction and metabolism. Recently, it was described that this brain area is critical for the development of whole-body aging and has impact on lifespan (Zhang et. al., 2013). Moreover, aging is associated with hypothalamic dysfunction, characterized by inflammation and impaired neurogenesis (Zhang et. al., 2013), defective autophagy (Kaushik et. al., 2012) and decreased Neuropeptide Y (NPY) levels (Higuchi et. al., 1988a; Gruenewald et. al., 1994; Vela et. al., 2003), which compromise its neuroendocrine function.

NPY is one of the most abundant peptides in the central nervous system, being mostly produced by the hypothalamus. Several findings suggest that NPY may play a role on the aging process. Aging is associated with reduced levels of NPY (Chiodera et. al., 2000), which has been correlated with neurodegenerative diseases (Rose et. al., 2009; Decressac et. al., 2010; Decressac et. al., 2012b; Duarte-Neves et. al., 2015). On the other hand, it has been shown that NPY has a neuroprotective role (Silva et. al., 2005; Alvaro et. al., 2008; Santos-Carvalho et. al., 2013; Duarte-Neves et. al., 2015). Moreover, transgenic rats overexpressing NPY have improved resistance to stress and increased mean lifespan (Michalkiewicz et. al., 2003). In addition, it has been shown that caloric restriction, a robust anti-aging strategy, does not increase lifespan in NPY knockout mice (Chiba et. al., 2014), suggesting that NPY may play a relevant role in lifespan. Given the key role of hypothalamus in the regulation of whole-body aging and that hypothalamic NPY levels decrease with age, modulation of hypothalamic NPY levels may act as a protective mechanism against impaired hypothalamic dysfunction associated with age, with impact on the aging process and lifespan.

The main objective of the present study was, therefore, to investigate whether modulation of hypothalamic NPY levels can delay aging. More specifically, we investigated whether hypothalamic NPY overexpression ameliorates the aging phenotype of a mouse model of human aging Zmpste24$^{-/-}$ mice.

This study showed that Zmpste24$^{-/-}$ mice have lower levels of hypothalamic NPY and alterations in hypothalamic neuronal structure as showed by the decrease in the levels of

MAP2, a dendritic marker, and NeuN, a neuronal marker. However, the levels of activated microglia (Iba1) decreased in Zmpste24$^{-/-}$ mice when compared to the wild-type control. In addition, when we compared Zmpste24$^{-/-}$ mice with the hypothalamic NPY overexpression group (Zmpste24$^{-/-}$+NPY mice), NPY was able to attenuate the neuroinflammatory process through the decrease of GFAP, an astrocyte marker, and pIκBα, a marker of NF-kB activation. Although the studies made showed that NPY decreased astrogliosis, no direct changes were observed in the number of hypothalamic neurons, suggesting that NPY may decrease neuronal death. In the peripheral tissues, overexpression of NPY in the hypothalamus induced a beneficial effect in the liver structure of Zmpste24$^{-/-}$ mice by decreasing pseudoinclusions, immune cell infiltration area, and the nuclear size. We also observed an increase in the number of hepatocytes concomitant with an increase in the protein levels of PCNA, a cell proliferation marker, and a decrease in the protein levels of the cell cycle repressor p53. However, no significant changes were observed in the parameters studied for the heart and kidney of Zmpste24$^{-/-}$ mice upon overexpression of hypothalamic NPY.

Overall, this study showed that NPY can, at least in part, have a beneficial effect in the hypothalamus of Zmpste24$^{-/-}$ mice. Additionally, among the peripheral organs studied which were liver, heart and kidney, only in the liver the beneficial role of NPY was stronger. In fact, there is evidence showing that the modulation of hypothalamic NPY could have a beneficial impact in the hypothalamus and in the periphery. Although, more studies are needed to understand the role of NPY as a strategy to revert age-related alterations, this study suggest that NPY may be a potential anti-aging strategy.

Keywords: Aging, Neuropeptide Y, Hypothalamus.

Resumo

O envelhecimento é caracterizado pela perda gradual das várias funções fisiológicas, levando ao fim da vida. Uma vez que a esperança média de vida humana aumentou, mas também a prevalência de doenças crónicas e declínio cognitivo, a investigação na área do envelhecimento foca-se em encontrar estratégias que aumentem a esperança média de vida e a qualidade de vida.

O hipotálamo é uma região cerebral importante na interacção neuroendócrina entre o sistema nervoso central e a periferia, controlando várias funções fundamentais à sobrevivência tais como o desenvolvimento, equilíbrio energético, sono, reprodução e metabolismo. Recentemente, foi descrito que esta região cerebral está envolvida no processo de envelhecimento global e tem impacto na esperança de vida (Zhang et. al., 2013). O envelhecimento está associado à disfunção do hipotálamo, que é caracterizado pela ocorrência de processos inflamatórios e alterações na neurogénese (Zhang et. al., 2013), alterações na autofagia (Kaushik et. al., 2012) e diminuição dos níveis de NPY (Higuchi et. al., 1988a; Gruenewald et. al., 1994; Vela et. al., 2003), que comprometem a sua função neuroendócrina.

O neuropeptídeo Y (NPY) é um dos peptídeos mais abundantes no sistema nervoso central, sendo maioritariamente produzido pelo hipotálamo. Várias evidências sugerem que o NPY pode desempenhar um papel no processo de envelhecimento. No envelhecimento ocorre decréscimo dos níveis de NPY (Chiodera et. al., 2000), que por sua vez tem sido relacionado com doenças neurodegenerativas (Rose et. al., 2009; Decressac et. al., 2010; Decressac et. al., 2012a; Duarte-Neves et. al., 2015; Duarte-Neves et. al., 2016). Por outro lado, diversos trabalhos mostraram que o NPY tem um papel neuroprotector (Silva et. al., 2005; Alvaro et. al., 2008; Santos-Carvalho et. al., 2013; Duarte-Neves et. al., 2015). Além disso, ratos transgénicos que sobreexpressam NPY apresentam uma melhoria da resistência ao stress e vivem mais tempo (Michalkiewicz et. al., 2003). Adicionalmente, tem sido mostrado que a restrição calórica, uma estratégia anti-envelhecimento, não aumenta a esperança de vida em murganhos que não expressam NPY (Chiba et. al., 2014), sugerindo que o NPY desempenha um papel relevante na longevidade. Tendo em conta que o hipotálamo tem um papel chave na regulação do envelhecimento de todo o corpo e que os níveis hipotalâmicos de NPY diminuem com a idade, a modulação dos níveis de NPY nesta área cerebral pode actuar como um mecanismo protector contra a disfunção do hipotálamo associada ao envelhecimento, tendo impacto no processo de envelhecimento e na esperança de vida.

Desta forma, o principal objectivo do presente estudo foi investigar se a modulação dos níveis hipotalâmicos de NPY podem retardar o envelhecimento. Concretamente, investigou-se se a

sobreexpressão de NPY no hipotálamo poderia melhorar o fenótipo de envelhecimento de um modelo animal de envelhecimento humano: o murganho Zmpste24$^{-/-}$.

Este estudo mostrou que os murganhos Zmpste24$^{-/-}$ apresentam baixos níveis de NPY no hipotálamo e alterações na estrutura neuronal do hipotálamo, nomeadamente a diminuição dos níveis de MAP2, um marcador de dentrites, e de NeuN, um marcador neuronal. Contudo os níveis de Iba1 diminuíram nos murganhos Zmpste24$^{-/-}$ quando comparados com o grupo controlo. Adicionalmente, quando se comparou os murganhos Zmpste24$^{-/-}$ com o grupo que sobreexpressa NPY (murganhos Zmpste24$^{-/-}$+NPY), o NPY foi capaz de atenuar o processo neuroinflamatório através da diminuição do GFAP, um marcador de astrócitos, e pIκBα, um marcador de activação do NF-kB. Apesar dos estudos realizados terem mostrado que o NPY diminuiu a astrogliose, não foram observadas alterações directas no número de neurónios no hipotálamo, sugerindo que o NPY pode estar a diminuir a morte neuronal. Nos tecidos periféricos, o NPY mostrou um efeito benéfico na estrutura do fígado dos murganhos Zmpste24$^{-/-}$ através da diminuição de pseudoinclusões, da área de infiltração por células imunes, e do tamanho dos núcleos. Também se observou um aumento no número de hepatócitos concomitante com o aumento dos níveis proteicos de PCNA, um marcador de proliferação celular, e uma diminuição dos níveis proteicos do repressor do ciclo celular p53. Contudo, não foram observadas alterações substanciais nos parâmetros estudados no coração e no rim dos murganhos Zmpste24$^{-/-}$ que sobreexpressam NPY no hipotálamo.

No geral, este estudo mostrou que o NPY hipotalâmico consegue, pelo menos em parte, ter um efeito benéfico no hipotálamo dos murganhos Zmpste24$^{-/-}$. Adicionalmente, entre os órgãos periféricos estudados, o fígado, o coração e o rim, apenas no fígado o efeito benéfico do NPY foi mais evidente. De facto, os resultados mostram que a modulação hipotalâmica de NPY pode ter um impacto benéfico, tanto no hipotálamo como na periferia. Apesar de serem precisos mais estudos para perceber o papel do NPY como estratégia para reverter as alterações relacionadas com o envelhecimento, este trabalho sugere que o NPY pode ser uma potencial estratégia anti-envelhecimento.

Palavras-chave: Envelhecimento, Neuropeptídeo Y, Hipotálamo.

Chapter I

Introduction

1.1. Aging

The number of aged people is increasing in almost every country in the world, having an impact in several sectors of society since it requires not only social but also economic shifts. This increase in longevity, especially in developed countries, may be due to an amelioration of public health conditions. Nevertheless, healthspan does not have a proportional increase and, thus, the aging process becomes a risk factor to develop some chronic diseases, being a new challenge to achieve as well as maintaining people healthy (Harkema et. al., 2016). Between 2015 and 2050 the global population of older persons (aged 60 years or over) is projected to grow from 901 million to 2.1 billion (United Nations).

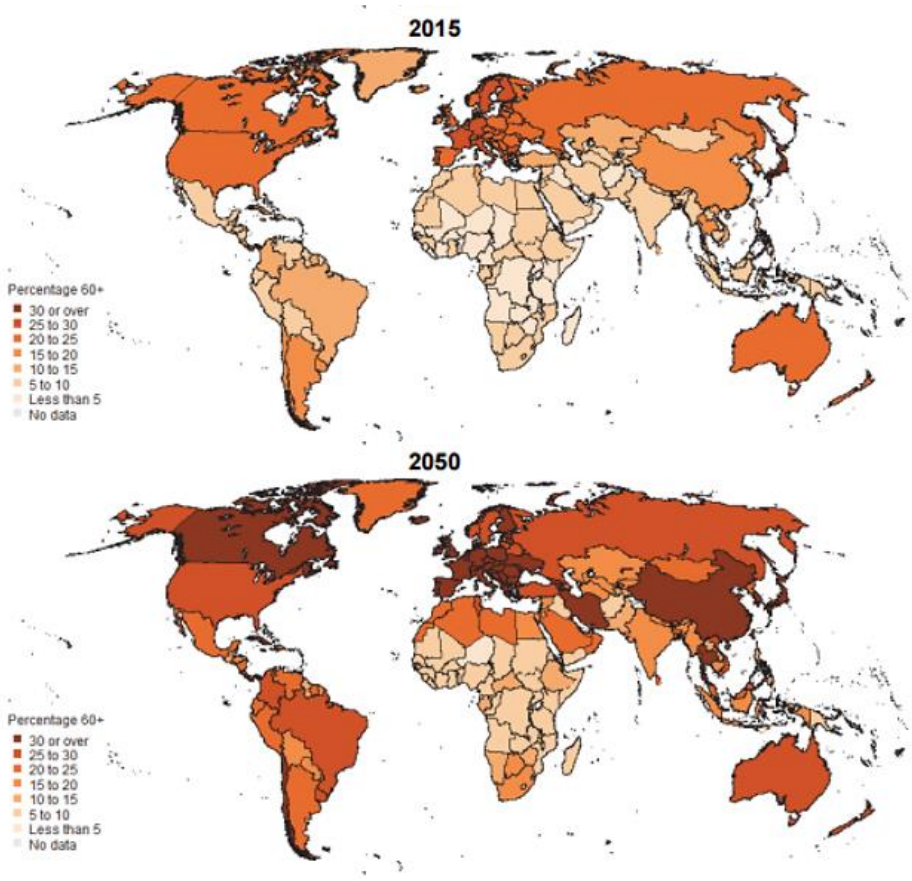

Figure 1.1 - Percentage of population aged 60 years or over in 2015 and projected for 2050. Data source: United Nations (2015).

Aging is a natural process defined as the gradual impairment of biological functions followed by a decreased capacity to respond to stress and by an increased propensity for morbidity and mortality (Marino et. al., 2010; Nicolai et. al., 2015). This is a complex process

characterized by a progressive failure in maintaining homeostasis of tissues which leads to age-related pathologies, namely cancer, cardiovascular diseases and neurodegenerative disorders (Nicolai et. al., 2015).

Some theories have been proposed to explain aging, but its molecular basis is not completely understood yet. Aging is, therefore, a process characterized by progressive failure of pathways in response to external and endogenous stresses leading to a decline of cellular homeostasis and cognitive impairment (Bishop et. al., 2010; Hartl, 2016). Even so, aging research has experienced some advances in the past few years, especially with the discovery that aging is controlled by genetic and biochemical processes conserved in evolution (Lopez-Otin et. al., 2013). One of the biggest challenge of aging research, now that lifespan has been increasing, is to understand the mechanisms underlying the aging process in order to increase healthspan as well as improve quality of life. In 2013, López-Otín and collaborators described and categorized nine cellular and molecular hallmarks of aging. These hallmarks are: genomic instability; telomere attrition; epigenetic alterations; loss of proteostasis; dysregulated nutrient sensing; mitochondrial dysfunction; cellular senescence; stem cell exhaustion; and altered intercellular communication (Lopez-Otin et. al., 2013).

Since the integrity and stability of DNA is often challenged by exogenous and endogenous threats, the first hallmark reported is genomic instability that occurs not only in premature aging diseases but also in normal aging, leading to the accumulation of genetic damage during life (Moskalev et. al., 2013). Point mutations and translocations are examples of genetic lesions. These lesions can be minimized due to the action of DNA repair mechanisms (Lord et. al., 2012). Besides, alterations in nuclear DNA including somatic mutations and copy number variations have been associated with aging (Faggioli et. al., 2012; Forsberg et. al., 2012). There is evidence that aging increases genomic damages due to deficiencies in DNA repair mechanisms and cause accelerated aging in mice (Hoeijmakers, 2009; Murga et. al., 2009; Gregg et. al., 2012). Mutations and deletions in aged mitochondrial DNA may also potentiate the aging process (Park et. al., 2011). Additionally, genomic instability can be caused by alterations in the nuclear architecture (called laminopathies), which results in premature aging syndromes (Worman, 2012). Genomic stability is regulated by protein complexes whose scaffold is provided by nuclear lamins (Liu et. al., 2005; Gonzalez-Suarez et. al., 2009). Alterations of nuclear lamina leads to the production of an aberrant prelamin A called progerin, which indeed have been detected during normal human aging (Scaffidi et. al., 2006; Ragnauth et. al., 2010). Experimental models have facilitated the identification of stress

pathways which occur in these nuclear architecture alterations, one of them is the activation of p53 (Varela et. al., 2005). Overall, genomic damage is involved in the aging process (Lopez-Otin et. al., 2013).

Another hallmark of aging that appears to be related with the accumulation of DNA damage is the telomere attrition, these chromosomal regions are susceptible to deterioration due to aging (Blackburn et. al., 2006). In fact, the accumulation of DNA damage is the most important cause for telomere attrition (Kirkwood, 2005), but telomere shortening is also observed during normal aging in humans and mice (Blasco, 2007). Telomere dysfunction, which includes telomere exhaustion and telomerase deficiency, accelerates aging. Telomeres are regions with restricted DNA repair, which causes higher propensity to DNA damage resulting in senescence and apoptosis (Fumagalli et. al., 2012; Hewitt et. al., 2012). Additionally, aging can be reverted, in telomerase-deficient mice, through telomerase activation (Jaskelioff et. al., 2011). In conclusion, telomere attrition was accepted as hallmark of aging because it accompanies normal aging, in pathological conditions accelerates aging and telomerase stimulation delay aging (Lopez-Otin et. al., 2013).

Epigenetic alterations are another hallmark and it can be defined as the set of changes, resulting from the action of environmental factors, which affect the way that cells express genes. There is evidence suggesting that aging is accompanied by epigenetic changes, which includes alterations in histones and DNA methylation (Fraga et. al., 2007; Han et. al., 2012). Although cells from patients and mice with progeroid syndromes present DNA methylation profiles similar to those found in normal aging (Shumaker et. al., 2006; Osorio et. al., 2010), there is no evidence that lifespan can be increased by changing DNA methylation (Lopez-Otin et. al., 2013). Histone modifications may also be implicated in aging, in fact, it is described that mice lacking sirtuin-6 present accelerated aging (Mostoslavsky et. al., 2006), while transgenic mice overexpressing sirtuin-6 have an increased lifespan when compared to the controls (Kanfi et. al., 2012). Taking into account these evidence, the understanding and manipulation of epigenome may be important to aging pathologies (Lopez-Otin et. al., 2013).

Protein homeostasis (called proteostasis) is maintained through some mechanisms such as chaperone-mediated protein folding, autophagy-lysosomal system and ubiquitin-proteasome system, each one has its function but all are implicated in the quality control of proteins. However, with age these mechanisms become dysregulated and proteins, which were functional, no longer have the correct conformation and tend to aggregate. This leads to

another hallmark, loss of proteostasis, which is characterized by a dysregulation in the mechanisms that are responsible for the stabilization of correctly folded proteins and for its degradation (Mizushima et. al., 2008; Koga et. al., 2011; Hartl, 2016). There is evidence that loss of proteostasis is common during the aging process (Koga et. al., 2011) and, in addition, the accumulation of misfolded or aggregated proteins is linked to age-related pathologies (Powers et. al., 2009). With aging, the synthesis of chaperones induced by stress becomes altered (Calderwood et. al., 2009). Moreover, the activities of autophagy-lysosomal and ubiquitin-proteasome systems also change with aging (Rubinsztein et. al., 2011; Tomaru et. al., 2012). Although proteostasis is involved in the aging process, there are manipulations that improve proteostasis and delay aging in mammals (Zhang et. al., 2008).

Insulin growth factor 1 (IGF-1) and insulin form the IIS signaling pathway, are involved in the aging control, together with its targets namely FOXO (forkhead box O) and MTOR (mechanistic target of rapamycin) (Fontana et. al., 2010; Kenyon, 2010; Barzilai et. al., 2012). Consequently, a decrease in its functions due to polymorphisms or mutations have been correlated with longevity, in humans and in model organisms (Fontana et. al., 2010; Kenyon, 2010; Barzilai et. al., 2012). Additionally, dietary restriction (DR) increases lifespan, being in accordance with the importance of deregulated nutrient-sensing as a hallmark of aging (Colman et. al., 2009; Fontana et. al., 2010; Mattison et. al., 2012). It is described that lifespan is extended in flies, worms and mice as a result of genetic manipulations that mitigate the signaling of the IIS (Fontana et. al., 2010). Additionally, GH and IGF-1 levels decrease with normal aging and in mouse models of premature aging (Schumacher et. al., 2008), in an attempt to extend lifespan but, instead, aggravates aging. Moreover, some interconnected nutrient-sensing systems are also involved in this aging process: MTOR (member of mTORC1 and mTORC2 multiprotein complexes), which senses high concentrations of amino acids; AMP-activated protein kinase (AMPK) and sirtuins, which detect low energy states (Houtkooper et. al., 2010). It has been shown that mTORC1 downregulation in yeast, worms and flies increases longevity (Johnson et. al., 2013). AMPK activation shuts off mTORC1 (Alers et. al., 2012), and increases lifespan in worms and mice upon metformin administration (medication for type 2 diabetes) (Onken et. al., 2010; Anisimov et. al., 2011; Mair et. al., 2011). Overall, the findings suggest that lifespan is extended with a decrease in nutrient signaling (Fontana et. al., 2010).

Aging is associated with a decrease in the efficacy of the respiratory chain, leading to the sixth hallmark called mitochondrial dysfunction. Some theories were proposed in this field, one of them is that progressive mitochondrial dysfunction, that characterizes aging, increases the production of reactive oxygen species (ROS), which consequently cause deteriorations in mitochondria (Harman, 1965). However, some studies described that in mice, an increase in mitochondrial ROS do not accelerate aging (Van Remmen et. al., 2003; Zhang et. al., 2009). On the other hand, studies in mice deficient in mitochondrial DNA show that alterations in the function of mitochondria can have an impact on aging independently of ROS (Edgar et. al., 2009; Hiona et. al., 2010). Although it is not well known whether the improvement of mitochondrial function can extend lifespan, its function have an important impact in the aging process (Lopez-Otin et. al., 2013).

Cells that experience different types of stress undergo a permanent arrest of proliferation called cellular senescence (Campisi, 2014), which is another hallmark of aging. The amount of senescent cells increases with aging, but its primary purpose is to prevent the propagation of damaged cells and this requires an efficient system for cell replacement involving clearance of aged cells (Lopez-Otin et. al., 2013). In aged organisms, the accumulation of senescent cells may be due to alterations in the cell replacement system that becomes inefficient (Lopez-Otin et. al., 2013). Senescence can be caused by telomere shortening (Bodnar et. al., 1998). The activation of p53 can have a beneficial response in order to prevent the propagation of damaged cells and its consequences on aging and senescence (Lopez-Otin et. al., 2013). In conclusion, cellular senescence is a response to damage that becomes altered and accelerates aging (Lopez-Otin et. al., 2013).

Aging is associated with a decline in the capacity of tissues to produce new cells, for example, with aging there is a decrease in hematopoiesis resulting in a diminished production of immune cells (Shaw et. al., 2010). Additionally, in aged mice, old hematopoietic stem cells (HSCs) have less cell divisions than young cells (Rossi et. al., 2007). This is correlated with the accumulation of damages in DNA (Rossi et. al., 2007), and with the increase in the expression of cell cycle arrest proteins (Janzen et. al., 2006). Overall, stem cell exhaustion is the result of several types of age-related damages (Lopez-Otin et. al., 2013).

Finally, the ninth hallmark is altered intercellular communication, being inflammation one of the age-related alterations in intercellular communication. Age-related inflammation may

result from several factors such as the accumulation of damage in tissue; failure of immune system; increase of pro-inflammatory cytokines; and alterations in autophagy (Salminen et. al., 2012). These alterations cause the production of IL-1β and NF-κB, as a result of activation of inflammatory pathways (Green et. al., 2011; Salminen et. al., 2012). It is described that in aged transgenic mice, the inhibition of NF-κB cause skin rejuvenation (Adler et. al., 2007). Overall, aging is associated with alterations in intercellular communication and its modulation can be beneficial to the aging process (Lopez-Otin et. al., 2013).

The study of the nine hallmarks of aging allows a detailed and better understanding of the mechanisms related with the aging process.

1.1.1. Aging in the brain

Aging on brain has effects that are widespread and have multiple origins, encompassing molecules, cells, vasculature, morphology and cognition (Peters, 2006). Structural changes include shrinkage in total brain volume and morphological changes in specific regions. Additionally, brain aging also involves alterations in metabolism, levels of enzymes, hormones, increased oxidative stress, altered protein processing and synaptic function, and these changes altogether lead to decline in physiological and cognitive functions (Thakur et. al.).

With aging the brain shrinks in volume, especially in the frontal cortex (Peters, 2006), which is accompanied by increase in ventricular volume (Anderton, 2002), reduction of synaptic spines, lower number of synapses (Dickstein et. al., 2007) and increase in blood-brain barrier permeability (Farrall et. al., 2009). Studies have suggested that these age-related reductions in brain weight were correlated with a decline in neuron number on cortex (Brody, 1955) and profound cell loss in the hippocampus of humans (Ball, 1977). However, more recently it was reported that profound loss of neurons does not significantly contribute to age-related cognitive impairments (Burke et. al., 2006). Instead, it depends on the maintenance of synaptic contacts between axons and dendritic spines (Hof et. al., 2004). In fact, decreases in spine density have been reported with brain aging in rodents (Leuba, 1983; Wallace et. al., 2007; Freeman et. al., 2008), non-human primates (Page et. al., 2002; Dumitriu et. al., 2010), and humans (Anderson et. al., 1996). The magnitude of spine loss seems to correlate with the degree of functional impairment (Mostany et. al., 2013). The hypothalamus, which is a brain region that plays important physiological functions has been linked to the control of systemic aging and lifespan (Zhang et. al., 2013). Therefore, the brain changes with increasing chronological age, less clear is the rate of change and the processes involved (Peters, 2006).

1.1.2. Molecular mechanisms of brain aging

Although the molecular basis of aging are not completely understood, some mechanisms have been reported to explain the aging process including increased mitochondrial dysfunction (Ames, 2004), oxidative stress (Serrano et. al., 2004) and accumulation of damaged proteins (Gray et. al., 2003).

Mitochondrial function has long been recognized to decline during aging (Bratic et. al., 2013), particularly in brain and muscle (Yankner et. al., 2008). In fact, it has been described that mitochondrial dysfunction can accelerate the aging process in mammals (Trifunovic et. al., 2004; Kujoth et. al., 2005; Vermulst et. al., 2008). Mitochondria are protected against endogenously generated reactive oxygen species (ROS), but an increase in the production of ROS is a consequence of alterations in the electron transport chain during aging. This protection against ROS is conferred by a number of antioxidants defenses such as manganese superoxide dismutase, periredoxins, and redox reactions mediated by cytochrome C and cytochrome oxidase (Yankner et. al., 2008). However, with aging there is a decrease in the action of these antioxidants leading to oxidative damage of mitochondrial proteins and deoxyribonucleic acid (DNA) (Yankner et. al., 2008).

The brain is affected by aging and, in addition, cells in nervous system experience increased amounts of oxidative stress and accumulation of damaged proteins (Mattson et. al., 2006). This accumulation of damaged and aggregated proteins is due to an age-related dysregulation in protein quality control which, in normal conditions, are mechanisms responsible for the stabilization of correct folded proteins. Studies have demonstrated that protein homeostasis is altered with aging (Koga et. al., 2011). The majority of damaged proteins are removed by autophagy (Bergamini et. al., 2003), however, this process fails as age increases leading to dysfunctional neurons and cell death (Bi et. al., 2000; Cuervo et. al., 2005; Meijer et. al., 2006). Additionally, dysregulated autophagy which results in protein aggregation are common characteristics of neurodegenerative diseases (Hara et. al., 2006; Rubinsztein et. al., 2011).

In order to increase lifespan and improve healthspan it is necessary to understand the molecular mechanisms that underlie normal aging and age-related pathologies.

1.1.3. Experimental models of human aging

Until recently, were scarce the experimental models to study the aging process. Since the molecular mechanisms that underlie human aging are not fully understood yet, have been

developed novel mouse models that exhibit multiple aging phenotypes and are expected to be important tools for aging research (Kuro-o, 2001).

Senescence-accelerated mice (SAM): was generated in 1970 and was developed by using several pairs of the AKR/J strain of mice based on premature onset of changes related with aging (Takeda et. al., 1997). SAM is a term used for three senescence-resistant strains (SAMR), and nine senescence-prone strains (SAMP), which serve as controls for SAMP. SAMP strains exhibit several aging phenotypes, such as amyloidosis, osteoporosis, malignant tumors and cataracts (Takeda et. al., 1991).

Lmna model: is a mouse model of aging that reproduce the deteriorations of Hutchinson-Gilford progeria syndrome (HGPS) and present the HGPS mutation, resulting of the replacement of wild-type mouse Lmna gene with an allele carrying the mutation c.1827C>T;p.Gly609Gly equivalent to the human LMNA gene mutation (Osorio et. al., 2011). These mice show reduced growth rates; weight loss; abnormal posture; lifespan of 103 days; cardiovascular alterations; bone alterations; nuclear abnormalities and progerin accumulation; and increase senescence in liver and kidney (Osorio et. al., 2011).

PolgA$^{mut/mut}$ mice: this is a mouse model to study the alterations in the replication and repair of mitochondrial DNA, being characterized by a mutation in Polg gene which is important for the function of DNA polymerase of mitochondria in mice (Trifunovic et. al., 2004; Harkema et. al., 2016). Despite not having an increase in ROS, these mice present phenotypic alterations similar to some age-related modifications including alopecia; cardiomyopathy; weight loss; accumulation of mutations in mitochondrial DNA; and osteoporosis (Mitchell et. al., 2015).

Zmpste24 deficient mice, a model of human aging: mutant mice deficient in a metalloproteinase called Zmpste24, which is a protein widely distributed in mammalian tissues, is a mouse model that has been already described (Pendas et. al., 2002). The Zmpste24 is involved in the lamin A maturation, which is an essential component of the nuclear envelope, and mice without this metalloproteinase exhibit several alterations similar to the human accelerated aging processes as is the case of Hutchinson-Gilford progeria syndrome (Marino et. al., 2010). Studies in Saccharomyces cerevisiae identified two genes, Ste24 and Rce1, involved in the cleavage of carboxyl-terminal amino acids from proteins who suffer isoprenylation (addition of hydrophobic molecules to a protein) and terminate with a CAAX sequence motif (Leung et. al., 2001). Ste24p cleaves the carboxyl-terminal -AAX from the yeast mating pheromone a-factor (farnesylated and carboxylmethylated peptide) and also cleaves the amino acid terminus of a-factor. Additionally, the mouse genome contains orthologue STE24, called Zmpste24.

Prelamin A is a farnesylated CAAX protein (a four-amino acid sequence at the carboxyl terminus of a protein) that goes through proteolytic processing steps, the removal of C-terminal –AAX and the cleavage of 15 residues from the C-terminus of the protein, both by Zmpste24, to form a mature lamin A (Bergo et. al., 2002). In 2002, Pendás and his colleagues developed this mouse model (by intercrossing) and they observed that the lack of Zmpste24 protein bring, after a few weeks of age, some differences when compared with their wild-type littermates such as: reduced growth rate; weight loss; abnormal posture (scoliosis) and an average lifespan of 5 months. After their death, the organs were assessed and they reported that Zmpste24 deficiency was associated with prelamin A processing defects as well as increase in the weight of kidneys in Zmpste24$^{-/-}$; cardiomyopathy; loss of subcutaneous fat layer; senescent cells in several organs; skin lesions; increased number of lipid droplets in hepatocytes and muscular dystrophy (Pendas et. al., 2002). Another study of Zmpste24 deficient mice reported some alterations in these animals including hair loss; fibrosis; spontaneous bone fractures in clavicle, sternum, zygomatic arch, mandible and humerus; muscle weakness; and defects in prelamin A processing (Bergo et. al., 2002). In 2008, one study using Zmpste24$^{-/-}$ mice showed that these mice, despite being a model of accelerated aging, exhibit an increase activation of basal autophagy instead of the characteristic decline that occurs with normal aging. Additionally, this increase is linked to MTOR pathway inhibition and AMPK activation.

1.2. Neuropeptide Y

The amino acid sequence of neuropeptide Y (NPY) was first isolated, in 1982, from extracts of porcine brain by Tatemoto and colleagues (1982) who showed that NPY is a 36 amino acid peptide with tyrosine residues (Y) and a carboxyl terminal amidation (Figure 1.2) (Tatemoto, 1982; Tatemoto et. al., 1982). This peptide is a member of NPY family, constituted by NPY which is expressed in central nervous system and in peripheral tissues; and peptide YY (PYY) and PP which are gut endocrine peptides (Larhammar, 1996). The presence of an α-amidated carboxyl terminus was determinant for the purification of NPY and, additionally, one important characteristic of the NPY-family peptides is that they exhibit certain amino acid residues necessary to adopt a specific three-dimensional structure namely the pancreatic polypeptide PP fold family (Chatzidimitriou-Dreismann et. al., 1996; Cerda-Reverter et. al., 2000).

Despite the large size, NPY is one of the most conserved peptides during evolution, while PYY and PP are more variable, and this occurs because many positions in NPY are more likely to be kept conserved due to binding to multiple receptor subtypes that differ in their points of interaction with the ligand (Larhammar, 1996).

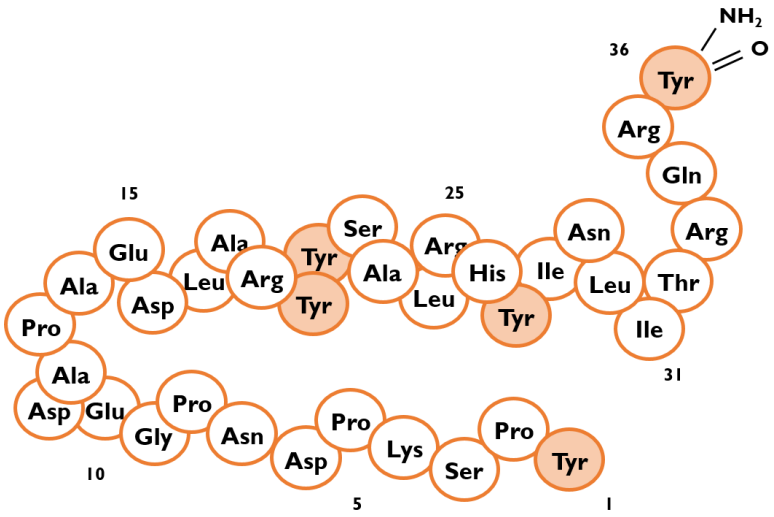

Figure 1.2 - Structure of human Neuropeptide Y, it has 36 amino acid peptides, several tyrosine residues (abbreviated by the letter Y) and an amidated carboxyl terminus. Adapted from (Schwartz et. al., 1990; Pruitt et. al., 2012).

1.2.1. Processing, localization and metabolization of Neuropeptide Y

In the human genome, the NPY gene is located in the chromosome 7 at the locus 7p15.1, divided into 4 exons which are separated for 3 introns (Pruitt et. al., 2012). But in mouse, the NPY gene is located in chromosome 6 locus 6 B3; 6 24.04 cM and in rat is located in chromosome 4, locus 4q24 (Pruitt et. al., 2012).

NPY is first synthesized as a precursor peptide and only becomes functional by post-translational modifications, which means that the precursor peptide is cleaved at specific sites leading to mature NPY. In the processing of NPY, four post-translational enzymatic events must occur for it to become active (Figure 1.3). In the ribosomes, the messenger ribonucleic acid (mRNA) is translated to form the pre-pro-NPY (with 97 amino acids), which is directed to the endoplasmatic reticulum where the signal peptide is removed by a signal peptidase to give the pro-NPY (removing 28 amino acids). The following processing step is the cleavage of the precursor pro-NPY at a dibasic site (Lys-Arg) by prohormone convertases giving origin to NPY_{1-39} and C-terminal peptide of NPY (CPON) which is released (Minth et. al., 1984; Silva et. al., 2002). NPY_{1-39} is then processed by carboxypeptidase B to form NPY_{1-37} which is thus cleaved by peptidylglycine α-amidating monooxygenase, removing the glycine amide donor and leading to the biologically active NPY_{1-36} commonly called NPY.

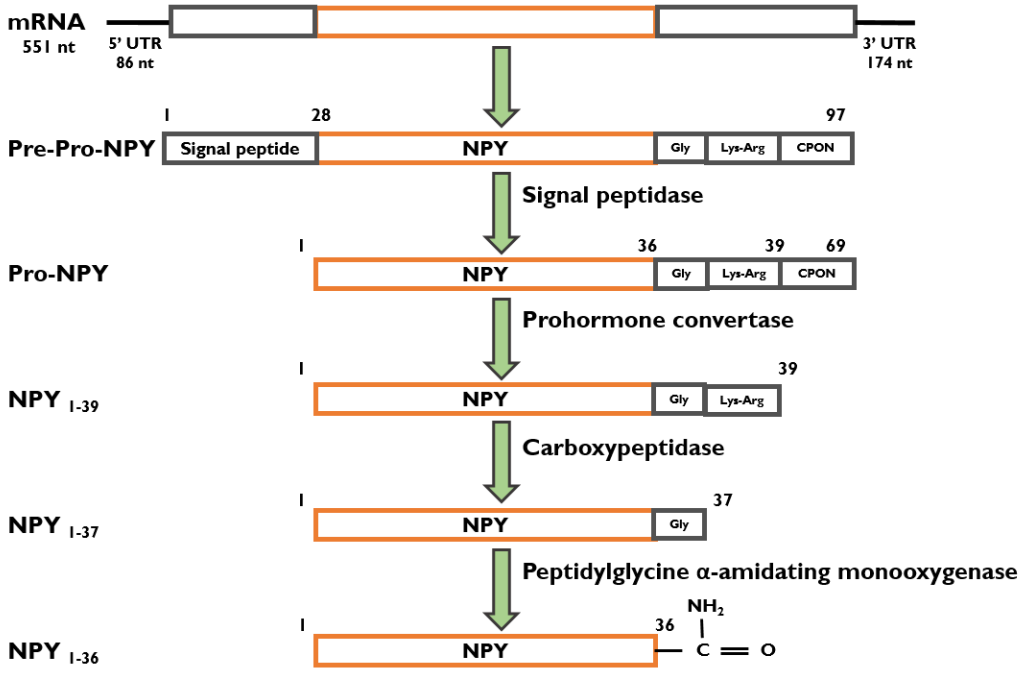

Figure 1.3 - Biosynthesis of NPY. UTR: untranslated region; nt: nucleotides; CPON: c-terminal peptide of NPY. Adapted from (Santos-Carvalho et. al., 2015).

The mature NPY can suffer additional metabolization processes through enzymatic cleavage by several enzymes, such as dipeptidyl peptidase-IV (DPP-IV) which is expressed on the cell surface and has an exopeptidase activity (cleaves the dipeptides from the N-terminal of polypeptides); aminopeptidase P which is a soluble proline-specific aminopeptidase that hydrolyses NPY at the N-terminal; and endopeptidase neutral 24-11, that cleaves peptides at the side of hydrophobic residues; the action of these three enzymes give rise to different metabolites with selective specificity to NPY receptors (Santos-Carvalho et. al., 2015). More specifically NPY can be cleaved by aminopeptidase P and DPP-IV giving origin to NPY_{2-36} and NPY_{3-36}, respectively (Figure 1.4), and these fragments are agonists of Y_2/Y_5 receptors (Silva et. al., 2003) which are able to regulate food intake among other functions as described in the Table 1.2 of section 1.2.2. Then, NPY_{2-36} and NPY_{3-36} can both be cleaved by endopeptidase neutral 24-11 to form two fragments, NPY_{1-20} and NPY_{31-36}, whose biological activity is not known yet (Medeiros et. al., 1994).

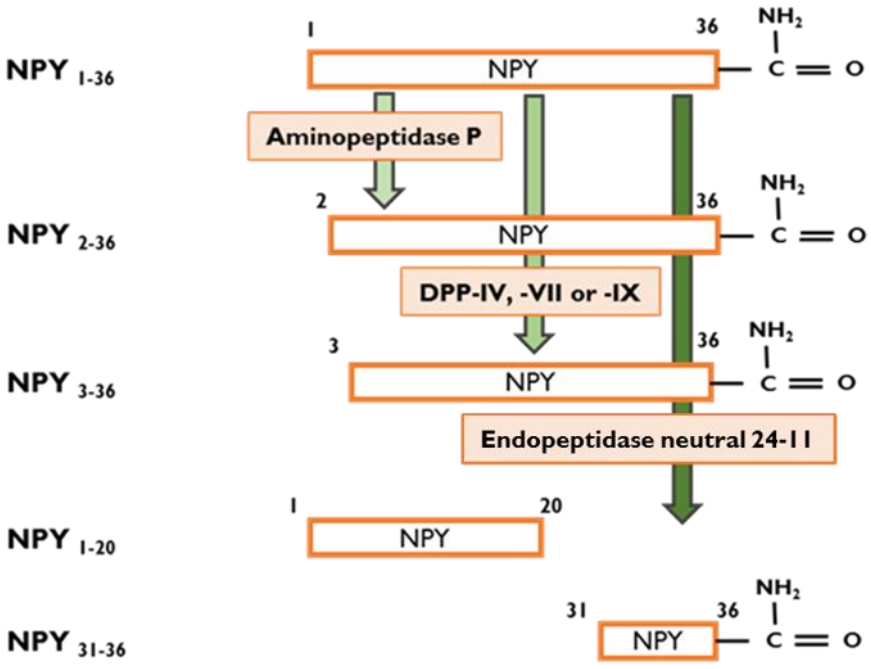

Figure 1.4 - Processing of NPY by several aminopeptidases to form NPY fragments. Adapted from (Botelho, 2015; Santos-Carvalho et. al., 2015).

NPY is widely expressed throughout the central and peripheral nervous system, exerting a number of important physiological functions (Decressac et. al., 2012a) as shown in Table 1.1.

Table 1.1 - Distribution of NPY in the body (Botelho, 2015; Santos-Carvalho et. al., 2015)

Localization of NPY	Distribution	References
Central Nervous System	Spinal Cord, mesencephalon, metencephalon	(Allen et. al., 1984; Halliday et. al., 1988; Pammer et. al., 1990)
	Hypothalamus, cortex, basal ganglia, thalamus, septum, striatum, amygdala	(Allen et. al., 1983; de Quidt et. al., 1986a; Silva et. al., 2005)
	Hippocampus	(Caberlotto et. al., 2000)
	Occipital lobe and temporal cortex	(Adrian et. al., 1983; Beal et. al., 1987; Delalle et. al., 1997)
Peripheral Nervous System	Sympathetic ganglion neurons	(Lundberg et. al., 1982)
Immunologic System	Spleen	(Lundberg et. al., 1989; Romano et. al., 1991)
	Thymus	(Kranz et. al., 1997)
Digestive system	Dental pulp	(Uddman et. al., 1984)
	Tongue	(Kusakabe et. al., 1998)
	Colon, enteric neurons, pylorus	(Rettenbacher et. al., 2001; Cox et. al., 2002; Lindestrom et. al., 2002; Anitha et. al., 2006; Zalecki, 2012)
	Liver	(Esteban et. al., 2001)
Cardiovascular system	Heart and endothelial cells	(Ahmed et. al., 1997; Jackerott et. al., 1997; Zukowska-Grojec et. al., 1998; Jacques et. al., 2003; Silva et. al., 2005)
Endocrine system	Pituitary gland	(Grunditz et. al., 1984; Jones et. al., 1989; Silva et. al., 2005)
	Thyroid gland	(Grunditz et. al., 1984)
	Adrenal Glands	(de Quidt et. al., 1986b; Pelto-Huikko, 1989; Fernandez-Vivero et. al., 1993; Cavadas et. al., 2001)
	Pancreas, Langerhans islets	(Jackerott et. al., 1997; Ponery et. al., 2000; Adeghate et. al., 2001; Lambert et. al., 2002)
	Adipose tissue and adipocytes	(Valet et. al., 1990; Rosmaninho-Salgado et. al., 2012)
Reproductive system	Ovary and corpus luteum	(Keator et. al., 2010)
	Testis	(Gong et. al., 2009)
	Corti organ	(Gomide et. al., 2009)
Sensory systems	Lacrimal glands	(Seifert et. al., 1996)
	Nasal mucosa	(Zhao et. al., 1998; Knipping et. al., 2003)
Hair and skin	Skin nerve fibers	(Johansson, 1986)
	Outer root sheath of hair follicles	(Johansson, 1986)
Bone, Muscles	Skeletal muscle	(Jonhagen et. al., 2006)
	Ligaments	(Jiang et. al., 2008)
	Periosteum Osteoblasts	(Togari et. al., 1997)

1.2.2. Neuropeptide Y receptors

The wide range of physiological effects of NPY are mediated by its receptors (Y_1; Y_2; Y_4; Y_5; and y_6), which belong to the G-protein coupled receptors (Decressac et. al., 2012a), and its distribution varies depending on the brain region as described in Table 1.1. The NPY receptors

can be organized into subfamilies: Y_1 subfamily includes the subtypes Y_1, Y_4 and y_6; the Y_2 subfamily contain the subtypes Y_2 and Y_7; and the Y_5 subfamily which is only formed by Y_5 itself (Larhammar et. al., 2004). NPY can act as a modulator of neurotransmission pre- and post-synaptically, all receptors are located post-synaptically but only Y_2 is located pre-synaptically and each of the receptors described above plays characteristic functions of NPY (Decressac et. al., 2012a). Additionally, the N-terminal is necessary for binding to the Y_1 receptor whereas the intact C-terminal is necessary for binding to Y_2 (Rose et. al., 2009). The several NPY receptors have different affinities for the elements of PP family, more properly the Y_1, Y_2 and Y_5 receptors have high affinity for NPY and PYY, but low affinity for PP (Blomqvist et. al., 1997). Y_4 receptor, instead, show high affinity for PP and low affinity for NPY and PYY (Blomqvist et. al., 1997).

The activation of NPY receptors is associated with different molecular signaling pathways Table 1.2. Y_1 and Y_2 receptors activation is transduced in several signals including inhibition of adenylate cyclase by Gi or Go protein activation; intracellular Ca^{2+} mobilization; modulation of K^+ and Ca^{2+} channels; mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K) pathways; protein kinase A (PKA) modulation; and protein kinase C (PKC) activation (Gerald et. al., 1995; Selbie et. al., 1995; Mannon et. al., 1998; Nie et. al., 1998; Sun et. al., 1998; Mullins et. al., 2002; Rosmaninho-Salgado et. al., 2012; Aveleira et. al., 2015). The activation of Y_4 receptor induces the inhibition of adenylate cyclase by Gi or Go protein activation; intracellular Ca^{2+} mobilization; modulation of K^+ and Ca^{2+} channels; MAPK pathway; and PKC activation (Bard et. al., 1995; Sun et. al., 1998; Mullins et. al., 2002). Y_5 receptor activation leads to signals transduction such as inhibition of adenylate cyclase by Gi or Go protein activation; PI3K pathway; MAPK pathway; PKA modulation; and PKC activation (Pellieux et. al., 2000; Mullins et. al., 2002; Igura et. al., 2011; Rosmaninho-Salgado et. al., 2012; Aveleira et. al., 2015).

Regarding the functions of this neuropeptide in the central nervous system, it is described that NPY is able of stimulate the proliferation of endothelial cells, through its Y_1 receptor (Zukowska-Grojec et. al., 1998); produce an anxiolytic effect in rats through its Y_1, Y_2 and Y_5 receptors (Sajdyk et. al., 1999; Redrobe et. al., 2003; Redrobe et. al., 2004; Morales-Medina et. al., 2012); increase appetite in rats through Y_1 receptor and regulate appetite by the activation of Y_2, Y_4 and Y_5 receptors (Corp et. al., 2001; Sainsbury et. al., 2002; Campbell et. al., 2003; Mashiko et. al., 2003; Morales-Medina et. al., 2012); promote neuroprotection against excitotoxicity in rat hippocampus by mediating neurodegeneration through Y_1, Y_2 and

Y_5 receptors (Silva et. al., 2003; Silva et. al., 2005). Additionally, in rat retinal cell cultures Y_2, Y_4 and Y_5 receptors inhibited cell death (Santos-Carvalho et. al., 2013); and regulate circadian rhythms by Y_2 receptor activation (Soscia et. al., 2005). Each one of the receptors show varied distributions not only across the central nervous system, but also in peripheral tissues. Both central Y_2 and peripheral Y_1 receptors act similarly to repress bone formation (Shi et. al., 2012). Thus, Y_1 receptors of the hypothalamus and periphery act in opposition, with central Y_1 acting to reduce adiposity, while peripheral Y_1 acts to stimulate proliferation of adipocyte (Shi et. al., 2012). Moreover, adipocytes also express Y_2 receptors and the action of peripheral Y_2 receptor in the white adipose tissue is opposite to the well-known anti-obesity action of hypothalamic Y_2 receptors (Shi et. al., 2012), promoting adipocyte proliferation and growth of abdominal fat (Kuo et. al., 2007). Y_1, Y_2 and Y_5 appear to be involved in the regulation of renal function, producing renal vasoconstriction in vitro through its Y_1 receptor, and can antagonize the effects of vasopressin by Y_2 receptors (Bischoff et. al., 1998).

Table 1.2 - Distribution and functions of mammalian NPY receptor family in the brain. Adapted from (Botelho et. al., 2015).

NPY receptor	Brain Localization	Examples of Functions in CNS	References
NPY Y_1	Hypothalamus, cerebral cortex, hippocampus, amygdala, thalamus.	Increases appetite; Anxiolytic and antidepressant effects; Proliferation; Alcohol consumption regulation; Neuroprotection.	(Zukowska-Grojec et. al., 1998; Sajdyk et. al., 1999; Corp et. al., 2001; Redrobe et. al., 2002; Silva et. al., 2002; Thiele et. al., 2002; Silva et. al., 2005)
NPY Y_2	Hippocampus, hypothalamus, thalamus, amygdale, brainstem and cortex.	Appetite regulation; Anxiolytic and Antiepileptic action; Neuroprotection; Learning and memory; Circadian rhythms regulation.	(El Bahh et. al., 2002; Sainsbury et. al., 2002; Redrobe et. al., 2003; Redrobe et. al., 2004; Silva et. al., 2005; Soscia et. al., 2005; Santos-Carvalho et. al., 2013)
NPY Y_4	Hypothalamus, frontal brain, hippocampus. Thalamus and amygdala.	Food intake regulation; Luteinizing hormone release; Neuroprotection.	(Horvath et. al., 2001; Campbell et. al., 2003; Silva et. al., 2005; Santos-Carvalho et. al., 2013)
NPY Y_5	Hypothalamus, thalamus, amygdala, hippocampus and striatum.	Appetite regulation, Anxiolytic and anticonvulsant effects, Neuroprotection; Circadian rhythms regulation.	(Mashiko et. al., 2003; Benmaamar et. al., 2005; Silva et. al., 2005; Morales-Medina et. al., 2012; Santos-Carvalho et. al., 2013)

1.2.3. Neuropeptide Y in the hypothalamus

NPY is an orexigenic neuropeptide widely distributed throughout the brain, being expressed at high levels in the hypothalamus (Minor et. al., 2009). Is from the hypothalamus that NPY exerts several physiological functions including food intake, energy expenditure, circadian rhythms and reproduction (Silva et. al., 2005; Kalra et. al., 2007).

The hypothalamus is the brain region responsible for regulate homeostasis in animals, it does so by integrating internal and external signals, processing them, then exerting regulatory autonomic signals and neuroendocrine releasing peptides to maintain homeostasis (Biran et. al., 2015). In humans, the hypothalamus is located above the pituitary gland and is composed of a number of interconnected cell groups (nuclei). Each nucleus contains several neuronal types, and these work in an orchestrated manner within and between nuclei to regulate physiological functions including metabolism, satiety, reproduction, circadian rhythm, and emotional responses (Machluf et. al., 2011; Pearson et. al., 2013). These functions are regulated by the paraventricular nucleus (PVN), dorsomedial nucleus (DMN), ventromedial nucleus (VMN), arcuate nucleus (ARC), lateral hypothalamic area (LHA) and suprachiasmatic nucleus (SCN) which are represented in Figure 1.5 (Kalra et. al., 1999).

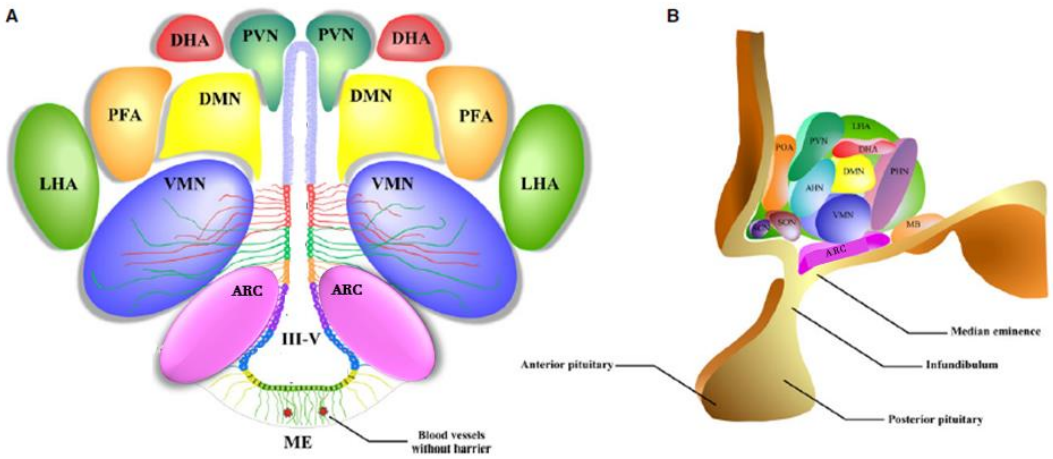

Figure 1.5 - Representation of the hypothalamic nuclei. (A) Coronal view of the approximated location of hypothalamic nuclei. (B) Sagital view of the hypothalamic nuclei distribution. PVN: paraventricular nucleus; DMN: dorsomedial nucleus; VMN: ventromedial nucleus; ARC: arcuate nucleus; DHA: dorsal hypothalamic area; LHA: lateral hypothalamic area; SCN: suprachiasmatic nucleus; SON: supraoptic nucleus; POA: preoptic area; ME: median eminence; III-V: third ventricle. Adapted from (Elizondo-Vega et. al., 2015).

An important mechanism for maintain life is the regulation of energy balance, a process that involves the hypothalamus. The primary region responsible for feeding control is the

hypothalamic ARC, but the hypothalamic PVN and DMN also have a role in regulation of energy balance (Kim et al., 2014). Leptin, insulin, and ghrelin are metabolic hormonal signals received by ARC neurons, which then are integrated through specific receptors (Schwartz et. al., 1996; Harrold et. al., 2008).

The activity of two opposing neuron populations within ARC is modified mainly by circulating factors, being one population the neurons expressing both neuropeptide Y (NPY) and agouti-related peptide (AgRP); and the other neurons expressing both proopiomelanocortin (POMC) and cocaine-amphetamine-regulated transcript (CART) (Minor et. al., 2009). The set NPY/AgRP neurons stimulate food intake (Shutter et. al., 1997; Broberger et. al., 1998) whereas POMC/CART neurons promote satiety (Elias et. al., 1998; Kristensen et. al., 1998).

1.2.4. Neuropeptide Y receptors in the hypothalamus

All of NPY receptors are expressed in the hypothalamus and, besides, the neurons of the arcuate nucleus (ARC) is where the NPY expression is stronger (Gray et. al., 1986) because is in these ARC neurons that NPY is synthesized and stored (Minor et. al., 2009). In the ARC, NPY Y_2 receptors are found both post- and pre-synaptically, while Y_1 and Y_5 receptors only have post-synaptically location (Broberger et. al., 1997; Fetissov et. al., 2004b; Ghamari-Langroudi et. al., 2005; Chee et. al., 2008). In other hypothalamic nuclei which receive NPY projections such as VMN, PVN, SCN and LHA, the expression of NPY Y_1 receptors has been reported (Larsen et. al., 1993; Broberger, 1999; Wolak et. al., 2003). The distribution of NPY Y_5 receptors is also present in hypothalamic nuclei that have NPY projections including the PVN, LHA and supraoptic nucleus (Gerald et. al., 1996; Nichol et. al., 1999; Wolak et. al., 2003; Morin et. al., 2006). In DMN and PVN nucleus, NPY Y_2 receptor is also present (Dumont et. al., 1998; Fetissov et. al., 2004a; Stanic et. al., 2006). Additionally, in the LHA nucleus is where NPY Y_4 receptors are present, especially in orexin-neurons which also receive NPY projections from the ARC (Horvath et. al., 1999; Campbell et. al., 2003). Therefore, NPY neurons and the presence of its receptors in several hypothalamic nuclei underlie the important role of NPY in the regulation of homeostasis (Fetissov et. al., 2004b).

NPY expression is influenced by diverse circulating factors, including glucocorticoids (Higuchi et. al., 1988b), insulin (Schwartz et. al., 1992) and leptin (Ahima et. al., 1996; Schwartz et. al., 1996; Baskin et. al., 1999). In addition, ghrelin, which is a gut hormone, can also modulate NPY expression (Kamegai et. al., 2001; Shintani et. al., 2001). Anorexigenic signals, such as leptin and insulin, have an appetite-suppressing influence on NPY. Leptin, which is a hormone produced by adipocytes, is important for maintain energy homeostasis acting as a signal to the hypothalamus (Jequier, 2002) and there is evidence that in a mouse model of obesity, the lack of leptin causes increased levels of NPY and food intake which can be reduced by leptin administration (Stephens et. al., 1995; Ahima et. al., 1996; Schwartz et. al., 1996). In mouse models of diabetes, which produce less insulin, the high levels of NPY can be normalized by insulin treatment (Williams et. al., 1989; White et. al., 1990; Jones et. al., 1992; Sahu et. al., 1997), meaning that insulin can decrease the expression of NPY in the ARC (Spanswick et. al., 1997). On the other hand, orexigenic signals being appetite stimulators, can activate the expression of NPY. Ghrelin, being produced by stomach (Ariyasu et. al., 2001), increases NPY levels (Kamegai et. al., 2001; Shintani et. al., 2001) to stimulate food intake.

1.2.5. Neuropeptide Y: a key player of the aging process?

Several studies have been made in order to understand how NPY is correlated with the aging process. Aging is associated with decreased levels of NPY in the hypothalamus, but also hippocampus and cortex (Gruenewald et. al., 1994; Vela et. al., 2003). In transgenic mouse models of Alzheimer and Huntington's disease it was shown that the levels of NPY are reduced (Rose et. al., 2009; Decressac et. al., 2010; Decressac et. al., 2012a) and, in the human brain suffering from neurodegenerative disorders the levels of NPY are altered (Duarte-Neves et. al., 2016). Additionally, it was shown that in humans a decline in NPY plasma levels is correlated with increasing age (Chiodera et. al., 2000) and long-lived female centenarians have high NPY plasma levels compared to younger women (Baranowska et. al., 2006). The hypothalamus is essential for the regulation of energy homeostasis (Berthoud, 2006) and it has been shown that this brain region plays an important role in regulating whole-body aging and longevity (Zhang et. al., 2013).

Caloric restriction (CR) is the only non-genetic, and the most consistent non-pharmacological intervention that extends lifespan in model organisms from yeast to mammals (Testa et. al., 2014). CR extends lifespan, at least in part, through the activation of autophagy (Donati, 2006; Hansen et. al., 2008; Blagosklonny, 2010). The major neuroendocrine effect of CR is the

increase of neuropeptide Y (NPY) in the hypothalamus, in response to low energy availability induced by CR (Minor et. al., 2009). It is described that the central modulation of NPY has physiological effects similar to those induced by CR, which are increase hyperphagia as well as glucocorticoid secretion and decrease body temperature, blood glucose levels and fertility (Minor et. al., 2009). Similar to animals submitted to CR, transgenic rat overexpressing hypothalamic NPY have increased resistance to stress and mean lifespan (Michalkiewicz et. al., 2003). There is evidence showing that NPY may be involved in the beneficial effects of CR, it has been shown that caloric restriction does not increase lifespan in NPY knockout mice, revealing a new role of NPY as a lifespan and aging regulator (Chiba et. al., 2014). And that NPY is involved in the tumorigenesis repression induced by CR (Minor et. al., 2011; Chiba et. al., 2014). In addition, it was recently shown that NPY receptors mediate the CR-induced autophagy in hypothalamic (Aveleira et. al., 2015) and cortical (Ferreira-Marques et. al., 2016) neurons.

Moreover, NPY has a neuroprotective role in different brain areas (Silva et. al., 2005; Santos-Carvalho et. al., 2013). In fact, there is evidence that NPY has a neuroprotective role against excitotoxicity in rat and mouse hippocampus and also in the retina (Silva et. al., 2005; Alvaro et. al., 2008).

Recently our group described that NPY can interfere with six of the nine hallmarks of aging, as shown in Figure 1.6, namely: loss of proteostasis, dysregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (Botelho et. al., 2015).

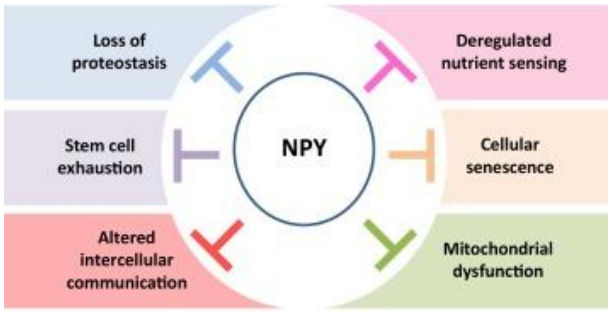

Figure 1.6 - NPY is able of inhibit or delay six of the nine hallmarks of aging (Botelho et. al., 2015).

NPY and Loss of Proteostasis: there are mechanisms responsible for maintaining protein homeostasis ensuring its quality. Being autophagy one of these mechanisms, it is responsible for the elimination of dysfunctional cellular components. With aging, autophagy becomes impaired, leading to the accumulation of aggregated macromolecules and to the aggravation of age-related diseases (Rubinsztein et. al., 2011). In fact, hypothalamic autophagy and NPY levels decrease with age (Chiodera et. al., 2000; Kaushik et. al., 2012). Recent studies show that NPY enhances autophagy in hypothalamic (Aveleira et. al., 2015) and cortical (Ferreira-Marques et. al., 2016) neurons. In addition, it was also shown that NPY mediates CR-induced autophagy in both hypothalamic and cortical neuros, further supporting its role as a CR mimetic. Nevertheless, to better understand the role of NPY of autophagy on whole-body aging more studies are needed (Botelho et. al., 2015).

NPY and Nutrient-Sensing Deregulation: sirtuins and GH are nutrient signaling pathways involved in aging (Barzilai et. al., 2012). In fact, the reduction of GH increases longevity (Barzilai et. al., 2012). Additionally, an increase in the levels of Sirt1 or a reduction of the IGF-1 activates the transcription factor FOXO, promoting longevity (Berdichevsky et. al., 2006; Kenyon, 2010). These pathways can mediate caloric restriction and NPY may have an impact in signaling pathways involving FOXO or GH (Botelho et. al., 2015). In fact, it was shown that in mammalian cells as well as in *Drosophila melanogaster*, NPY can activate FOXO through the upregulation of dual-specificity tyrosine phosphorylation-regulated kinase 1a (Dyrk1a) (Hong et. al., 2012). The effects of NPY on GH levels are variable and, in rats, NPY injection reduces the levels of circulatory GH through Y_1- or Y_2- receptors (Harfstrand et. al., 1987; Suzuki et. al., 1996), while in pigs or golfish cells, NPY stimulates the secretion of GH (Peng et. al., 1993; Barb et. al., 2005). Being NPY an orexigenic peptide and the hypothalamus a regulator of metabolism, NPY may have an effect on metabolic pathways (Botelho et. al., 2015).

NPY and Mitochondrial Dysfunction: the loss of mitochondrial integrity and function has an impact on aging, however this topic remains a challenge in aging research. There is evidence suggesting that NPY has an important role in the function of mitochondria (Botelho et. al., 2015). It has been suggested that the form NPY_{17-36} is located in the outer membrane of the mitochondria, affecting the energetic balance of mitochondrial membranes (Kaipio et. al., 2009). Finally, an increase in fatty acid transport and β oxidation is a result of the lack of NPY

Y_1 receptor (Zhang et. al., 2010), and this suggest that NPY is involved in the protection of mitochondria, although more studies are needed (Botelho et. al., 2015).

NPY and Cellular Senescence: cellular senescence is the gradual deterioration of cell function due to stress episodes, being characteristic of the aging process. Cellular senescence is involved in tumour suppression and tissue repair and, therefore, senescence depends on proteins that respond to DNA-damage (Campisi, 2014). However, cell senescence may be a beneficial process in order to eliminate old or cancer cells, but when the DNA damage response is persistent cell cycle is inhibited (Campisi, 2013). As cells or tissues become senescent the cell proliferation is blocked and some studies suggest that NPY changes cell proliferation, for example, it increases the proliferation of endothelial cells (Zukowska-Grojec et. al., 1998). Since NPY seems to be able to alter the proliferation of cells (Lopez-Otin et. al., 2013), is crucial more studies related to the role of NPY in cellular senescence (Botelho et. al., 2015).

NPY and Stem Cell Exhaustion: with aging, tissues decrease their regenerative capacity due to several damages, leading to stem cell decline, but its rejuvenation may reverse the aging phenotype, and NPY seems to have a role in this process (Botelho et. al., 2015). Through the activation of Y_1 or Y_5 receptors, NPY is able to maintain the self-renewal of human stem cells (Son et. al., 2011). Additionally, it is described that NPY has a neuroprotective effect in retina (Santos-Carvalho et. al., 2015) and a proliferative effect in hippocampus (Baptista et. al., 2012). Overall, the age-associated damages triggered by stem cell exhaustion may be delayed with the capacity of NPY to stimulate cell proliferation (Botelho et. al., 2015).

NPY and Intercellular Communication: a decrease in intercellular communication and an increase in inflammation occur during aging. Studies have demonstrated that NPY may have the ability to increase intercellular communication in osteoblasts (Ma et. al., 2013); inhibit the GnRH excitability (Roa et. al., 2012); and act as anti-inflammatory agent (De la Fuente et. al., 2001; Puerto et. al., 2005; Ferreira et. al., 2010; Li et. al., 2014). In conclusion, NPY may play a role in the regulation of inflammatory response (Botelho et. al., 2015).

Taking into account all of this evidence, NPY has an impact and can interact in various age-related mechanisms, although more studies are needed to understand clearly how this interaction occurs.

1.3. Objectives

It is well known that aging is characterized by the loss of various physiological functions, leading to the end of lifespan. However, the average human life expectancy has increased, but also the prevalence of chronic diseases and cognitive decline. Taking this into account, aging research is now focused in finding strategies that increase both lifespan and healthspan.

The hypothalamus, which is known to have a role in the aging process, is involved in the control of several survival functions such as development, growth, energy balance, sleep, reproduction and metabolism. Additionally, it was described that this brain area is responsible for the development of whole-body aging and has impact on lifespan (Zhang et. al., 2013). Despite being one of the most abundant peptides in the brain, NPY is predominantly expressed in the hypothalamus (Minor et. al., 2009), where it is responsible for several physiological functions including food intake, energy expenditure, circadian rhythms and reproduction (Silva et. al., 2005; Kalra et. al., 2007).

CR, which is the reduced intake of food without malnutrition, is one of the robust anti-aging intervention (Testa et. al., 2014). Additionally, the major neuroendocrine effect of caloric restriction is the increase of neuropeptide Y (NPY) in the hypothalamus (Minor et. al., 2009). Given that NPY may act as a mimetic of CR, since they share several common functions (Minor et. al., 2009), and that the levels of NPY decrease with age in brain regions including the hypothalamus (Gruenewald et. al., 1994; Vela et. al., 2003), the modulation of hypothalamic NPY levels may be a strategy to delay aging. In fact, it has been shown that CR does not increase lifespan in NPY knockout mice, revealing a new role of NPY as a lifespan and aging regulator (Chiba et. al., 2014).

The main objective of the present study is, therefore, to investigate whether modulation of hypothalamic NPY levels can delay aging. To this end, we will investigate whether hypothalamic NPY overexpression, using adenoassociated viral (AAV) gene transfer technology, ameliorates the aging phenotype of a mouse model of human aging (Zmpste24$^{-/-}$ mice (Bergo et. al., 2002; Pendas et. al., 2002)). Specifically, we will investigate the effect of NPY on i) hypothalamic aging (age-related alterations in hypothalamic neuropeptides, neuronal structure and inflammatory markers); and ii) peripheral aging (age-related alterations in histology of target organs such as liver, heart and kidney).

Overall, we expect with this project to provide NPY as an innovative therapeutic strategy to delay aging. Moreover, the results will also contribute to a better understanding of hypothalamus as key player of whole-body aging progress.

Chapter II

Materials and Methods

2.1. Animals

Zmpste24$^{-/-}$ mice (C57BL/6 background) were generated and genotyped in the laboratory of Prof. Carlos López-Otín (University of Oviedo, Spain) as previously described (Pendas et. al., 2002). Adult male C57BL/6 mice (wild-type mice) with 5-6 months of age were obtained from Charles River, Spain. Zmpste24$^{-/-}$ mice (2 month old) were randomly divided into two groups: Zmpste24$^{-/-}$ mice (saline-injected Zmpste24$^{-/-}$ mice; control group) and hypothalamic NPY overexpressing Zmpste24$^{-/-}$ mice (Zmpste24$^{-/-}$+NPY). Mice were housed two per cage, under 12 hours light/dark cycles, in a temperature/humidity controlled room with ad libitum access to water and a standard chow diet.

To manipulate the endogenous expression of NPY, recombinant adenoassociated virus (AAV)-NPY vectors were injected by bilateral stereotaxic injection in the arcuate nucleus of the hypothalamus (Zmpste24$^{-/-}$+NPY mice), as descried below. The control group (Zmpste24$^{-/-}$ mice) received a saline solution (0.9% NaCl) injection instead of viral vectors.

The experimental endpoint of this experiment was 4 months after AAV injection. As NPY is a potent orexigenic neuropeptide, to avoid major weight changes, Zmpste24$^{-/-}$+NPY mice were pair-fed (given the same amount of food that saline-treated Zmpste24$^{-/-}$ mice ate, daily - approximately 4-5 g/day). Each mouse was weighted every other day, for weight control.

All experimental procedures were performed in accordance with European Community directive for the care and use of animals in laboratory (2010/63/EU) translated to the Portuguese law in 2013 (Decree-law 113/2013). Moreover, all the people working with animals have received appropriate education (FELASA course) as required by the Portuguese authorities. In addition, animals are housed in our licensed animal facility (International Animal Welfare Assurance number 520.000.000.2006). The present study is included in a project approved and financed by the Portuguese Science Foundation that approved the utilization of animals for this project (reference PTDC/SAU-FCF/099082/2008).

2.1.1. Overexpression of Neuropeptide Y in the mouse hypothalamic arcuate nucleus

Recombinant AAV particles were generated as described before (Malik et. al., 2005; Shevtsova et. al., 2005; Sousa-Ferreira et. al., 2011). AAV-1/2 chimerical capsids, containing recombinant plasmids with NPY cDNA under a neuronal specific promoter, the human synapsin promoter, were injected in mice hypothalamic arcuate nucleus (ARC), in order to induce constitutive NPY overexpression. The human synapsin promoter in the viral vector guarantees that only

mature neurons will express the transgene. The proximal region of the synapsin promoter is sufficient for directing neuron-specific gene expression. This proximal region is highly conserved between mouse and human (Malik et. al., 2005; Shevtsova et. al., 2005). Mice were anesthetized with an intraperitoneal injection of ketamine/xylazine (100 mg/kg and 10 mg/kg, respectively) and placed on a stereotaxic frame. The ARC was defined by using The Paxino's Mouse Brain Atlas. Injection was performed bilaterally into the ARC: 0.5 mm lateral to the middle line, 1.58 mm posterior to the bregma and −5.8 mm ventral to the brain surface. The Zmpste24$^{-/-}$+NPY mice received 3.6 × 10^{9} v.g. per side of AAV-hSyn-NPY, in a final volume of 1.5 µL per side. The control group, Zmpste24$^{-/-}$ mice received saline solution (0.9% NaCl). Injection was performed at a rate of 0.5 µL/min with a 10 µL-Hamilton syringe attached to an automatic Pump Controller (WPI). Needle was kept in place for 5 min to minimize backflow. Mice were allowed to recover for 2 days.

2.1.2. Tissue and blood collection

Mice were euthanized four months after the stereotaxic injections, by an intraperitoneal administration lethal dose of sodium thiopental (B. Braun, Hesse, Germany).

Animals from both groups were randomly selected either for collection hypothalamic or liver tissue for protein extraction, or for whole brain, liver, heart and kidney removal for immunohistochemistry or histological procedures. For tissue lysates after decapitation, hypothalami or liver were collected and stored at -80°C until use. For immunohistochemistry or histology, animals were intracardially perfused with 4% (w/v) paraformaldehyde/0.1 M phosphate buffered saline (PBS; 137 mM NaCl; 2.7 mM KCl; 10 mM Na_2PO_4; 1.8 mM KH_2PO_4; pH 7.4) fixative solution and, after decapitation, whole brain, liver, heart and kidneys were removed, fixed with 4% paraformaldehyde (w/v) in PBS for 24 hours at 4°C, and cryopreserved in 30% sucrose/PBS solution (w/v) for 72 hours, at 4°C. After that, the organs were removed from the sucrose solution and stored at -80°C until use.

2.2. Western Blotting

Whole hypothalamic and liver tissue were lysed on ice in RIPA (radio-immunoprecipitation assay) buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA; 1% Triton X-100; 0.5% deoxycholate; 0.1% sodium dodecyl sulphate (SDS); 200 µM phenylmethylsulphonylfluoride (PMSF): 1 mM dithiothreitol (DDT); 1 mM Na_3VO_4; 10 mM NaF), supplemented with mini

protease inhibitor cocktail tablet (Roche), with a microcentrifuge tube adapted pestle (Eppendorf). Lysates were sonicated on ice, and the insoluble material was pelleted by centrifugation for 10 minutes at 16,000×g at 4°C. The protein concentration of each sample was determined by the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology). Samples were then equalized for protein concentration (60µg/35µl) to ensure equal amount of protein and volume loading. The samples were denaturated by adding 6x concentrated sample buffer (0.5 M Tris, 30% glycerol, 10% SDS, 0.6 M DTT, 0.012% bromophenol blue) and heating for 5 minutes at 95°C. Samples were stored at -20°C until use. Equal amounts of total protein were loaded per lane separated by electrophoresis in 4-10% and 8-12% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE) on a Tris-Bicine buffer (25 mM Tris; 25 mM Bicine; 1% (w/v) SDS; pH 8.3), first at 70V for 10 minutes, and then at 120-140V for 90 minutes. Then, the protein samples were transferred electrophoretically from the gels to previously methanol-activated Polyvinylidene Fluoride (PVDF) membranes, in CAPS transfer buffer (10 mM CAPS, pH 11.0; 10% (v/v) methanol), at a current of 750 mA during 2 hours and 30 minutes, at 4°C. The membranes were blocked with 5% low-fat milk in Tris-buffered saline (137 mM NaCl; 20 mM Tris-HCl; pH 7.6) containing 0.1% Tween 20 (TBS-T) for one hour at room temperature (RT) and then incubated with the primary antibodies (diluted in TBS-T with 5% (w/v) BSA or milk), overnight, at 4°C. The primary antibodies used at a dilution of 1:500 were: mouse anti-PCNA (Santa Cruz Biotechnology) and mouse anti-phospho-p53 (Cell Signaling). Afterwards the membranes were washed three times with TBS-T and incubated, for 1 hour at room temperature, with the alkaline-phosphatase-linked secondary antibody, specific to rabbit or mouse immunoglobulin G (Pierce) in a 1:10,000 dilution in TBST with 5% (w/v) BSA or milk according to the primary antibody used. The membranes were then washed in TBS-T and the immunoreactive protein bands were visualized using the ECF substrate (GE Healthcare). The membranes were reprobed with a monoclonal β-Actin (Sigma-Aldrich) antibody in a 1:5,000 dilution in TBS-T with 5% (w/v) non-fat milk, overnight at 4 °C. After being washed in TBS-T, the membranes were incubated with an alkaline phosphatase-linked secondary antibody, specific to mouse IgG (GE Healthcare), in a dilution of 1:5,000 in TBS-T with 5% (w/v) non-fat milk, for one hour at RT. The optical density of the bands was quantified with the Quantity One Software (Bio-Rad). The results obtained were normalized to β-Actin and were expressed as the relative amount compared to control.

2.3. Immunohistochemistry

For immunohistochemistry purposes, brains were cut at a cryostat-microtome (Leica CM3050S, Leica Microsystems Nussloch GmbH, Nußloch, Germany) in 30 µm coronal sections. Slices were collected and stored in 48-well trays, free floating in 0.1 M PBS supplemented with 0.12 µmol/L sodium azide. The plates were stored at 4°C until immunohistochemical processing. Brain sections with 30 µm of thickness were washed twice with PBS and blocked and permeabilized in PBS with 10% GS and 0.3% (v/v) TX-100, for one hour at RT. Brain slices were then incubated with a polyclonal rabbit anti-NPY antibody (1:6,000; Sigma-Aldrich), monoclonal mouse anti-MAP2 (Microtubule Associated Protein 2) antibody (1:500; Sigma-Aldrich), monoclonal mouse anti-NeuN (Neuronal Nuclear Protein) antibody (1:500; Chemicon), polyclonal rabbit anti-GFAP (Glial Fibrillary Acidic Protein) antibody (1:1,000, Dako), polyclonal rabbit anti-Iba1 (Ionized Calcium-Binding Adapter Molecule) antibody (1:315; WAKO) in blocking solution, overnight at 4 °C. The sections were then washed in PBS and incubated with goat anti-rabbit Alexa-Fluor 594-, goat anti-mouse Alexa Fluor 594-, or goat anti-rabbit Alexa Fluor 488-conjugated secondary antibodies, for two hours at RT. Nuclei were counterstained with Hoechst 33342 (2 µg/ml; Invitrogen). After incubation, brain sections were washed in PBS and mounted in slides with Mowiol® mounting medium (Sigma-Aldrich). In the end of the procedure, these slides were analyzed on a Zeiss Axiovert fluorescence microscope (Carl Zeiss) or Axio Imager Z2 (Carl Zeiss) and Axio Observer inverted microscope (Carl Zeiss).

2.3.1. Quantification of NPY, MAP2, NeuN, GFAP and Iba1 immunoreactivity in the mouse hypothalamic ARC

Coronal sections of approximately equal spacing were sampled over the anterior–posterior extent of the hypothalamic ARC (Bregma -1.34 to -2.54). To define the ARC region it was used The Paxino's Mouse Brain Atlas. For each mouse, the ARC of one hemisphere was delimited and the integrated density (the product of area and mean grey value; arbitrary units) of 6 sections was measured under 20x magnification using the Fiji (Fiji is Just ImageJ) software (National Health Institute, Bethesda, MD, USA). The integrated density values were summed to yield total integrated density values for each animal, and the mean of the total integrated density values was calculated for each experimental group. Analyses were done on one hemisphere from each section. The results are expressed as the relative amount compared to control.

2.3.2. Quantification of the number of NeuN- and Iba1-positive nuclei in the mouse hypothalamic ARC

The number of NeuN- and Iba1-positive cells was quantified in the arcuate nucleus of the hypothalamus. A cell was considered positive when there was co-localization of NeuN (red) with Hoechst (blue) or Iba1 (green) with Hoechst (blue). For each mouse, the ARC of one hemisphere was delimited and the number of positive cells of 6 sections was counted manually under 20x magnification using the Fiji (Fiji is Just ImageJ) software (National Health Institute, Bethesda, MD, USA). The number of positive cells was summed to yield total number of positive cells for each animal, and the mean of the total number of positive cells was calculated for each experimental group. Then, the values were normalized to the control group. Analyses were done on one hemisphere from each section.

2.4. Histological analysis

The tissues previously fixed and cryopreserved in 30% sucrose/PBS solution were placed in a solution of 10% neutral buffered formalin for 24 hours, then they passed through several steps for paraffin blocks inclusion: 1 hour at ethanol 70%; two series of ethanol 95%, 40 minutes each; two series of ethanol 100%, 1 hour each; two series of xylene, 1 hour each and two series of paraffin, 1 hour each. In the end, we proceeded to the inclusion in paraffin blocks. Samples were sectioned at 3 µm thickness with microtome (Thermo Scientific HM325). Hematoxylin-eosin staining was performed according to the manufacturer guidelines. The paraffin sections were deparaffinized in xylene and rehydrated in 100% ethanol and then in 95% ethanol. The slides were incubated with Hematoxylin Solution modified acc. to Gill III (Merck Millipore), and then washed in distilled water. Then the sections were counterstained with Eosin Y-solution 0.5% aqueous (Merck Millipore), washed with distilled water and finally they were dehydrated in 95% ethanol, 100% ethanol and xylene. Mounting medium (Thermo Scientific) was added to the tissue sections and a cover slip was placed carefully avoiding bubbles and were left to dry overnight. The nuclei were stained blue and the cytoplasm was stained red, in order to detect structural alterations in the tissue.

2.4.1. Liver quantification

The number of hepatocytes per area and the size of immune cells infiltration were quantified in the liver using Fiji software (National Health Institute, Bethesda, MD, USA). To quantify the

number of hepatocytes, we used images acquired in the Axio Imager Z2 (Carl Zeiss) with immersion objective of 63x oil. The hepatocytes were counted in at least thirteen fields for each mouse and the hepatocyte density was estimated by dividing the number of hepatocytes by the tissue area.

To the size of immune cells infiltration, we used images acquired in the Axioskop 2 Plus (Carl Zeiss) with a magnification of 20x. The area of each infiltration cluster was assessed in 3 spaced sections of each mouse.

2.4.2. Heart quantification

The nuclear length and the cross-sectional area of myocytes were evaluated using images acquired in the Axio Imager Z2 (Carl Zeiss) with immersion objective of 63x oil. For the nuclear length quantification cardiomyocyte nuclei were measured along longitudinal length of cells where we drew a line for each nucleus corresponding to its length using the Fiji software (National Health Institute, Bethesda, MD, USA). The myocyte cross-sectional area was also measured using the Fiji software. For each parameter we performed measurements in 3 fields per animal.

2.4.3. Kidney quantification

The cortical thickness, renal corpuscle area and glomerular density were measured using Fiji software. For the cortical thickness were used mosaic images acquired in the Axio Imager Z2 (Carl Zeiss) at a magnification of 5x. In each kidney were performed three measurements in distinct regions of cortex in middle longitudinal section of the organ and the mean of the three measurements was obtained in one kidney for each animal.

The renal corpuscle area analysis were performed using images acquired in the Axio Imager Z2 (Carl Zeiss) at a magnification of 10x. It was assessed in three fields for each mouse, namely in the same kidney evaluated to cortical thickness.

To analyze the glomerular density we used images acquired in the Axio Imager Z2 (Carl Zeiss) at a magnification of 10x. The glomerular density was estimated by dividing the number of renal corpuscles by the tissue area. The analysis was performed in eighteen fields for each animal.

2.5. Statistical analysis

All the results are expressed as mean ± standard error of the mean (SEM). Data were analyzed using Student's unpaired *t* test with one-tailed *p* value when comparing two groups only. A value of $p<0.05$ was considered significant. For all statistical analysis it was used Prism 6.0 (GraphPad Software).

Chapter III

Results

3.1. Age-induced alterations in the hypothalamus of Zmpste24$^{-/-}$ mice

The aging process causes various structural and functional changes in tissues. With age, the brain area hypothalamus shows neuroinflammatory markers and a reduction in the levels of Neuropeptide Y (NPY) (Gruenewald et. al., 1994; Rozovsky et. al., 1998; Vela et. al., 2003). We hypothesized that the Zmpste24$^{-/-}$ mice, being a model of premature aging, could have some alterations in the hypothalamus. Therefore, we investigated several parameters related to the structure of the arcuate nucleus (ARC) of the hypothalamus.

3.1.1. Zmpste24$^{-/-}$ mice show decreased levels of NPY in the arcuate nucleus of the hypothalamus

We assessed NPY immunoreactivity in the ARC of Zmpste24$^{-/-}$ mice, comparing to age matched wild-type mice, by immunohistochemistry, as shown in Figure 3.1 A, and NPY immunoreactivity is 40% lower in the ARC of Zmpste24$^{-/-}$ mice compared to wild-type mice (Figure 3.1 B; 60.3 ± 5.4% of Wild-type).

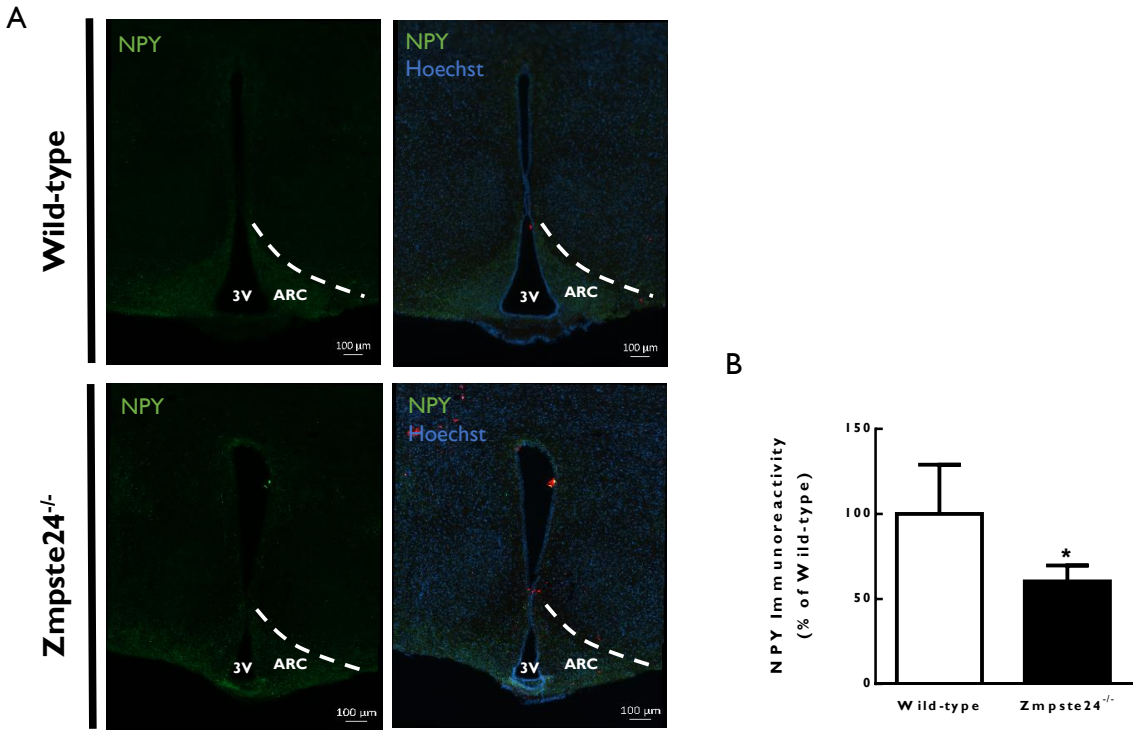

Figure 3.1 - NPY levels decrease in the hypothalamic ARC of Zmpste24$^{-/-}$ mice. (A) Representative images of NPY immunoreactivity in the ARC of Wild-type mice group (control group; Wild-type) and in the Zmpste24 deficient mice group (Zmpste24$^{-/-}$). (B) Quantification of NPY immunoreactivity through the anterior-posterior length of the mouse ARC. The results represent the mean ± SEM and are expressed as percentage of Wild-type. *p< 0.05, significantly different from the Wild-type mice group as determined by Student's unpaired t test. n=3 mice per group. 3V, third ventricle; ARC, Arcuate Nucleus.

3.1.2. Zmpste24$^{-/-}$ mice show decreased levels of MAP2 and NeuN in the hypothalamic ARC

The hypothalamus is responsible for maintain the body homeostasis, which requires the intervention of several neurons, but the responsiveness of these neurons declines during aging, compromising the physiological functions of the body (Chen et. al., 2015). To understand whether aging alters Zmpste24$^{-/-}$ mice hypothalamic neuronal structure, we evaluated the immunoreactivity of two neuronal markers: MAP2, a dendritic marker, and NeuN, a marker of mature neurons, by immunohistochemistry (Figure 3.2 A and C, respectively). The quantification of MAP2 and NeuN immunoreactivity was done through the anterior-posterior length of the hypothalamic ARC (Figure 3.2 B and D, respectively). We observed 41% lower levels of MAP2 immunoreactivity in the ARC of Zmpste24$^{-/-}$ mice when compared to the Wild-type mice group (Figure 3.2 B; 58.6 ± 12.9% of Wild-type). Regarding NeuN immunoreactivity, we observed 59% lower levels of NeuN in the hypothalamic ARC of Zmpste24$^{-/-}$ mice when compared to the Wild-type mice group (Figure 3.2 D; 41.0 ± 6.4% of Wild-type). A decrease in NeuN immunoreactivity led us to hypothesize that Zmpste24$^{-/-}$ mice have a decrease in the number of neurons within hypothalamic ARC. However, upon the quantification of the number of NeuN-positive cells in the hypothalamic ARC (Figure 3.2 E), we did not observed any statistical differences between the two groups of mice. Nevertheless, a decrease in both ARC MAP2 and NeuN immunoreactivity suggest that Zmpste24$^{-/-}$ mice show alterations in hypothalamic neuronal structure.

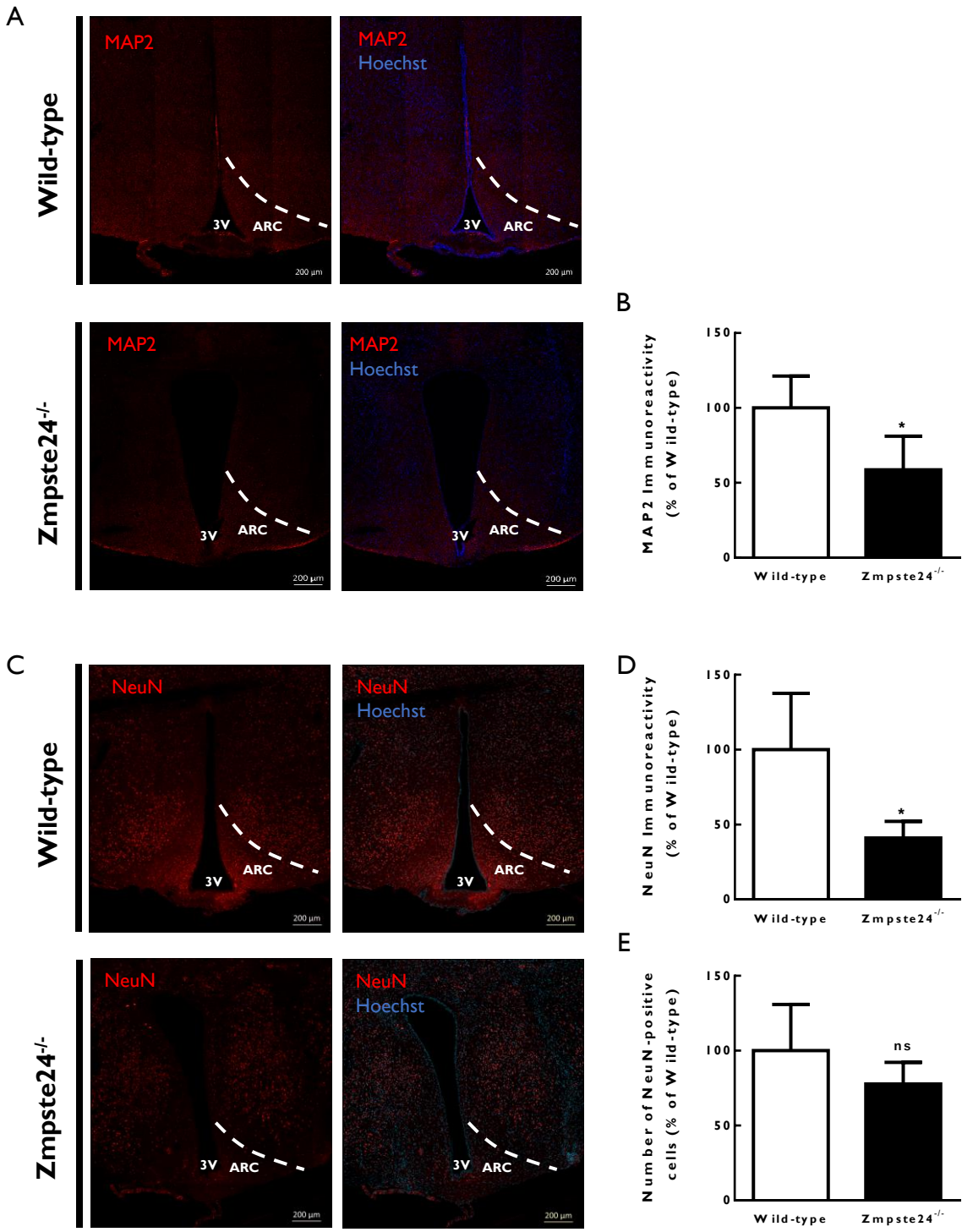

Figure 3.2 - Zmpste24$^{-/-}$ mice show lower levels of MAP2 and NeuN immunoreactivity in the hypothalamic ARC. (A and C) Representative images of MAP2 (A) and NeuN (C) immunoreactivity in the mouse hypothalamic ARC, in the Wild-type mice group (control group; Wild-type) and in Zmpste24 deficient mice group (Zmpste24$^{-/-}$). Quantification of MAP2 (B) and NeuN (D) immunoreactivity through the anterior-posterior length of the mouse ARC. (E) Quantification of NeuN-positive cells in the mouse ARC. The results represent the mean ± SEM and are expressed as percentage of Wild-type. ns, not statistically different; *p< 0.05, significantly different from the Wild-type mice group, as determined by the Student's unpaired *t* test. n=3-5 mice per group. 3V, third ventricle; ARC, Arcuate Nucleus.

3.1.3. Effect of aging on hypothalamic inflammation in Zmpste24$^{-/-}$ mice

In the adult brain there is a balance between pro-inflammatory and anti-inflammatory cytokines, but with increased age this balance becomes dysregulated, leading to an increase of neuroinflammation (Sparkman et. al., 2008). To investigate if Zmpste24$^{-/-}$ mice show inflammatory alterations in the hypothalamus, we evaluated the levels of two neuroinflammatory markers: GFAP, an astrocyte marker, and Iba1, a marker of microglia, by immunohistochemistry, as shown in Figure 3.3 A and C, respectively. The quantification of GFAP and Iba1 immunoreactivity was done through the anterior-posterior length of the mouse hypothalamic ARC (Figure 3.3 B and D, respectively). We did not observed significant statistical differences in GFAP immunoreactivity in the ARC of Zmpste24$^{-/-}$ mice when compared to the Wild-type mice group (Figure 3.3 B). However, we observed a decrease of 32% of Iba1 immunoreactivity in the hypothalamic ARC of Zmpste24$^{-/-}$ mice compared with the Wild-type mice group (Figure 3.3 D; 68.5 ± 5.2% of Wild-type), and also a decrease of 28% in the number of Iba1-positive cells within hypothalamic ARC of Zmpste24$^{-/-}$ mice (Figure 3.3 E; 71.9 ± 5.8% of Wild-type).

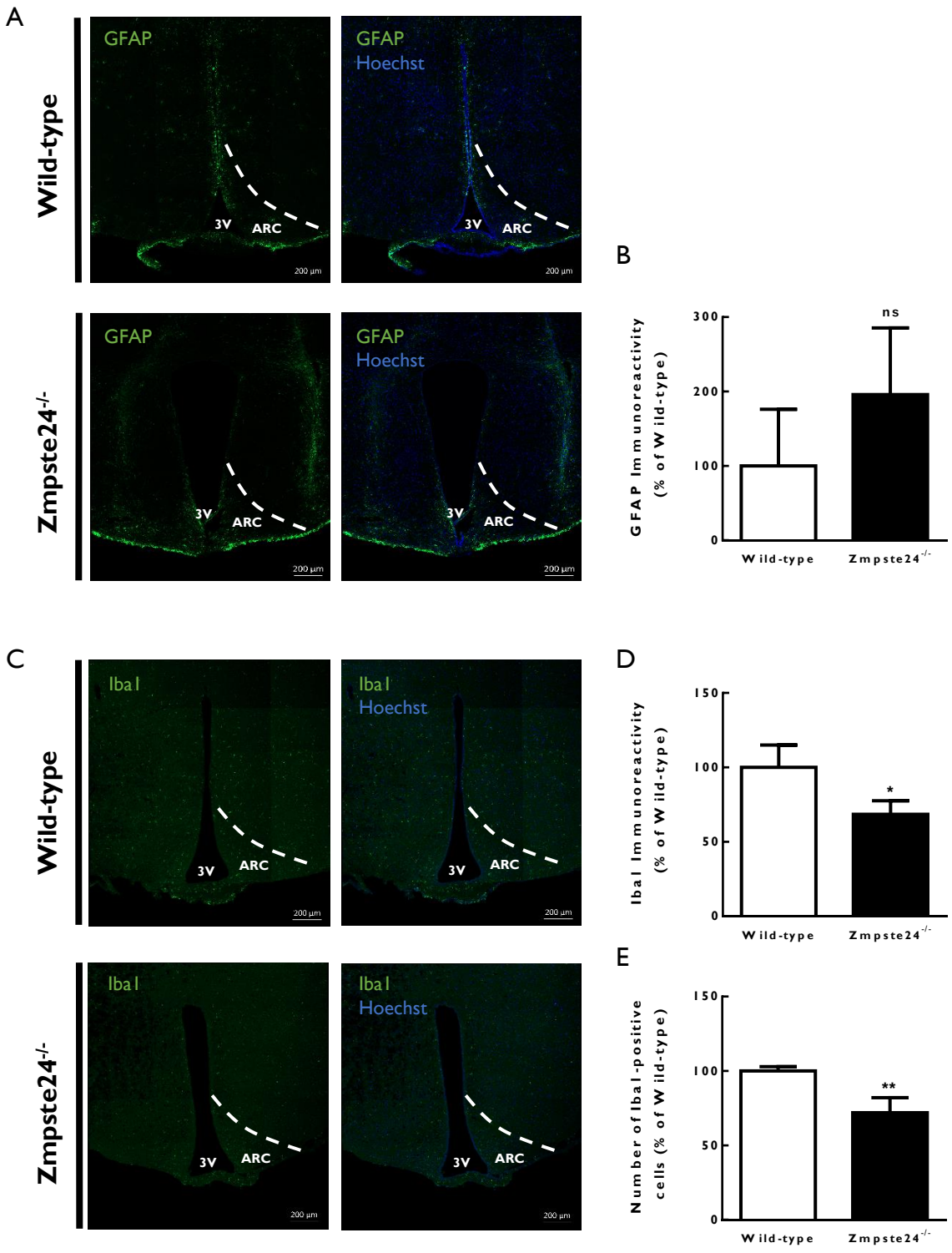

Figure 3.3 - Zmpste24$^{-/-}$ mice show lower levels of microglia cells marker (Iba1) in the hypothalamic ARC. (A and C) Representative images of GFAP (A) and Iba1 (C) immunoreactivity in the mouse hypothalamic ARC, in the Wild-type mice group (control group; Wild-type) and in the Zmpste24 deficient mice group (Zmpste24$^{-/-}$). (B and D) Quantification of GFAP (B) and Iba1 (D) immunoreactivity through the anterior-posterior length of the mouse hypothalamic ARC. (E) Quantification of the number of Iba1-positive cells in the mouse hypothalamic ARC. The results represent the mean ± SEM and are expressed as percentage of Wild-type. ns, not statistically different; *p< 0.05, **p< 0.01, significantly different from the Wild-type mice group, as determined by Student's unpaired t test. n=3-4 mice per group. 3V, third ventricle; ARC, Arcuate Nucleus.

3.2. Impact of modulation of hypothalamic NPY levels on Zmpste24$^{-/-}$ mice aging phenotype

There is evidence that the hypothalamus is responsible for the whole-body aging and lifespan control (Satoh et. al., 2013; Zhang et. al., 2013). Some evidence suggest that NPY is able to interfere in six of the nine hallmarks of aging, which are: loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (Botelho et. al., 2015). Moreover, aging is associated with decreased levels of NPY (Gruenewald et. al., 1994; Vela et. al., 2003). We previously showed that hypothalamus in Zmpste24$^{-/-}$ mice have lower NPY levels. Taking all into account, we hypothesized that increasing hypothalamic NPY levels in the hypothalamus could delay the aging phenotype of Zmpste24$^{-/-}$ mice.

3.2.1. Overexpression of NPY in the hypothalamus

In order to investigate the effect of hypothalamic NPY on aging phenotype of Zmpste24$^{-/-}$ mice, NPY was overexpressed in the ARC of the hypothalamus by gene transfer technology using recombinant adeno-associated viral vectors (rAAV), in Zmpste24$^{-/-}$ mice. Mice were injected with rAAV encoding NPY under a neuronal-specific promoter (Schoch et. al., 1996) (hypothalamic NPY overexpressing Zmpste24$^{-/-}$ mice group; Zmpste24$^{-/-}$ + NPY), or saline (control group; Zmpste24$^{-/-}$), by bilateral stereotaxic injection in each hypothalamic ARC. After 4 months, we assessed the expression of NPY in the hypothalamic ARC, by immunohistochemistry, as shown in Figure 3.4 A. As expected, we observed that the overexpression of NPY increased ~250% NPY immunoreactivity in the hypothalamic ARC of Zmpste24$^{-/-}$ mice compared to Zmpste24$^{-/-}$ mice (Figure 3.4 B; 348.9 ± 95.5% of Zmpste24$^{-/-}$).

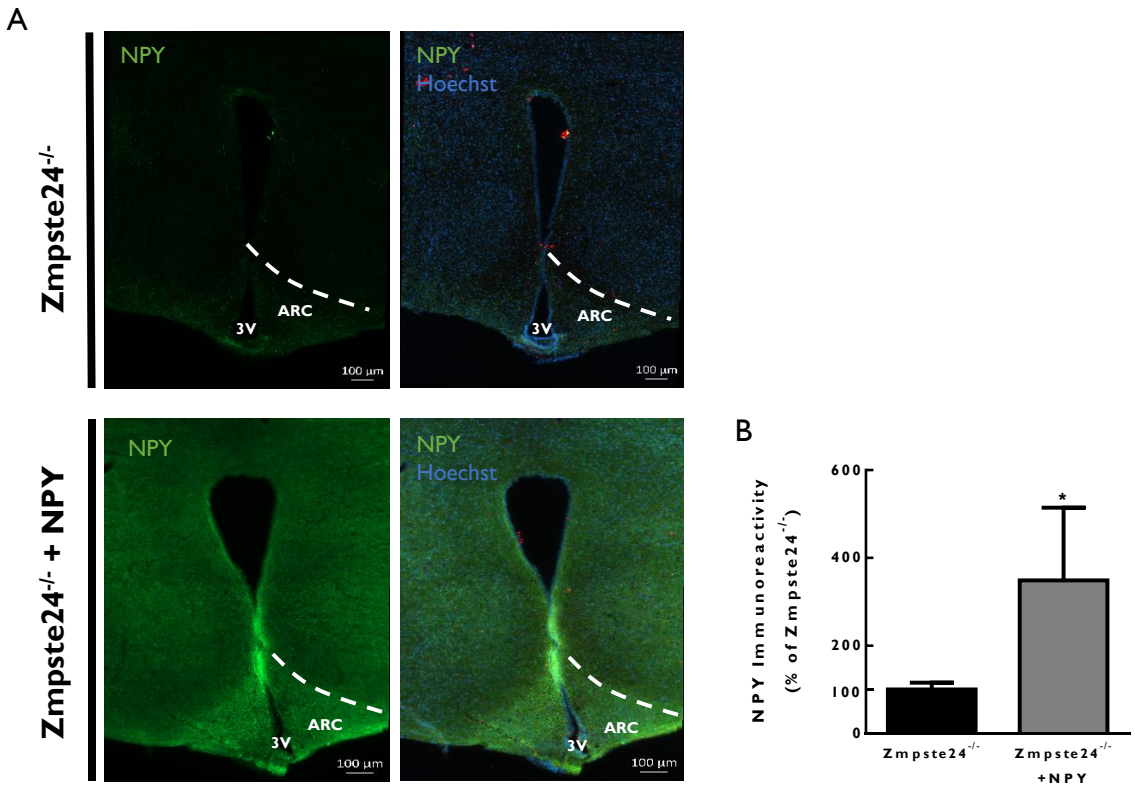

Figure 3.4 - NPY overexpression was modulated through bilateral injection of rAAV in the hypothalamic ARC of Zmpste24$^{-/-}$ mice. (A) Representative images of NPY immunoreactivity in the mouse hypothalamic ARC, in the Zmpste24$^{-/-}$ mice group (control group; Zmpste24$^{-/-}$) and hypothalamic NPY overexpressing Zmpste24$^{-/-}$ mice group (Zmpste24$^{-/-}$ + NPY). (B) Quantification of NPY immunoreactivity through the anterior-posterior length of the mouse hypothalamic ARC, 4 months after injection. The results represent the mean ± SEM and are expressed as percentage of Zmpste24$^{-/-}$. *p< 0.05, significantly different from the Zmpste24$^{-/-}$ mice (control group) as determined by Student's unpaired t test. n=3 mice per group. 3V, third ventricle; ARC, Arcuate Nucleus.

3.2.1. Impact of hypothalamic NPY overexpression on age-related hypothalamic impairments in Zmpste24$^{-/-}$ mice

3.2.1.1. Effect of hypothalamic NPY modulation in hypothalamic neuronal markers in Zmpste24$^{-/-}$ mice

To understand whether hypothalamic NPY overexpression had any beneficial effect in the hypothalamic neuronal markers, we assessed the immunoreactivity of the neuronal markers MAP2 and NeuN, by immunohistochemistry, 4 months upon injection, as shown in Figure 3.5 A and C, respectively. We did not observe any alterations in the immunoreactivity of both markers in the ARC upon hypothalamic NPY overexpression in Zmpste24$^{-/-}$ mice. In addition, when we quantified the number of NeuN-positive cells in hypothalamic ARC, we did not observe any difference in the number of hypothalamic neurons between Zmpste24$^{-/-}$ and Zmpste24$^{-/-}$+NPY mice. However, hypothalamic NPY overexpression increased the protein content of NeuN (Figure 3.5 F) in the hypothalamus of Zmpste24$^{-/-}$ mice (124.8 ± 5.5% of

Zmpste24$^{-/-}$), as determined by Western blotting analysis. The results related to NeuN are different, the immunohistochemistry compared with the Western blotting analysis, because the first was quantified only in the hypothalamic ARC and the second includes the whole hypothalamus.

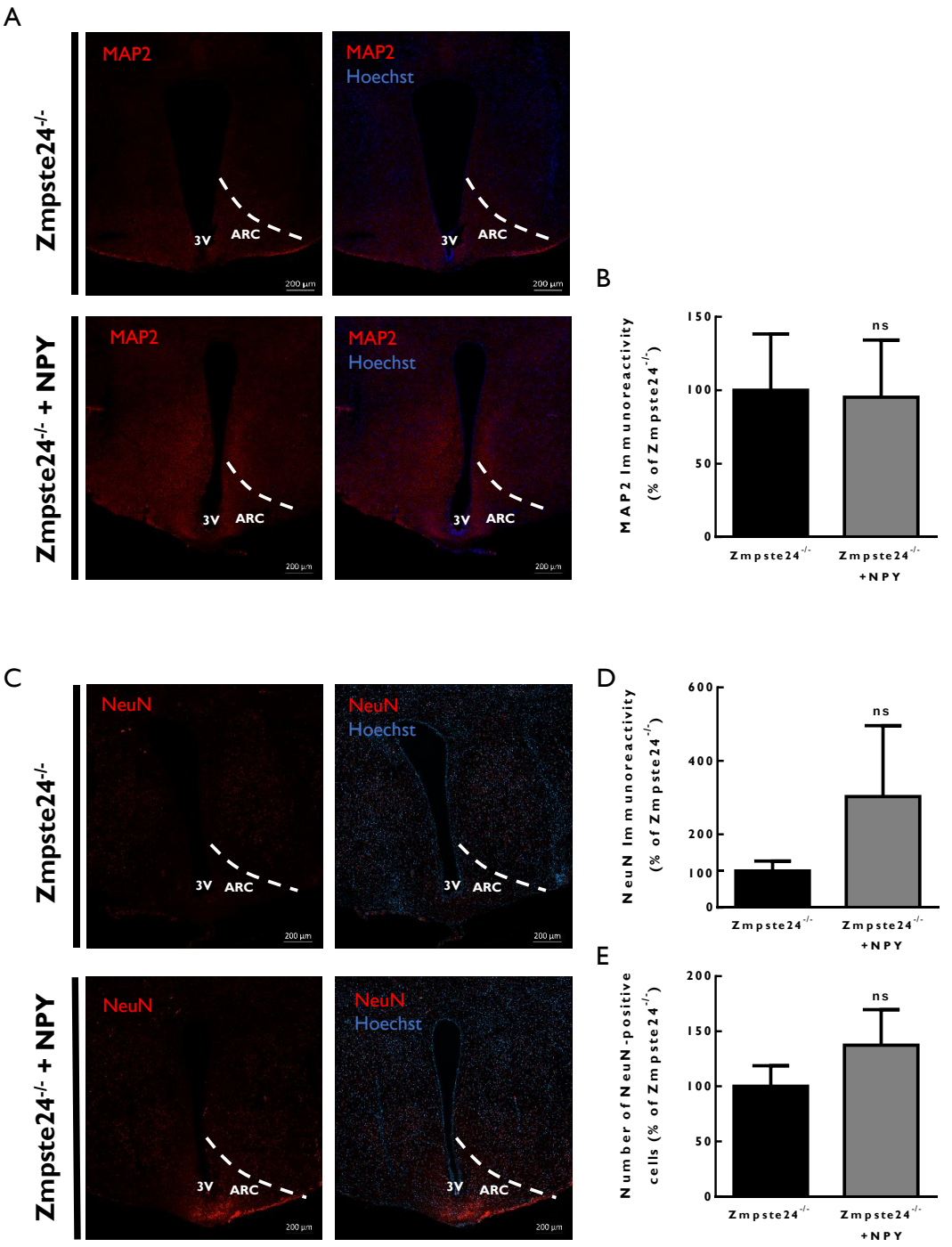
A
Zmpste24-/-
Zmpste24-/- + NPY
MAP2
MAP2
Hoechst
3V
ARC
200 µm
B
MAP2 Immunoreactivity (% of Zmpste24-/-)
150
100
50
0
ns
Zmpste24-/-
Zmpste24-/- +NPY
C
NeuN
NeuN
Hoechst
D
NeuN Immunoreactivity (% of Zmpste24-/-)
600
400
200
100
E
Number of NeuN-positive cells (% of Zmpste24-/-)
F
Zmspte24-/-
Zmspte24-/- +NPY
NeuN
β-Tubulin
48 kDa
46 kDa
50 kDa
NeuN/Tubulin (% of Zmpste24-/-)
*

Figure 3.5 – Effect of hypothalamic NPY overexpression in hypothalamic neuronal markers in Zmpste24$^{-/-}$ mice. (A and C) Representative images of MAP2 (A) and NeuN (C) immunoreactivity in the hypothalamic ARC, in the Zmpste24$^{-/-}$ mice group (control group; Zmpste24$^{-/-}$) and hypothalamic NPY overexpressing Zmpste24$^{-/-}$ mice group (Zmpste24$^{-/-}$+NPY), 4 months after injection. (B and D) Quantification of MAP2 (B) and NeuN (D) immunoreactivity through the anterior-posterior length of the mouse hypothalamic ARC. (E) Quantification of the number of NeuN-positive cells in the hypothalamic ARC. (F) Whole hypothalamic lysates from Zmpste24$^{-/-}$ and Zmpste24$^{-/-}$+NPY mice were assayed for NeuN and β-Tubulin immunoreactivity by Western blotting. Representative Western blots are presented above the graph. The results represent the mean ± SEM and are expressed as percentage of Zmpste24$^{-/-}$. ns, not statistically different; *$p<0.05$, significantly different from Zmpste24$^{-/-}$ mice group (control group), as determined by Student's unpaired t test. n=3-5 mice per group. 3V, third ventricle; ARC, Arcuate Nucleus.

3.2.1.2. Hypothalamic NPY overexpression decreases neuroinflammation markers in the hypothalamus of Zmpste24$^{-/-}$ mice

It has been shown that NPY is a player in mediating functional interaction between the nervous systems and the immune system (Dimitrijevic et. al., 2013), exhibiting modulatory properties in neuroinflammation, through the inhibition of excessive microglial activity (Malva et. al., 2012). Therefore, we investigated, by immunohistochemistry, the effect of hypothalamic NPY overexpression on markers of astrocytes (GFAP) and activated microglia cells (Iba1) in the hypothalamus of Zmpste24$^{-/-}$ mice. We observed that hypothalamic NPY overexpression decreased by ~62% the levels of GFAP immunoreactivity (37.9 ± 9.8% of Zmpste24$^{-/-}$) in the hypothalamic ARC of Zmpste24$^{-/-}$ + NPY mice. A decrease in the hypothalamic protein content of GFAP (Figure 3.6 C) was also observed in Zmpste24$^{-/-}$+NPY mice when compared to the Zmpste24$^{-/-}$ (70.1 ± 3.6% of Zmpste24$^{-/-}$), as determined by Western blotting analysis. Regarding Iba1, we did not observe alterations in both Iba1 immunoreactivity (Figure 3.6 E) and number of Iba1-positive cells (Figure 3.6 F) in the hypothalamus of Zmpste24$^{-/-}$+NPY mice. We also investigated the effect of hypothalamic NPY overexpression in the phosphorylation status of IκBα. IκBα is an NF-κB ligand which is released upon its phosphorylation leading to NF-κB activation. NF-κB is activated when an immune response to inflammation is needed. Recently it was demonstrated that aging is associated with hypothalamic NF-κB activation, meaning that aging increases inflammation in the hypothalamus (Zhang et. al., 2013). As shown in Figure 3.6 G, hypothalamic NPY overexpression decreased the levels of pIκBα in the hypothalamus of Zmpste24$^{-/-}$+NPY mice when compared to Zmpste24$^{-/-}$ mice (76.5 ± 3.2% of Zmpste24$^{-/-}$). This observation suggests that NPY may decrease the NF-κB activation in the hypothalamus and consequently have a positive effect in the hypothalamic inflammation.

Overall, these results suggest that NPY may ameliorate the neuroinflammation in the hypothalamus of Zmpste24$^{-/-}$ mice.

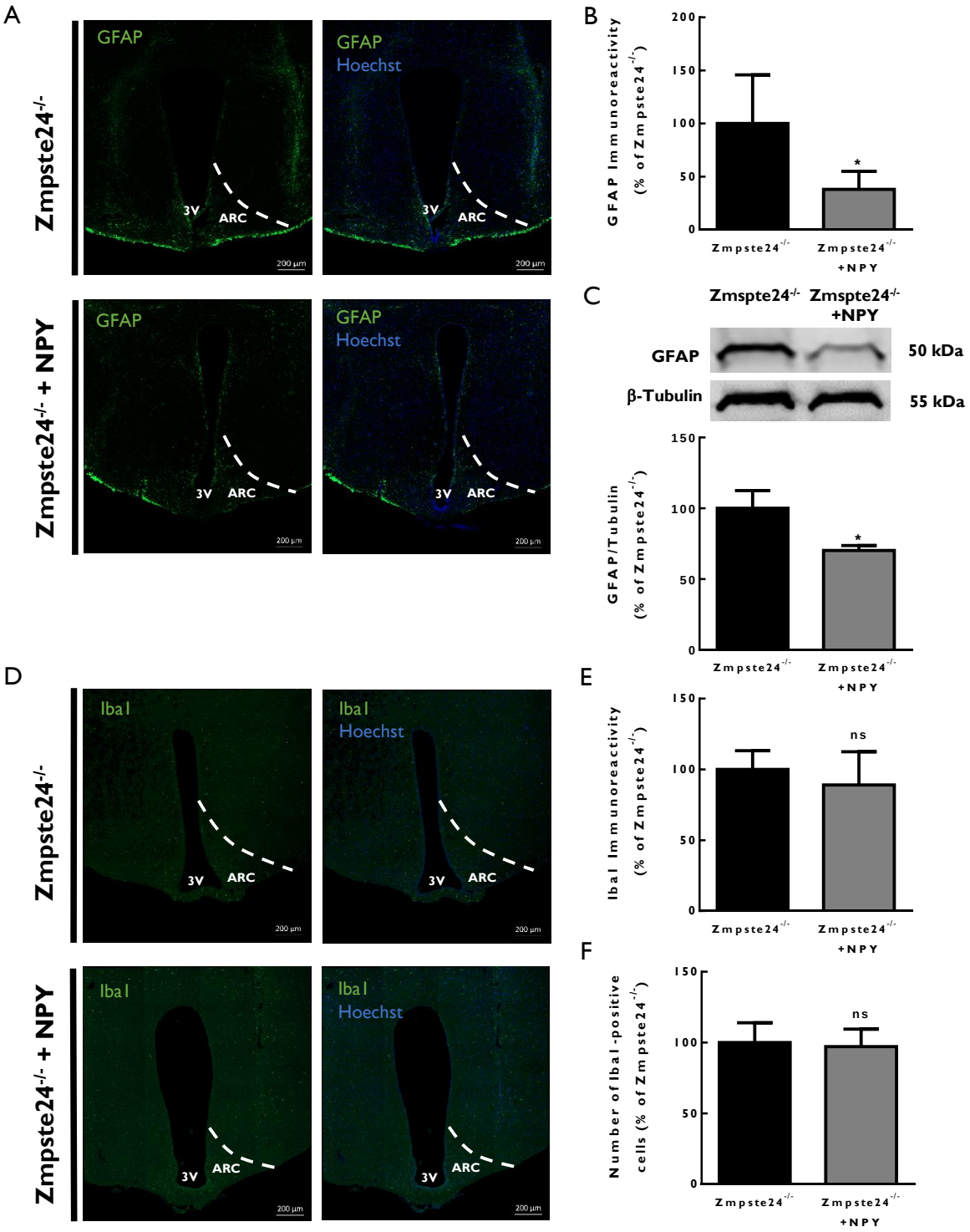
A
Zmpste24-/-
Zmpste24-/- + NPY
GFAP
GFAP
Hoechst
3V
ARC
200 µm
B
GFAP Immunoreactivity (% of Zmpste24-/-)
200
150
100
50
0
*
Zmpste24-/-
Zmpste24-/- +NPY
C
Zmspte24-/-
Zmspte24-/- +NPY
GFAP
β-Tubulin
50 kDa
55 kDa
GFAP/Tubulin (% of Zmpste24-/-)
150
100
50
0
*
Zmpste24-/-
Zmpste24-/- +NPY
D
Zmpste24-/-
Zmpste24-/- + NPY
Iba I
Iba I
Hoechst
3V
ARC
200 µm
E
Iba I Immunoreactivity (% of Zmpste24-/-)
150
100
50
0
ns
Zmpste24-/-
Zmpste24-/- +NPY
F
Number of Iba I-positive cells (% of Zmpste24-/-)
150
100
50
0
ns
Zmpste24-/-
Zmpste24-/- +NPY
G
Zmspte24-/-
Zmspte24-/- +NPY
pIκBα
β-Tubulin
40 kDa
55 kDa
pIκBα/Tubulin (% of Zmpste24-/-)
150
100
50
0
***
Zmpste24-/-
Zmpste24-/- +NPY

Figure 3.6 - Hypothalamic NPY overexpression decreases levels of GFAP and pIκBα in the hypothalamus of Zmpste24$^{-/-}$ mice. (A and D) Representative images of GFAP (A) and Iba1 (D) immunoreactivity in the mouse hypothalamic ARC, in Zmpste24$^{-/-}$ mice group (control group; Zmpste24$^{-/-}$) and hypothalamic overexpressing Zmpste24$^{-/-}$ mice group (Zmpste24$^{-/-}$+NPY). (B and E) Quantification of GFAP (B) and Iba1 (E) immunoreactivity through the anterior-posterior length of the mouse hypothalamic ARC. (F) Quantification of the number of Iba1-positive cells in the hypothalamic ARC. (C and G) Whole hypothalamic lysates from Zmpste24$^{-/-}$ and Zmpste24$^{-/-}$+NPY mice were assayed for GFAP (C) and pIκBα (G) and β-Tubulin immunoreactivity by Western blotting. Representative Western blots are presented above the respective graphs. The results represent the mean ± SEM and are expressed as percentage of Zmpste24$^{-/-}$. ns, not statistically different; *p< 0.05, ***p< 0.001, significantly different from the Zmpste24$^{-/-}$ mice, as determined by Student's unpaired t test. n=3-6 mice per group. 3V, third ventricle; ARC, Arcuate Nucleus.

3.2.3. Impact of hypothalamic NPY modulation on peripheral key metabolic organs

Zmpste24 deficient mice exhibit multiple histopathological defects (Marino et. al., 2010) such as cardiomyopathy, muscular dystrophy, lipodystrophy, reduced growth rate and weight loss (Pendas et. al., 2002). As demonstrated before, the hypothalamus is a brain region crucial for the regulation of the aging process and taking into account that NPY has a beneficial effect in several hallmarks of aging, we aim to evaluate the effect of the modulation of hypothalamic NPY levels in several peripheral key organs.

3.2.3.1. Effect of hypothalamic NPY overexpression in the histological structure of liver

The liver is an important metabolic organ which, with aging, is considered to have less capacity to respond to a variety of injuries and suffer functional and structural changes (Harkema et. al., 2016). Liver histological alterations induced by aging include: decrease of volume; increased lobular size; fibrosis; lipid accumulation; decreased hepatocyte number; pseudoinclusions (intranuclear inclusions); increased nuclear size (anisokaryosis); polyploidy, which are cells that contain 4, 8, 16 or more times the haploid chromosome complement and is a process that occurs through failed cytokinesis (cell division) (Duncan et. al., 2010); and pseudocapillarization, characterized by thickening of endothelial cells and decreased endothelial pores, all of this together leads to an impaired function of liver (Gregg et. al., 2012; Harkema et. al., 2016). To evaluate the effect of hypothalamic NPY overexpression in the liver structure of Zmpste24$^{-/-}$ mice, we performed histological analysis of liver using paraffin sections from both Zmpste24$^{-/-}$ and Zmpste24$^{-/-}$+NPY mice by hematoxylin-eosin (HE) staining. After the analysis (Figure 3.7 A), we observed that Zmpste24$^{-/-}$+NPY mice showed some alterations

in the liver structure such as: smaller hepatocytes; increased hepatocyte number; decreased nuclear size; and do not exhibit pseudoinclusions (cytoplasmatic invaginations into the nucleus making it appear intranuclear inclusions). In addition, we measure the number of hepatocytes and the infiltration area. The liver consists mainly of hepatocytes but also has a pool of immune cells (macrophages, T cells, B cells and neutrophils), which protect the organism by playing a major regulatory role in inducing and sustaining inflammatory conditions. With aging there is an increase in the infiltration area of immune cells in response to an increase inflammation (Singh et. al., 2008). We observed that infiltration in the liver occurred in both groups of mice, hypothalamic NPY overexpressing Zmpste24$^{-/-}$ mice showed, Figure 3.7 B, a lower liver infiltration area (267700 ± 18189 mm^2) when compared to the control group Zmpste24$^{-/-}$ mice (391309 ± 39248 mm^2). This observation suggest that the overexpression of hypothalamic NPY reduced the infiltration area. As shown in Figure 3.7 C we also observed an increase in the number of hepatocytes in the liver of Zmpste24$^{-/-}$+NPY mice (15.9 ± 0.5 number/μm^2) when compared to the control group Zmpste24$^{-/-}$ mice (13.8 ± 0.4 number/μm^2), suggesting that hypothalamic NPY overexpression may induce cell proliferation in the liver. To support this hypothesis, we measure the protein content of PCNA, a cell proliferation marker, in the liver of Zmpste24$^{-/-}$ and Zmpste24$^{-/-}$+NPY mice, by Western blotting. As shown in Figure 3.7 D, the levels of PCNA increased in the liver of Zmpste24$^{-/-}$+NPY mice when compared to Zmpste24$^{-/-}$ mice (179.5 ± 14.7% of Zmpste24$^{-/-}$), supporting the hypothesis that hypothalamic NPY can regulate cell proliferation in peripheral organs such as the liver. We also evaluated the levels of phosphorylated p53 (pp53), a tumor suppressor protein that plays a major role in the cellular response to DNA damage and other genomic aberrations. Activation of p53 by phosphorylation can lead to either cell cycle arrest or apoptosis (Maclaine et. al., 2009; Kruiswijk et. al., 2015; Chen, 2016). A decrease in the levels of pp53 was observed in the liver of Zmpste24$^{-/-}$+NPY mice (Figure 3.7 E; 66.1 ± 9.6% of Zmpste24$^{-/-}$) may suggest that the repressor effect of p53 in cell proliferation or apoptosis induction is diminished in hypothalamic NPY overexpressing mice. These results show that NPY has a beneficial effect the liver of Zmpste24$^{-/-}$ mice, since it can improve its structure.

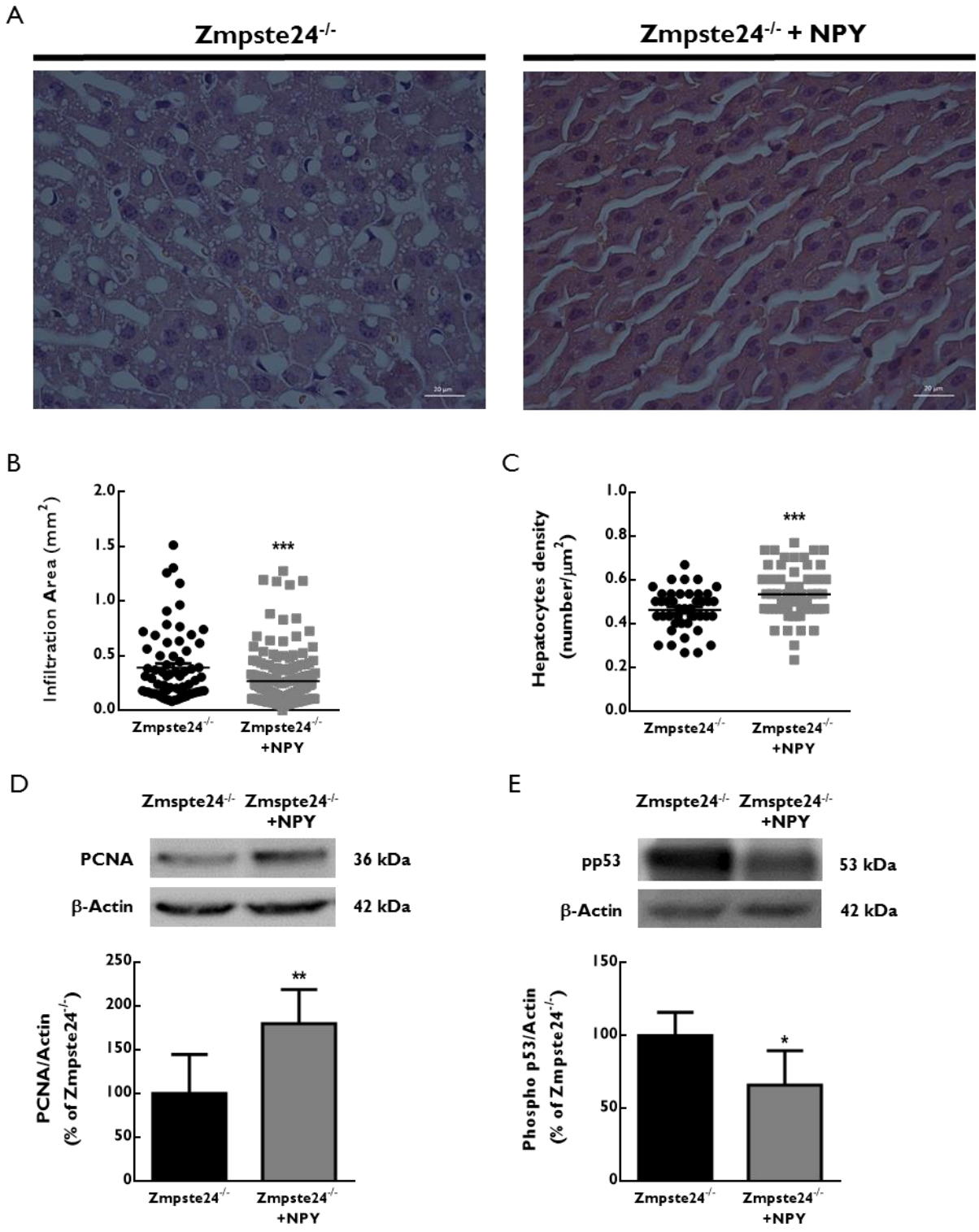

Figure 3.7 - Effect of hypothalamic NPY modulation in the liver structure of Zmpste24$^{-/-}$ mice. (A) Representative images of hematoxylin and eosin staining of liver paraffin sections of Zmpste24$^{-/-}$ mice group (control group; Zmpste24$^{-/-}$) and hypothalamic NPY overexpressing Zmpste24$^{-/-}$ mice group (Zmpste24$^{-/-}$+NPY). (B) Quantification of the infiltration area (immune cell clusters), in mice liver sections. (C) Quantification of hepatocytes density, determined by counting the number of hepatocytes per area. The results are presented in scatter plots of all the analyzed values and are expressed in mm^2 (B) and number/µm^2 (C) and the transverse black line represent the mean of all observed values. n=3-4 mice per group; all infiltration areas were assessed from 3 sections per animal, and for hepatocytes density, at least 13 fields per animal were examined. (D and E) Liver lysates from Zmpste24$^{-/-}$ and Zmpste24$^{-/-}$+NPY mice were assayed for PCNA (D), phospho-p53 (E) and β-actin by Western blotting. Representative Western blots are presented above the respective graphs. The results

represent the mean ± SEM and are expressed as percentage of Zmpste24$^{-/-}$. *p< 0.05, **p< 0.01, ***p< 0.001, significantly different from the Zmpste24$^{-/-}$ mice (control group), as determined by Student's unpaired t test. n=5 mice per group.

3.2.3.2. Effect of hypothalamic NPY overexpression in the heart histology

As Zmpste24 is highly expressed in the heart and since Zmpste24 deficient mice have several abnormalities in the heart being probably the cause of their death (Pendas et. al., 2002), we also decided to analyze the effect of hypothalamic NPY overexpression in the heart histological structure of Zmpste24$^{-/-}$ mice. As shown by the representative images of HE staining of heart cross-sectional paraffin slices (Figure 3.8 A), we did not observe any evident differences in the heart histological structure between Zmpste24$^{-/-}$ and Zmpste24$^{-/-}$+NPY mice. It is described in other mouse model of accelerated aging, that the cardiomyocytes are smaller and their nuclei have an abnormal elongation (Mounkes et. al., 2003; Muchir et. al., 2009). In order to evaluate the effect of hypothalamic NPY overexpression on these parameters, we measured the nuclear length and the cross-sectional area of cardiomyocytes. We did not observe differences in cardiomyocyte nuclear length (Figure 3.8 B) from Zmpste24$^{-/-}$+NPY mice when compared to the Zmpste24$^{-/-}$ mice. However, we observed a decrease in the cardiomyocyte cross-sectional area (Figure 3.8 C) in the heart of Zmpste24$^{-/-}$+NPY mice (71.1 ± 2.3 μm^2) when compared to the control group Zmpste24$^{-/-}$ mice (91.0 ± 3.1 μm^2). In the aging heart the characteristic structural changes include thickening of the left ventricular wall, cardiac myocyte hypertrophy, focal areas with increased amounts of collagen, and reduced numbers of cardiac myocytes (Harkema et. al., 2016). Additionally, in Zmpste24$^{-/-}$ mouse model some age-related changes are reported such as muscle degeneration; lymphocytic infiltration; interstitial fibrosis and intracellular vesicles (Pendas et. al., 2002). A decrease in the cross-sectional area suggest that NPY may decrease the hypertrophy of cardiac myocytes. More studies are needed to evaluate the beneficial role of NPY in the heart of Zmpste24$^{-/-}$ mice.

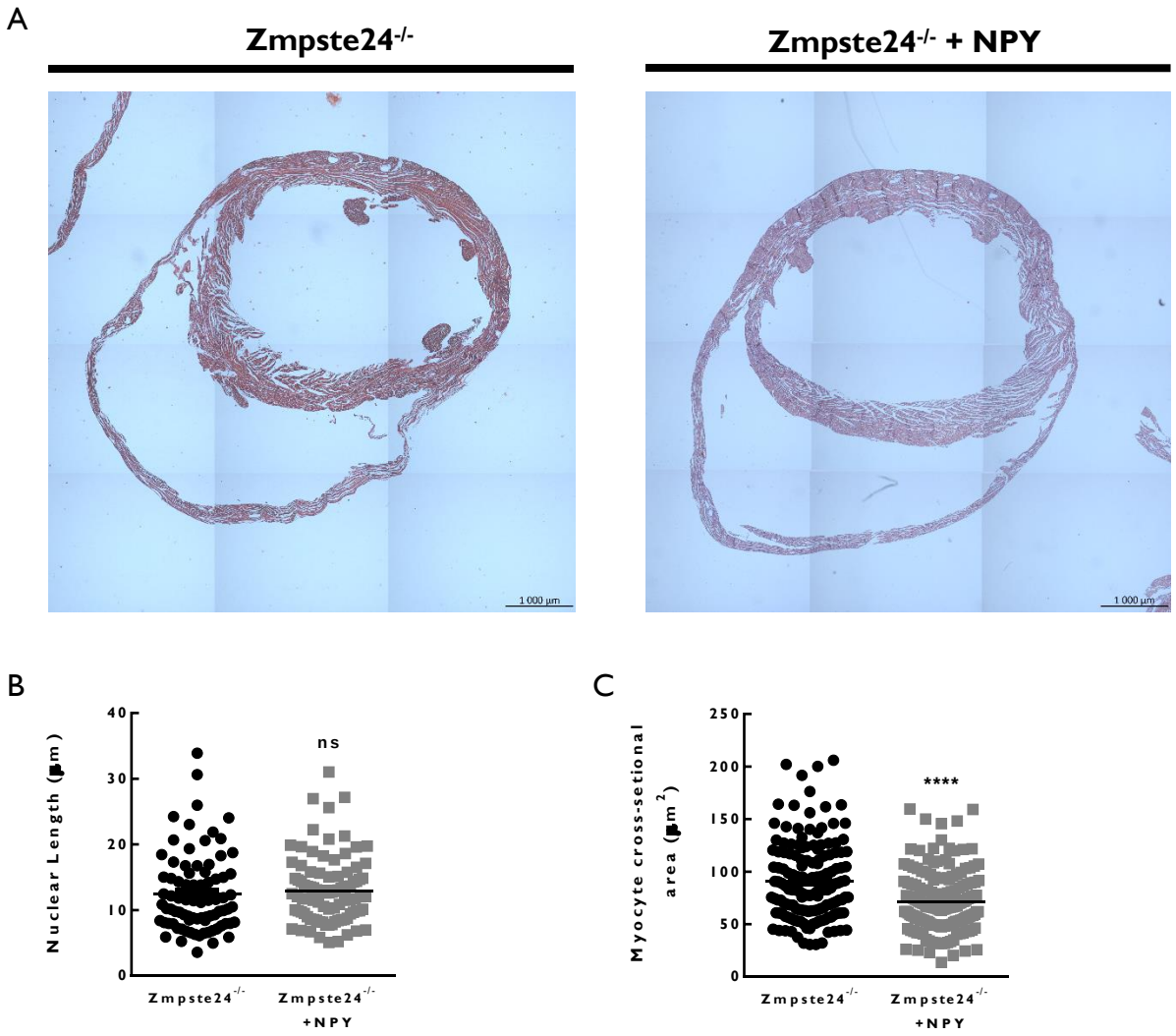

Figure 3.8 - Effect of hypothalamic NPY modulation in the heart of Zmpste24$^{-/-}$ mice. (A) Representative images of hematoxylin and eosin staining of heat cross-sectional heart paraffin slices of Zmpste24$^{-/-}$ mice group (control group; Zmpste24$^{-/-}$) and hypothalamic NPY overexpressing Zmpste24$^{-/-}$ mice group (Zmpste24$^{-/-}$+NPY). (B) Quantification of the nuclear length of the cardiomyocyte longitudinal fibers. (C) Quantification of cross-sectional area of the cardiomyocytes fibers. The results are presented in scatter plots of all the analyzed values and are expressed in µm (B) mm^2 (C) and the transverse black line represent the mean of all observed values. ns, not statistically different; *p< 0.05, ****p < 0.0001, significantly different from the Zmpste24$^{-/-}$ mice (control group), as determined by Student's unpaired t test. n=3-4 mice per group; for each parameter 3 fields were assessed per animal.

3.2.3.3. Effect of hypothalamic NPY overexpression in the histological structure of the kidney

Age leads to a functional decline of kidney. The modifications in the aged kidney include: decrease in size; increase in the renal corpuscle diameter; increase renal cysts; increase mesangial expansion; and renal vasculature changes, for example hypertrophy and arteriolosclerosis, which can lead to a decrease in the number of glomeruli (Pendas et. al., 2002; Lim et. al., 2012; Yabuki et. al., 2014; Harkema et. al., 2016). The Zmpste24$^{-/-}$ mice also have alterations in the kidneys like the increase in the weight of the kidneys and increase in senescent cells (Pendas et. al., 2002). We evaluated the effect of hypothalamic NPY overexpression in the kidney structure of Zmpste24$^{-/-}$ mice. As shown by the representative images of HE staining of kidney longitudinal paraffin sections (Figure 3.9 A), we did not observe any differences in the histological structure of the kidney in Zmpste24$^{-/-}$+NPY mice when compared to Zmpste24$^{-/-}$ mice. We evaluated the cortical thickness (Figure 3.9 B) and the area of renal corpuscles (Figure 3.9 C), but we did not observe any significant differences in Zmpste24$^{-/-}$+NPY mice when compared to Zmpste24$^{-/-}$ mice. However, we observed a decrease in the glomerular density (Figure 3.9 D) in the kidney of Zmpste24$^{-/-}$+NPY mice (8.9 ± 0.4 number/mm^2) when compared to the Zmpste24$^{-/-}$ mice (10.1 ± 0.5 number/mm^2). This decrease in the number of glomerulus may be due to alterations in the renal vasculature of these mice, however, more studies are needed regarding the kidney function to understand if this decrease is physiological and also compare these results with a Wild-type group.

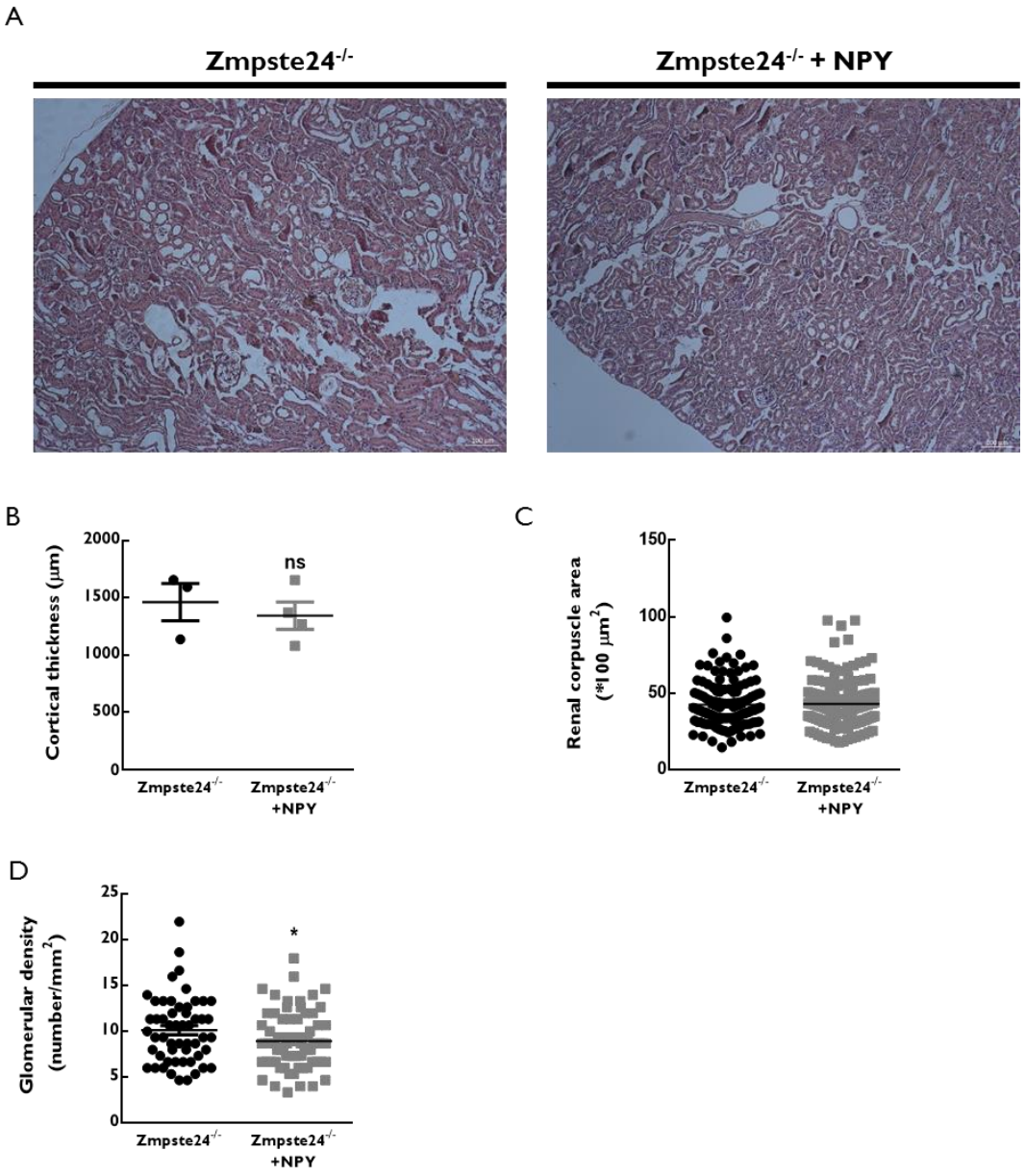

Figure 3.9 - Effect of hypothalamic NPY overexpression in the histopathological structure of the liver. (A) Representative images of hematoxylin and eosin staining of kidney longitudinal paraffin slices of Zmpste24$^{-/-}$ mice group (control group; Zmpste24$^{-/-}$) and hypothalamic NPY overexpressing Zmpste24$^{-/-}$ mice group (Zmpste24$^{-/-}$ +NPY). (B) Quantification of cortical thickness in longitudinal sections through the middle of the kidney. (C) Quantification of glomerular density, which was quantified by counting the number per area. The results are presented in scatter plots of all the analyzed values and are expressed in µm (B); *100 µm^2 (C); number/mm^2 (D) and the transverse black line represent the mean of all observed values. ns, not statistically different; *p< 0.05, significantly different from the Zmpste24$^{-/-}$ mice (control group), as determined by Student's unpaired t test. n=3-4 mice per group; for cortical thickness 1 section per animal was assessed; for renal corpuscle areas 3 fields were assessed per animal; and for glomerular density 18 fields were assessed per animal.

Chapter IV

Discussion

Aging is a natural process characterized by an impairment of normal body functions, a decreased capacity to respond to stress and, consequently, an increased predisposition to develop age-related pathologies such as cardiovascular diseases, diabetes, cancer and neurodegenerative disorders (Nicolai et. al., 2015). Nowadays the society experiences low rates of birth and death, which results in higher proportions of older people bringing socioeconomical issues due to the prevalence of chronic diseases. Moreover, the aging process involves a multiplicity of mechanisms and the understanding of its molecular basis is not fully completed (Kirkwood, 2005). The brain is one of the most affected organs by the aging process and the hypothalamus also becomes altered with aging (Zhang et. al., 2013).

Caloric restriction (CR) is the most robust anti-aging intervention known to increase lifespan and healthspan from yeast to mammals (Masoro, 2006; 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 disease (Colman et. al., 2009). Additionally, the major neuroendocrine effect of caloric restriction is the increase of NPY in the hypothalamus (Minor et. al., 2009), which recently has been demonstrated to be a brain region responsible for the whole-body aging (Zhang et. al., 2013). Aging is mainly associated with decreased levels of NPY in several brain regions such as hypothalamus, hippocampus and cortex (Gruenewald et. al., 1994; Vela et. al., 2003) and, in humans, a decline in NPY plasma levels is correlated with increasing age (Chiodera et. al., 2000). Additionally, a reduction of NPY levels is associated with some neurodegenerative diseases (Decressac et. al., 2012a; Duarte-Neves et. al., 2015; Duarte-Neves et. al., 2016). Besides, an increase in the levels of NPY can have physiological effects similar to those induced by CR (Minor et. al., 2009). Central administration of NPY has been shown to induce hyperphagia (Stanley et. al., 1986; Beck et. al., 1992) and lower blood glucose levels in both humans and rats (Ahlborg et. al., 1994; Marks et. al., 1997; Bischoff et. al., 1998). Additionally, transgenic rats overexpressing NPY show improved resistance to stress and increased mean lifespan (Michalkiewicz et. al., 2003). 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).

Although there is no effective strategies to stop or delay the alterations that occur with aging, the main focus of aging investigation is to understand its molecular basis with the aim of improve the human health. Taking into account that NPY can act as a mimetic of CR (Minor et. al., 2009), the modulation of NPY levels can have beneficial impacts in the aging process.

Zmpste24 knockout (Zmpste24$^{-/-}$) mice were used in this study because it is a mouse model of premature aging and it was not known if this mouse model of aging has hypothalamic alterations as described in natural aged mice (Zhang et. al., 2013). In this study, we show that Zmpste24$^{-/-}$ mice have alterations in the hypothalamus. These alterations include decreased levels of NPY in the hypothalamus, and decreased immunoreactivity of neuronal and neuroinflammatory markers. A decrease of hypothalamic NPY levels in Zmpste24$^{-/-}$ mice is in accordance with what is described in the literature, since it is described that aging is associated with decreased levels of NPY in several brain regions such as hypothalamus, hippocampus and cortex (Gruenewald et. al., 1994; Vela et. al., 2003). In humans, a decline in NPY plasma levels is correlated with increasing age (Chiodera et. al., 2000). Being the hypothalamus responsible for the regulation of the aging process (Zhang et. al., 2013), a decrease in hypothalamic NPY levels may cause alterations in the function of this brain region and have implications in the whole-body aging. The present study also show that Zmpste24$^{-/-}$ mice have alterations in hypothalamic neuronal structure, as shown by the decrease in the levels of MAP2 and NeuN immunoreactivity in the hypothalamic ARC of these mice. MAP2 is exclusively associated with the cytoskeleton in somal and dendritic compartments of neurons, being important for microtubule stability, dendritic development and function, and for neuronal plasticity. A reduction of MAP2 immunoreactivity in hypothalamic neurons may be due to a reduction in dendritic arborization, which may lead to neuronal dysfunction, or neuronal cell death. Concomitant with MAP2 loss, we also observed a decrease in the immunoreactivity of NeuN, a neuronal marker, within the hypothalamus of Zmpste24$^{-/-}$ mice. Although we did not find a significant decrease in the number of NeuN-positive cells, our results suggests that the number of hypothalamic neurons is decreased in Zmpste24$^{-/-}$ mice. This decrease may be caused by cell death or a decrease in the proliferation of neuronal progenitor cells. Neurogenesis is a natural process by which new cells are generated from a small population of multipotent stem cells in the adult nervous system (Marr et. al., 2010). There is evidence that neurogenesis decreases with aging in brain regions of mice such as hippocampus (Kuhn et. al., 1996; Drapeau et. al., 2003; Drapeau et. al., 2008). Overall, our results suggest that the hypothalamus of these mice have a decrease in the number of neurons which can result in neurogenesis commitment or cell death, but more studies are needed to evaluate these processes. An increase of neuroinflammation accompanies the aging process and it is known that aging cause astrogliosis through the increasing of GFAP levels and this increase may be a result of astrocyte activation or astrocyte proliferation (Rozovsky et. al., 1998). In fact, an

increase in GFAP immunoreactivity in the hypothalamus of Zmpste24$^{-/-}$ mice is observed suggesting astrogliosis, however this observation is not significant probably because the reduced number of individuals studied. To verify this result it is necessary to increase the number of mice in the study. Since the results obtained do not have a significant increase of GFAP immunoreactivity, the Zmpste24$^{-/-}$ mice do not show signs of astrogliosis. As shown in the human brain, the accumulation of functional and morphological alterations over time also can cause microglial cell death (Streit, 2004; Streit et. al., 2009; Lourbopoulos et. al., 2015). Although in the young brain microglia plays a neuroprotective role, in the aged brain it may react abnormally becoming neurotoxic (Luo et. al., 2010). Additionally, after a damage, may be triggered an inflammatory process involving the recruitment of immune cells (Lourbopoulos et. al., 2015). In 2005 it was described that the regulation of microglial proliferation changes with aging, particularly, after an injury, the proliferation of microglia in old rats is maintained higher than in young rats (Conde et. al., 2006). Controversially to what is described in the literature, where it was shown that the number of microglial cells increase in an age-dependent manner (Zhang et. al., 2013), our results show a decrease in Iba1 suggesting that Zmpste24$^{-/-}$ mice have less microglia and, consequently, a lower immune response to neuronal damage. The decrease in microglia within hypothalamus in Zmpste24$^{-/-}$ mice is probably due to a decrease in microglia proliferation; a decrease in the recruitment of immune cells such as macrophages and monocytes from the periphery; or to death of microglial cells due to changes in the hypothalamus. In addition, something in the development of these mice can also cause a lower amount of microglia within hypothalamus. Therefore, more studies are needed to understand why there is a neuroinflammation decrease in the hypothalamus of these mice, by investigating if the alterations seen are due to microglial cell death, decreased in cell proliferation, or less immune response, together with increasing the number of mice in the study.

This set of results indicate that Zmpste24$^{-/-}$ mice have hypothalamic alterations due to the aging process, by showing a decrease in the NPY levels and in the number of neurons, which indicates that the hypothalamus of these mice may have a commitment in neurogenesis and/or cell death. Unexpectedly, these mice show a decrease in neuroinflammation probably because hypothalamic alterations caused cell death, lower immune response and less cell proliferation.

NPY can be a CR mimetic candidate since they share several similar effects such as increase hyperphagia and glucocorticoid secretion as well as decrease body temperature, blood glucose levels and fertility (Minor et. al., 2009), and increase autophagy (Aveleira et. al., 2015;

Botelho et. al., 2015). In order to investigate if the NPY was able of improve the results discussed above related with the aging process, we studied its effect by overexpressing NPY in the hypothalamic ARC of Zmpste24$^{-/-}$ mice giving rise to a new group called Zmpste24$^{-/-}$ +NPY mice. As expected the levels of NPY increased in the hypothalamus of Zmpste24$^{-/-}$ +NPY mice, and remained elevated during the duration of the study (4 months). Preliminary results in our group suggest that an increase of endogenous hypothalamic NPY levels rescue Zmpste24$^{-/-}$ mice phenotype, such as body weight loss, lipodystrophy, alopecia and memory impairment (results not showed). Moreover, several studies described that NPY plays a role in neuroprotection in different brain areas such as hippocampus (Silva et. al., 2003; Silva et. al., 2005; Santos-Carvalho et. al., 2013). This neuroprotective role seems to have a beneficial effect in neurodegenerative disorders including Alzheimer's disease, Parkinson's disease and Huntington's disease (Rose et. al., 2009; Decressac et. al., 2012a; Duarte-Neves et. al., 2016). There is evidence that NPY produces a neuroprotective effect by decreasing cell death processes in retina, hippocampus, cerebellum and striatum (Silva et. al., 2005; Alvaro et. al., 2008; Goncalves et. al., 2012; Santos-Carvalho et. al., 2013; Duarte-Neves et. al., 2015; Duarte-Neves et. al., 2016); decreasing inflammation (De la Fuente et. al., 2001); and increasing neurogenesis in mice hippocampus (Decressac et. al., 2011). Regarding MAP2 immunoreactivity, NeuN immunoreactivity and number of NeuN-positive cells, our results do not show a neuroprotective effect of NPY in the hypothalamus of Zmpste24$^{-/-}$ mice contrarily to what is described in the hippocampus and other brain regions. However, hypothalamic NPY overexpression increased the protein content of NeuN in the hypothalamus of Zmpste24$^{-/-}$+NPY mice. Although there is an increase in NeuN content upon hypothalamic overexpression, the results observed are not sufficient to support our hypothesis that hypothalamic NPY overexpression is neuroprotective. Nevertheless, these results may be due to a low number of mice tested, thereby we have to increase the number of animals to confirm these results. Our results show that NPY is able to reduce the gliosis and inflammatory process, by decreasing the levels of GFAP immunoreactivity in the hypothalamic ARC of Zmpste24$^{-/-}$+NPY mice when compared with the control group Zmpste24$^{-/-}$ mice, decreasing the protein content of GFAP and pIκBα in the hypothalamus of Zmpste24$^{-/-}$+NPY mice. The decrease in GFAP means that NPY was able to decrease astrogliosis, production of cytokines and neuron damage in the hypothalamus of Zmpste24$^{-/-}$ +NPY mice when compared with the control group Zmpste24$^{-/-}$ mice. Regarding Iba1, NPY has no beneficial effect in both immunoreactivity and number of Iba1-positive cells in the

hypothalamus of Zmpste24$^{-/-}$ mice. It has been shown that NPY exhibit modulatory properties in neuroinflammation through the inhibition of excessive microglial activity (Malva et. al., 2012). Additionally, previous studies showed that NPY is able of protect rat retinal cells against necrosis and apoptosis induced by glutamate, suggesting that this peptide have a protective effect against these cell death processes (Santos-Carvalho et. al., 2013). In fact, the Iba1 immunoreactivity and the number of Iba1-positive cells do not show a significant decrease, probably because NPY may be inhibiting microglial cell death. Furthermore, it was demonstrated that, upon inflammation, hypothalamic NF-κB is activated (through the release of IκBα) in association with increasing age (Zhang et. al., 2013). Our data suggest that NPY is able to decrease NF-κB activation in the hypothalamus, resulting of a decreased observed in the levels of pIκBα, having thus a positive effect in the hypothalamic inflammation of Zmpste24 mice. Therefore, NPY can attenuate the neuroinflammatory process that occurs in the hypothalamus of Zmpste24$^{-/-}$ mice.

Taking into account that this set of results is a comparison between Zmpste24$^{-/-}$+NPY mice and the control group Zmpste24$^{-/-}$ mice, they show that NPY can, at least in part, have a beneficial effect in this mouse model of aging. Preliminary results suggest that NPY rescued Zmpste24$^{-/-}$ mice phenotype, such as body weight loss, lipodystrophy, alopecia and memory impairment. Additionally, NPY can attenuate the neuroinflammatory process by decreasing the levels of markers such as GFAP and pIκBα. However, we did not observed a neuroprotective effect of NPY.

As described in the literature the hypothalamus is responsible for the regulation of whole-body aging and being NPY a hypothalamic neuropeptide, is from this brain region that NPY is involved in the regulation of many homeostatic functions (Fetissov et. al., 2004b; Zhang et. al., 2013). Despite being predominantly expressed through the central nervous system, the neuropeptide Y also exhibits its functions in peripheral organs such as the cardiovascular system, the gastrointestinal tract and the kidney (Bischoff et. al., 1998; Balasubramaniam, 2002). Taking into account this evidence we decided to evaluate the effect of hypothalamic NPY modulation in peripheral organs namely liver, heart and kidney. Additionally, in this mouse model of premature aging it was already described some histopathological alterations that occur due to the aging process (Marino et. al., 2010).

We show that NPY has a beneficial effect in the liver of Zmpste24$^{-/-}$ mice, since it can improve some histopathological features namely increasing hepatocytes density, and decreasing

immune infiltration, probably due to an increase in cell proliferation and decrease in DNA damage and cell cycle arrest. Accordingly to what is described in the literature the aged liver show some characteristic alterations including loss of organ volume; increased lobular size; fibrosis; lipid accumulation; decreased hepatocyte number; pseudoinclusions; increased nuclear size; polyploidy and pseudocapillarization (Gregg et. al., 2012; Harkema et. al., 2016). We observed, through qualitative and quantitative analysis, that the liver of Zmpste24$^{-/-}$ mice have some of these reported age-related alterations. From qualitative analysis, we observed alterations such as lipid accumulation; and pseudoinclusions and NPY was able to improve some of these alterations in the liver structure, it decreased the existence of pseudoinclusions; decreased the nuclear size; and it increased the number of hepatocytes, suggesting that NPY can improve the liver structure. It is described that acute or chronic liver injury is often associated with the activation, proliferation, and fibrogenic response of hepatic stellate cells (the liver's principal fibrogenic cells) and it is reported that in cirrhosis there is an increase in the levels of NPY (Oben et. al., 2003). Additionally, others showed that during the regulation of liver fibrosis, NPY can promote proliferation of hepatic stellate cells, but NPY do not alter the collagen gene expression (Oben et. al., 2004). Given the fact that was already seen a proliferative effect of NPY in hepatic stellate cells, NPY may have the same effect on hepatocytes once it was observed an increase in the number of hepatocytes and in the hepatocytes per area when NPY is overexpressed in the hypothalamus of Zmpste24$^{-/-}$ mice. The proliferative effect of NPY, in the liver of Zmpste24$^{-/-}$+NPY mice is in agreement with the increase in PCNA observed. Following these beneficial effects, we show that hypothalamic overexpression of NPY decreased the area of immune cell infiltration. This decrease is concomitant with the literature where it is reported that NPY can modulate immune cell trafficking; T helper cell differentiation; cytokine secretion; and natural killer cell activity, and since the immune cells are capable of producing NPY, this peptide can regulate immune cell functions (Dimitrijevic et. al., 2013). Besides, systemic immune stimulation with lipopolysaccharide (LPS) decreases the expression of NPY in the rat hypothalamus (Kim et. al., 2007), so with hypothalamic NPY overexpression probably the accumulation of immune cell infiltrations due to aging may decrease. We also observed a decrease in the levels of phospho-p53, a tumor suppressor protein that plays a major role in the cellular response to DNA damage. It is described that Zmpste24$^{-/-}$ mice show hyperactivation of the tumor suppressor p53 which causes accelerated aging and, particularly in the liver, these mice show an upregulation of p53 target genes (Varela et. al., 2005). In 2005, Varela *et al*. also showed

that the decline in tissue-specific functions of Zmpste24$^{-/-}$ mice was due to an abnormal activation of p53, for this they targeted this abnormal activated p53 by generating transgenic mice lacking the Zmpste24 and p53 (Zmpste24$^{-/-}$p53$^{-/-}$) and these transgenic mice rescued their phenotype. The decrease observed in phospho-p53 levels in the liver suggest that the overexpression of NPY may be diminishing the upregulation of p53 target genes which is involved in the stress signaling pathway and, thus, decreasing DNA damage. p53 plays a role in the determination of cell response to several types of stress including DNA damage and hypoxia (Kruiswijk et. al., 2015). Once p53 becomes activated the inhibition of cell proliferation can occur, through the induction of cell death and senescence (Chen, 2016). Additionally, p53 activates p21 to cause cell cycle arrest and the inhibition of DNA replication also can occur through the interaction between p21 and PCNA (Chen, 2016). Therefore, the increase of proliferation seen in the liver of Zmpste24$^{-/-}$ mice may be supported by the decrease of p53 levels, meaning that NPY decreases events of cell death and cell cycle arrest. Overall, NPY seems to have a positive effect in the structure of the liver of Zmpste24$^{-/-}$ mice. However, it is necessary further investigation of the role of NPY in the liver to support this results, it should be assessed tissue fibrosis through histological procedures such as Masson Trichrome; some autophagy markers how is the case of LC-3B and p62; also parameters related to liver function. In addition, these studies should include an age-matched wild-type mice control group.

Another peripheral organ that we studied was the heart, which is described to be affected in Zmpste24$^{-/-}$ mice. Accordingly to what is described in the literature, the aging process in the heart causes some characteristic alterations including congestion of blood vessels; higher deposition of connective tissue in the ventricle muscle and fibrosis (Gottwald et. al., 1997; Hacker et. al., 2006). Additionally, others showed in another mouse model of aging that an abnormal elongation of nuclei in cardiomyocytes occur due to the alterations caused by accelerated aging (Mounkes et. al., 2003). There is evidence that in the heart of Zmpste24$^{-/-}$ mice some histological age-related alterations occur including the increase senescence; muscle degeneration; fibrosis; lymphocytic infiltration and intracellular vesicles (Bergo et. al., 2002; Pendas et. al., 2002). However, our study show that the overexpression of hypothalamic NPY was not able to interfere with the studied parameters, only in the cross-sectional area of cardiomyocytes was observed a decrease. As cells become senescent they change their morphology to a large flattened one (Zhu et. al., 2013) and with aging cardiomyocytes become

hypertrophic which is characterized by an increase in cell size (Frey et. al., 2004; Harkema et. al., 2016). This decrease in the cardiomyocyte cross-sectional area observed in Zmpste24$^{-/-}$ +NPY mice may be due to a decrease in cell senescence and/or cardiomyocyte hypertrophy. It has been shown that NPY is present in this organ and have other regulatory roles including maintenance of cardiac contraction; compensatory or detrimental remodeling of myocardial tissue upon ischemic conditions and promotion of angiogenesis to regenerate myocardium after ischemic injury (McDermott et. al., 2007). Nevertheless, in the most of the parameters studied we did not observed differences in the heart of Zmpste24$^{-/-}$ mice compared with Zmpste24$^{-/-}$+NPY mice, but more studies are needed such as evaluate tissue fibrosis, ventricle thinning, cell senescence, muscle degeneration and infiltration of immune cells in order to investigate whether NPY can or not improve the heart structure of Zmpste24$^{-/-}$ mice.

Finally, we studied the kidney which is responsible for the elimination of drugs and its loss of function during aging influences the response to pharmacotherapy (Harkema et. al., 2016). It is described that the aging process in the kidney causes alterations including: increased diameter of renal corpuscle; increased mesangial area; increased apoptotic cells; increased cystic renal corpuscle and fibrosis (Gagliano et. al., 2000; Lim et. al., 2012; Yabuki et. al., 2014). In Zmpste24$^{-/-}$ mice the changes reported by others were: a decrease in kidney size; increase senescence staining in proximal and distal tubules and collecting ducts of kidney (Pendas et. al., 2002; Varela et. al., 2005). Qualitatively, we did not observe any histological alterations in the kidney of these mice upon hypothalamic NPY overexpression. Quantitatively, our results show that the cortical thickness and the renal corpuscle area do not change after the overexpression of hypothalamic NPY. Upon hypothalamic NPY overexpression it was observed a decrease in the number of glomerulus per area, but other studies need to be made to see if this decrease is physiological. Overall, this data suggest that hypothalamic NPY overexpression do not have a beneficial effect in the parameters studied, but other parameters should be investigated such as cell senescence, inflammatory cell infiltration, fibrosis and apoptotic cells.

This set of results show the effect of NPY in some peripheral organs, but only in the liver the beneficial role of NPY was stronger. However, more studies need to be done and since it is described that NPY can delay cellular senescence (Botelho et. al., 2015), seems necessary the evaluation of senescence mechanisms in these organs.

There is evidence showing that the modulation of hypothalamic NPY could have a beneficial impact in the hypothalamus and in the periphery. However, more studies are needed to understand the role of NPY as a strategy to revert age-related alterations, lifespan extension and healthspan improvement.

Chapter V
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