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MicroRNA based therapeutic approaches for obesity

Dissertação de Mestrado apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia Farmacêutica, realizada sob orientação da Professora Doutora Cláudia Cavadas (Faculdade de Farmácia, Universidade de Coimbra) e Doutora Lígia Sousa-Ferreira (Centro de Neurociências e Biologia Celular)

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Cover Note: Immunohistochemical analysis of neuropeptide pro-opiomelanocortin (POMC) and neuronal injury marker heat shock protein 70 (HSP70) in the mouse hypothalamus. Representative image of POMC (green), HSP70 (red) and nuclear marker DAPI (blue) in coronal sections of mice arcuate nucleus from animal fed with high fat diet with control microRNA overexpression in the hypothalamus.

The present work was performed in the “Neuroendocrinology and Aging Group”, at the Center for Neuroscience and Cell Biology, University of Coimbra, under scientific guidance of Professor Cláudia Cavadas and Doctor Lígia Sousa-Ferreira.
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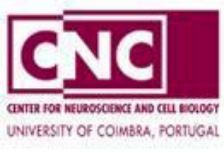

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Table of Contents

Abbreviations

3V - Third Ventricle

Ago2 - Argonaute 2

AGRP - Agouti-Related Protein

ANS - Autonomous Nervous System

ARC - Arcuate Nucleus

BBB - Blood Brain Barrier

BSA - Bovine Serum Albumin

BMI - Body Mass Index

CART - Cocaine and Amphetamine-Regulated Transcript

CNC - Central Nervous System

CRH - Corticotropin

DGCR8 - DiGeorge Syndrome Chromossomal Region 8

DMN - Dorsomedial Nucleus

DsRNA – Double Stranded RNA

EDTA - Ethylenediamine Tetraacetic Acid

ELISA - Enzyme-Linked Immunosorbent Assay

ER – Endoplasmatic Reticulum

GFAP - Glial Fibrillary Acidic Protein

GFP - Green Fluorescent Protein

GI - Gastrointestinal Tract

GRH – Growth Hormone Receptor

HDL - High-density Lipoprotein

HEK 293 - Human Embryonic Kidney 293 cells

HFD - High Fat Diet -

HSP - Heat Shock Protein

Iba1 - Ionized calcium-binding adapter molecule 1

IGF-1 - Insulin-like Growth Factor 1

IKBKB/IKKβ - Inhibitor of Nuclear factor kappa-B kinase subunit beta

IL6 - Interleukin 6

InsR - Insulin Receptor

KO – Knock Out

LepR - Leptin Receptor

LHA - Lateral Hypothalamic Area

Lin28 - Protein lin-28 homolog A

MBH - Mediobasal Hypothalamus

MC3R/MC4R – Melanocortin ¾ Receptor

MCH – Melanin Concentrating Hormone

ME - Medial Eminence

miRISC miRNA-induced silencing complex

miRNAs, miRs - microRNAs

mRNA - Messenger RNA

NTS - Nucleus of the Solitary Tract

NF-κB - Nuclear Factor kappa B

NPC - Neural Progenitor Cell

NPY - Neuropeptide Y

NSC - Neural Stem Cell

ORX - Orexins

OXY - Oxitocin

PBS - Phosphate buffered saline

PCNA - Proliferating Cell Nuclear Antigen

PFA - Paraformaldehyde

PGK - Phosphoglycerate Kinase 1

POMC - Pro-Opiomelanocortin

pre-miRNA - Precursor microRNA

pri-miRNA - Primary microRNA

PVN - Paraventricular Nucleus

RNA - Ribonucleic Acid

SOX2 - (Sex determining region Y)- box 2

SGZ - Subgranular zone

SVZ - SubVentricular Zone

TLR – Toll Like Receptor

TRBP – Trans Activation Response RNA-Binding Protein

TRH – Tyrotropin

UTR – Untranslated Region

VMN - Ventromedial Nucleus

WAT - White Adipose Tissue

XPO5 – Exportin 5

Abstract

Obesity causes cellular injury in the hypothalamus, a region of the brain responsible for maintenance of body homeostasis and feeding behaviour. This injury includes neuro-inflammation, cell death and impaired neurogenesis. However, inhibition of hypothalamic inflammation in obesity results in improved hepatic and adipose tissue metabolic parameters.

MicroRNAs are small endogenous non-coding RNA molecules that regulate several biological processes. These molecules are being investigated as therapeutic strategies for some diseases. Let-7 microRNAs are interesting candidates for a central anti-obesity therapy because they attenuate inflammation (in macrophages), control peripheral glucose homeostasis and promote neuronal differentiation of stem cells.

The aim of the present work is to investigate microRNA based therapeutic approaches for the prevention of obesity. This project is divided in two specific goals: investigate if let-7 microRNA can prevent peripheral metabolic alterations induced by obesity in mice and explore the possibility of let-7 microRNA prevent hypothalamic injury induced by obesity in mice. To achieve our goal, let-7 or control microRNA (miR-neg) were over-expressed in the mouse hypothalamus using lentiviral vectors and the mice were exposed to high-fat diet (HFD) for 7 weeks. Control group of mice exposed to chow diet (low fat content) were also included.

Metabolic parameters evaluated included serum cholesterol, triglycerides and hormones levels, adipocytes diameter and liver steatosis. Hypothalamic injury was appraised using immunohistochemistry technique to assess neuro-inflammation (astrocytes activation and microglia number, neuronal injury, neurogenesis (number of neuroprogenitor cells) and neuropeptides levels.

In our study, we found that let-7 microRNA was beneficial in several peripheral metabolic parameters that are altered from normal levels in obesity. Mostly, in insulin and leptin levels, total cholesterol and triglyceride levels, white adipose tissue expansion and liver pathology, the treated mice group showed a potential melioration. For central alterations at the hypothalamus that occurs in this pathology, we show here that the overexpression of this microRNA has a potential protective role against the development of obesity.

These findings achieved by this project validate let-7 microRNA as new innovative target for obesity.

Keywords: Obesity, MicroRNA, Let-7, Hypothalamus, Metabolism.

Resumo

A obesidade causa lesões celulares no hipotálamo, uma região do cérebro responsável pela homeostase corporal e comportamentos alimentares. Estas lesões incluem neuro-inflamação, morte celular e comprometem a neurogénese. Todavia, a inibição da inflamação hipotalamica na obesidade resulta no melhoramento de parâmetros metabólicos hepáticos e do tecido adiposo.

MicroRNAs são moléculas RNA endógenas não codificantes que regulam vários processos biológicos. Estas moléculas têm sido investigadas como estratégias terapêuticas para diversas doenças. Os microRNAs let-7 são candidatos a uma terapia central anti-obesidade porque atenuam a inflamação (em macrófagos), controlam a homeostase periférica da glucose e promovem a diferenciação neuronal de células estaminais.

O objetivo do trabalho aqui apresentado é investigar uma estratégia terapêutica baseada em microRNAs para prevenção da obesidade. Este trabalho está dividido em duas partes: investigar se o microRNA let-7 consegue prevenir alterações periféricas induzidas pela obesidade em murganhos e explorar a possibilidade de o microRNA let-7 prevenir lesões hipotalamicas induzidas pela obesidade. Para atingir este objetivo, let-7 ou miRNA de controlo (miR-neg) foram sobre-expressos no hipotálamo do murganho utilizando vetores lentivirais e os murganhos foram expostos a uma dieta hiperlipídica durante 7 semanas. Grupos de controlo expostos a uma dieta normal (baixo teor de gordura) também foram incluídos.

Os parâmetros metabólicos avaliados incluem colesterol, triglicerídeos e níveis de hormonas leptina e insulina, diâmetro dos adipócitos e alterações hepáticas. As lesões hipotalamicas foram avaliadas utilizando a técnica de imunohistoquimica para averiguar neuro-inflamação (ativação de astrócitos, numero de células de microglia), neurogénese (numero de células neuroprogenitoras) e níveis de neuropeptídeos.

Neste presente estudo, demonstramos o papel benéfico microRNA let-7 em vários parâmetros periféricos bioquímicos, usualmente diferentes de níveis basais, na obesidade. Maioritariamente, nos níveis de insulina e leptina, níveis de colesterol total e triglicerídeos, expansão de tecido adiposo e patologia hepática, o grupo de animais tratados com o microRNA demostraram um possível melhoramento. Quanto a alterações hipotalamicas que ocorrem nesta condição, mostramos aqui que a sobre-expressão deste microRNA tem um papel protetor contra o desenvolvimento da obesidade.

Estes resultados alcançados deste projeto, validam o microRNA let-7 como um novo alvo inovador para a obesidade.

Palavras Chave: Obesidade, Let-7, MicroRNA, Hipotálamo, Metabolismo.

Chapter I:
Introduction

1. Obesity

Obesity is a metabolic condition that has emerged as a major health problem (1). This condition is considered a major public health problem that the world today faces because diet-induced obesity can lead to several chronic diseases, such as diabetes, cardiac disease and cancer (2).

Obesity is the result of an imbalance between energy intake and energy expenditure, which leads to a deregulation of the body homeostatic state (3). As reported by the World Health Organization (WHO), individuals can be classified according to their weight in: normal weight (19 ≤ Body Mass Index (BMI) < 25 kg/m^2), overweight (25 ≤ (BMI < 30 kg/m^2), and obese (30 ≤ BMI < 40 kg/m^2).

There are several obesity-associated morbidities, such as high triglycerides and low high-density lipoprotein (HDL) cholesterol, type 2 diabetes, metabolic syndrome, coronary artery disease, stroke, non-alcoholic fatty liver disease, and other social problems, such as social isolation and depression (4).

According to WHO, in 2014, around 2 billion adults were overweight and 600 million were obese (BMI ≥30 kg/m^2). Furthermore, 13% of the world's adult population were obese, being 11% men and 15% women. Estimates from World Obesity Federation and WHO showed that by 2025, 177 million adults worldwide will be overweight.

1.1 Peripheral alterations occurring in obesity

A hallmark of obesity is the expansion of adipose tissue and its ability to undergo remodelling in order to achieve its role as the major energy-storing organ (5). However, obesity also promote alterations in other systems and organs, as described above. Hormones, such leptin and insulin, have the ability to release the most robust signals to certain neurons located in the hypothalamus in agreement with body energy storages in the periphery, possibly triggering physiological responses (6).

Regarding triglycerides and cholesterol, not all obese individuals are hypertriglyceridemic, but there is a correlation between obesity and the amount of triglycerides present in the serum and with low levels of plasma HDL-cholesterol.

There is an interrelationship between obesity and ectopic fat deposition in the liver (7). It was described that in rats with high fat diet (HFD) regime, the hepatocytes shows a cytoplasm filled with small vacuoles, which were uniform in size and smaller than the centrally located nucleus (8).

1.1.1 Adipose tissue

The adipose tissue is a storage place of fatty acids, has a role in lipids and glucose metabolism and also produces a large number of hormones and cytokines, e.g. angiotensinogen, tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), adiponectin, leptin, among others (9). Adiposity is the fraction of total body mass comprised of neutral lipid stored in adipose tissue (10) and it is correlated with physiological parameters such as blood pressure, systemic insulin sensitivity, serum triglyceride levels, leptin concentrations and accumulation of macrophages and CD8 effector T-cells (10). Another parameter that is altered with obesity is the secretory function of adipocytes and macrophages, together with chronic low-grade inflammation and an increase risk to develop insulin resistance (9).

It is known that high caloric diet regime progressively increases the size of adipocytes, induce a significant level of adipocyte hypertrophy and greatly increases fat mass, which is associated with adipocyte hypertrophy (7). With increasing obesity there is an induction of white adipose tissue (WAT) expansion, which is tightly associated with adipose inflammation (11) and there are several proinflamatory factors produced in adipose tissue (11). It has been described that with adipose tissue expansion, macrophages infiltrate in this tissue and there are an increase of adipocytokines production and of inflammation (9).

To summarize, this endocrine organ is not only a storage reservoir of fat, but also has a metabolic role that changes with increasing obesity (11).

1.1.2 Leptin

Leptin is an adipocyte-derived cytokine that have a major influence on energy balance. It is a mediator of long-term regulation of energy balance, having an effect of suppressing food intake (12). Leptin, released by adipocytes, modulates the function of neurons in the arcuate nucleus (ARC) exerting its inhibitory effect in neurons that release neuropeptide Y (NPY) and agouti-related protein (AGRP) – orexigenic neuropeptides. At the same time, leptin stimulates neurons that release pro-opiomelanocortin (POMC) – anorexigenic neuropeptides that suppress appetite (5).

1.1.3 Insulin

Insulin is a hormone with a key role in blood glucose levels regulation, preventing hyperglycemia, and also regulating food intake. Low levels of insulin, observed in fasting, leads to the activation of NPY and AGRP neurons and, therefore, increase hunger and decrease energy expenditure. In contrast, after a meal the levels of insulin increase, giving rise to the inhibition of NPY and AGRP neurons and stimulating POMC neurons, promoting satiety (6).

1.1.4 Liver

A continued high caloric intake induces metabolic inflammation and activation of insulin-responsive peripheral tissues that leads to changes in WAT, liver and skeletal muscle (10).
The regulation of hepatic gluconeogenesis is precise and has a role in the maintenance of whole-body glucose homeostasis. Then, it has a responsibility for under physiological conditions to avoid oscillations in the blood levels of glucose. And because parasympathetic vagal fibers conduct signals from neurons that are sensible to leptin and insulin, hypothalamic resistance to these hormones, can lead to impaired hepatic glucose production (13).
It has been proven that the distribution of significant liver histopathology in the morbidly obese patient is highly correlated with the degree of impaired glycemic status (14). Another study showed that hepatic steatosis, the buildup of fat in the liver, were common in morbidly obese (15). Also, abnormal liver function and portal inflammation is related to hepatic fibrosis, that together with histologic features reflect biological significance of the chronic inflammatory condition in the obese population (15). It is described that inflammatory markers in the liver increase in rats over the course of HFD (5).
Furthermore, the presence of hepatocyte fat vacuoles can be considered steatosis and arrives from any alteration of the lipid transport and lipoprotein secretion that may cause enlargement of normal lipid droplets. Hyperinsulinemia promotes triglyceride synthesis and hepatocellular fat accumulation, which seems to be correlated with higher values of BMI.
Although many genetic, environmental, and other factors seem to play a role in the liver pathogenesis and also, depending on the degree of inflammation, these named changes may oscillate between liver pathology or even hepatic steatosis (16).

1.2 Central alterations occurring in obesity

Several studies showed that the hypothalamus has a role in neuronal turnover and glial activation and also, that the arcuate nucleus (ARC) is particularly vulnerable to obesity (5). Both of these findings raised the hypothesis that obesity can result, in part, from hypothalamic injury.

Diet-induced obesity will cause the activation of inflammatory pathways in brain areas that are the central control of food intake and body homeostasis. These areas include mediobasal hypothalamus (MBH), ARC and median eminence (ME). Diet-induced inflammation in the hypothalamus occurs before metabolic disturbances in peripheral tissues (1) suggesting a role in modelling normal metabolic physiology associated with over-nutrition.

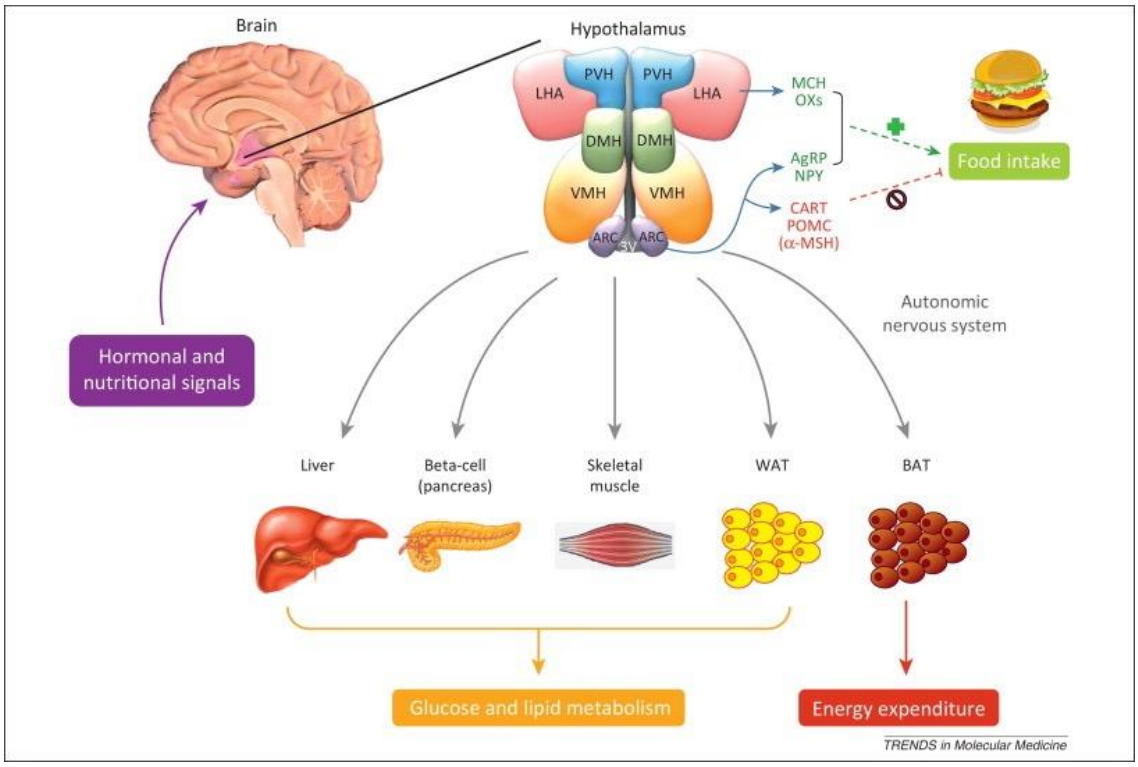


Figure 1.1 - Hypothalamic regulation of whole-body energy balance and metabolism. There is a dynamic circuit between the peripheral metabolic environment and central brain mechanisms. Food availability, energy stores and nutritional requirements have influence on specific nuclei of the hypothalamus and any modification on certain hormones, via the autonomous nervous system (ANS), hypothalamic nuclei respond and emit functional changes to peripheral tissues. Adapted from López *et al.*, 2013 (17).

2. Hypothalamus and Regulation of Food intake

The hypothalamus is a brain region, just below the thalamus, that plays a crucial role in body energy homeostasis through a network of neuronal circuits of various nuclei (18). It represents 1% of encephalic mass and has several nuclei: ARC, Paraventricular Nucleus [PVN], Lateral Hypothalamic Area [LHA], Ventromedial Nucleus [VMN] and Dorsomedial Nucleus [DMN].

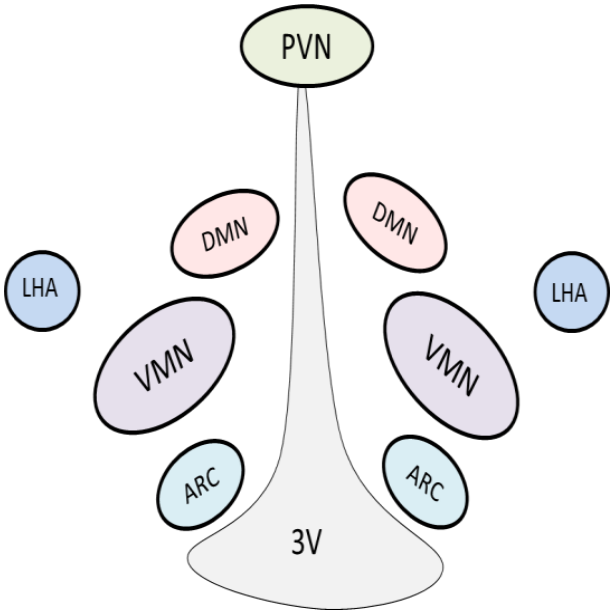


Figure 1.2 - Organization of the hypothalamic nuclei. ARC - Arcuate Nucleus, PVN - Paraventricular Nucleus, LHA - Lateral Hypothalamic Area, VMN - Ventromedial Nucleus, and DMN - Dorsomedial Nucleus. Adapted from Leal, 2015 (19).

The ARC nucleus of the hypothalamus is adjacent to the ME that have a defective blood-brain barriers (BBB).The ARC has the main function of regulate food intake as it integrates signals from brainstem and the periphery (20). It has two different neuronal populations: neurons co-expressing neuropeptide Y (NPY) and agouti-related peptide (AGRP) that are orexigenic neuropeptides and neurons co-expressing proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) that are anorexigenic neuropeptides (21). Orexigenic neuropeptides stimulate food intake and anorexigenic neuropeptides repress it. NPY is a 36 amino acid peptide member of the pancreatic polypeptide family and it is one of the most abundant peptides found in the CNS. In the ARC, NPY is the most potent orexigenic peptide that increases food intake and decreases energy expenditure. Therefore, NPY leads to a state of positive energy balance. The other orexigenic neuropeptide is AGRP, also involved in body weight homeostasis. POMC/CART neurons are exclusively located in the ARC, where this neuropeptides repress food intake and increase energy expenditure (21).

These neurons present in the ARC are first-order neurons, whereas they answer to peripheral signals and project that information to the second order neurons, that are located in other hypothalamic nuclei, for example PVN, LHA, VMN and DMN (22).

PVN is a hypothalamic nucleus responsible for stress control, metabolism, growth, reproduction, immune, and other autonomic functions, for example gastrointestinal, renal and cardiovascular functions (22). There are afferent inputs to this nucleus from many integrative centers of the hypothalamus (ARC, suprachiasmatic nucleus), pons (lateral parabrachial nucleus) and medulla (dorsal motor nucleus of the vagus, ventrolateral medulla). The neurons present in PVN synthesize and secrete neuropeptides with a catabolic action, such as corticotrophin-releasing hormone, thyrotropin-releasing hormone, somatostatin, vasopressin, and oxytocin. It is known that PVN has a inhibitory role in food intake and weight gain (22).

LHA makes connection between the hypothalamus and all major hypothalamic nuclei. Neurons present in LHA are the largest in the hypothalamus and are organized topographically (23). This hypothalamic area is also considered a feeding center with two neuronal population that produces orexigenic neuropeptides: melanin concentrating hormone (MCH) and orexin. MCH acts as endogenous orexigenic molecule, and orexin is involved in glucose sensing and the regulation of sleep-awake cycles (22).

In the VMN there are projections coming from the ARC and going to the DMN, LHA and also as brainstem regions. These projections are AGRP/NPY and POMC/CART neurons, suggesting a role of VMN in food intake signalling. The VMN presents neurons that are sensible to glucose and leptin, and a brain-derived neurotrophic factor (BDNF), which suggests that this hypothalamic area generates satiety and maintains glucose homeostasis (22).

Lastly, the DMN receives several NPY terminals and α-MSH terminals originating from the ARC. It is known that lesion or destruction of this nucleus leads to hyperfagia and obesity (22).

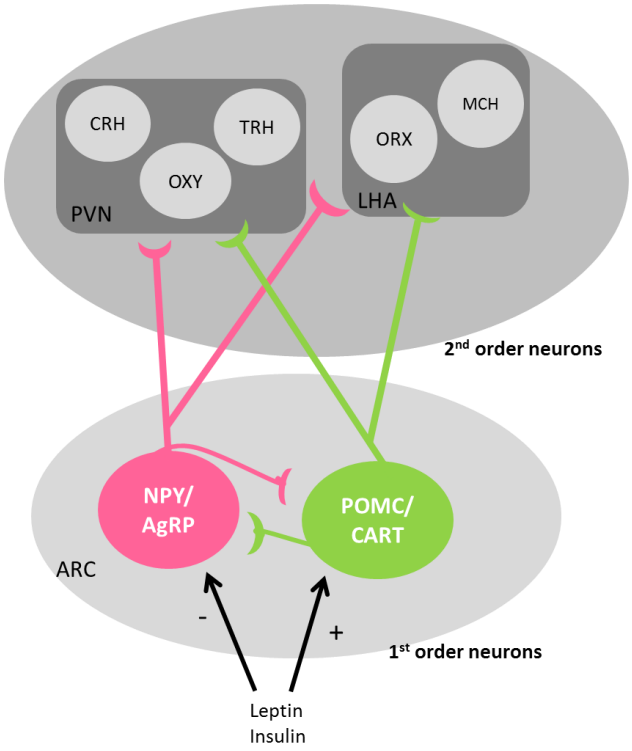
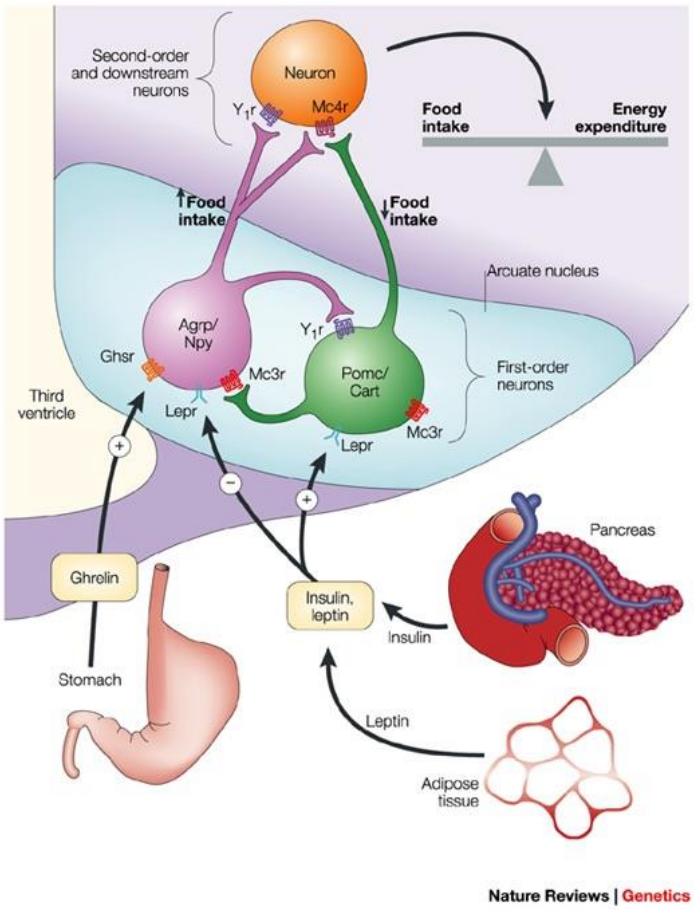


Figure 1.3 - Schematic representation of NPY/AGRP and POMC/CART neurons in the ARC of hypothalamus and their interaction with other hypothalamic nuclei. NPY/AgRP neurons and POMC/CART neurons are inhibited and stimulated, respectively by both insulin and leptin. Through the projection of second-order neurons in the PVN and LHA, feeding-related peptides make the connection to the neuroendocrine system, brainstem and higher centers of the brain. CRH - Corticotropin – releasing hormone; TRH - Thyrotropin – releasing hormone; MCH – Melanin-concentrating hormone; ORX – Orexins; OXY - Oxytocin. Adapted from Leal, 2015 (19).

Although other nuclei are involved in feeding regulation, the ARC receives inputs as circulating hormones and nutrients (3). The metabolic signals coming from the periphery includes hormones and gastrointestinal peptides vary before and after a meal. For example, after a meal adipose-derived hormone leptin activates leptin receptors in the ARC neurons to increase the expression and release of POMC and reduce the expression and release of NPY (24). Projections originated in the ARC reach the PVN, which contains high levels of melanocortin receptors, namely the melanocortin receptor-4.

Figure 1.4 - Schematic representation of regulation of energy balance. As said above, there are two



sets of neurons in the arcuate nucleus — AGRP/NPY and POMC/Cart neurons — that are regulated by circulating hormones. Insulin and leptin are hormones that inhibit AGRP/NPY neurons and stimulate adjacent POMC/CART neurons. Therefore, lower insulin and leptin levels are predicted to activate AGRP/NPY neurons, while inhibiting POMC/CART neurons. Ghrelin is a circulating peptide secreted from the stomach that is able to activate AGRP/NPY neurons, thereby stimulating food intake. GHRS - growth hormone receptor; Lepr - leptin receptor; Mc3r/Mc4r - melanocortin 3/4 receptor; Y_1r - neuropeptide Y1 receptor. Adapted from Barsh *et al.*, 2002 (25)

Satiety signals are linked from the gastrointestinal (GI) tract to the brain by nucleus of the solitary tract (NTS), through the sensory vagus nerve. Therefore, the brainstem is another brain area involved in regulation of food intake. As referred before and to summarize, activation of the neurons co-expressing POMC triggers the release of α-MSH from POMC axon terminals, which leads to suppressed food intake and increased energy expenditure. By contrast, promotion of the activity of ARC NPY/AGRP neurons causes the release of AGRP, which antagonizes the effect of α-MSH (26).

Peripheral organs release hormones and nutrients that modulate the hypothalamic activity in order to maintain body homeostasis. Leptin is a hormone produced by the adipose tissue in proportion to fat storage, activates POMC anorexigenic neurons while inhibits NPY/AGRP orexigenic neurons, which results in the decrease of appetite. Insulin, released by pancreatic β-cells, also modulates hypothalamic activity. Insulin is released when there is an energy state, and it activates POMC anorexigenic neurons and inhibits NPY/AGRP orexigenic neurons.

From the GI, specifically from the stomach, the initiation of the meal increase the release of ghrelin that reaches the ARC and activates NPY/AGRP orexigenic neurons, promoting food intake (2).

The hypothalamus has a glucose-sensing capacity, so AGRP and POMC neurons are able to sense nutrients and there is evidence that there is reciprocal regulation (2).

In general, peripheral and metabolic signals, although they fluctuate within normal physiological parameters, have a direct influence in the hypothalamus, specifically in the ARC neurons, regulating hypothalamic neurocircuitry.

2.3 Hypothalamic inflammation

The brain is considered an immuno-privileged organ because the BBB restricts the access of several particles, such as immune cells. This characteristic limits the adaptive immune response (T and B cell activation, antigen presentation, among others) but the innate immune response is able to occur throughout the brain (toll like receptors, TLR), activating the main inflammatory transcription factor Nuclear Factor kappa β (NFkβ). An inflammatory stimulus in one cell type can originate cytokines and neuropeptides that may change the action of cells nearby (1).

The excess of peripheral and metabolic signals can lead to deregulation of the hypothalamic feeding circuits, which is implicated in the metabolic inflammation in the MBH. The hypothalamic dysfunction is a central characteristic in the development of obesity and, therefore the activation of inflammatory pathways in the hypothalamus have been investigated leading to its description in rats on a HFD regime. So, it has been described that in rodents exposed to HFD, after the first days of the diet there is activation of pro-inflammatory factors in the hypothalamus, such as cytokines,

TNF-α, suppressor of cytokine signaling 3 (SOCS3) and interleukins 1β (IL-1β) and IL-6 (1). It was also described that rats exposed to HFD for three days showed a complex pro-inflammatory gene expression"on-off-on" pattern, with elevated hypothalamic levels of Il6, TNFα, SOCS3, Ikbkb, and Ikbke mRNA.

Moreover, diet has certain components that directly activates TRL4 (Toll like receptor 4), for example saturated fatty acids, that on the other hand activates TNF-α, IL-1β and IL-6. Associated with TLR4 is the activation of IKKβ/NFkβ pathway, which is involved in the process of leptin and insulin resistance through SOCS3 activity (27). In obesity, SOCS3 activity is high and, therefore, leads to an increase of leptin activity that triggers leptin resistance (28).

It is described that HFD also triggers gliosis, which is the process of activation of microglia and astrocytes. The activation of microglial cells was shown by Iba1 (ionized calcium-biding adapter molecule 1) and the activation of astrocytes is detected by GFAP (glial fibrillary acidic protein) in the rat hypothalamus (1). In this study, Thaler, J.P., *et al.* observed that microglial number increased by day three of HFD exposure and remained high during two weeks.

2.4 Hypothalamic neurogenesis

Neurogenesis is a complex process that generates new neurons occurring in high rates during the embryonic state (24).

The CNS is a large network of interconnecting cells that form functional circuits. Neural stem cells (NSCs) are cells that generate the central nervous system (CNS) during embryonic development, leading to the formation of circuits, having the ability to generate neural and glia linages (29). With the regulation of several transcription factors, NSCs proliferate originating neuroprogenitor cells (NPCs), with a proliferative ability and maintaining an undifferentiating state. NSC differentiation can be seen as a two-step process, early differentiation - progenitors are first formed - and terminal differentiation - generation of neurons, astrocytes and oligodendrocytes.

In the adult brain, neurogenesis occurs in sub-ventricular zone (SVZ) and sub-granular zone (SGZ) of the hippocampus (24). NPCs generated in these specific brain regions then migrate to the olfactory bulb and the dentate gyrus, where they originate new neurons, integrating pre-existing circuits. Therefore, this process represents a functional response of the body in response to daily oscillations of state (24). Through the embryonic period, there is the development of hypothalamic feeding circuits. Some POMC neuronal progenitors give rise to neuronal populations expressing

NPY (30). Other cell populations also are generated in this period, such as ependymal cells (tanycytes) and cuboid ciliated cells that line the third ventricle (3V) (24).

Adult neurogenesis has been proven to exist in the hypothalamus and, thus, the hypothalamus contains NPCs with the ability to generate new neurons responsible for regulation of food intake (24). Therefore, a neurogenic niche in the hypothalamus is relevant in feeding circuits, not only because the proximity of hypothalamic NPCs to nutritional cues, but also because neurogenic process contribute to the physiological regulation of food intake and body weight (24).

2.5 Autophagy

Normal cellular development and growth require a balance between protein synthesis and degradation. Autophagy is a cellular degradation process for long-lived proteins and a highly conserved catabolic process essential to cellular survival. It is characterized by the sequestration of cytoplasm and excess organelles that are no longer useful for cellular activity, into double-membrane vesicles and delivered to the degradative organelle, the lysosome, for breakdown (31). In this way, the cellular structure and functions are maintained. Autophagy may imply cellular stress, particularly endoplasmic reticulum (ER) and oxidative stress, both of them are induced by over nutrition (3).

In rats exposed to HFD it was observed the increase of autophagosomes and the up-regulation of chaperones, which results from the ER stress (1). This suggest an attempt of defense strategy to HFD, although it is not enough to revert the effect of a continuous exposure. It has been described, after 20 weeks of HFD exposure the percentage of POMC neurons detectable with autophagosomes increased. POMC neurons are anorexigenic neurons, therefore have an important role in protection against obesity, because they potently reduce food intake through the production of biologically active peptides in a tissue-specific manner.

3. MicroRNAs

MicroRNAs (miRNAs) are single-stranded and natural non-coding RNAs, with approximately 22 nucleotides (*nt*), that regulate downstream and post-transcriptionally messenger RNA (mRNAs) by binding to a site in the 3' untranslated region (UTR) of target mRNAs (32).
The first two miRNAs that were known are *lin-4* and *let-7* were found in the nematode *Caenorhabditis elegant.* These molecules are highly conserved across species, being 60% of miRNA *loci* in common between mouse and human (33). The miRNAs are key players in diverse biological processes, such as stem-cell differentiation, heart development, insulin secretion, apoptosis, aging and immunity (34). With these biological regulation aspects, their involvement in several pathologies, including cancer and metabolic diseases.

3.1 - Biogenesis of microRNAs

The first step of the process of miRNAs occurs in the nucleus, where the primary miRNA (pri-miRNA) is located. This first transcript contains a hairpin structure with the mature miRNA, then being considered the precursor miRNA (pre-miRNA). In the nucleus takes place the transcription of miRNA transcription unit, by Drosha coupled to DGCR8 (DiGeorge syndrome critical region 8), a RNA polymerase type II, originating a double-stranded pre-miRNA (dsRNA). The function of DGCR8 is to ensure the proper position of Drosha's catalytic site, positioning the complex correctly for cleavage. The pre-miRNA previously formed is transported to the cytoplasm by Exportin 5 (XPO5), where is cleaved by Dicer (cytoplasmic RNase III) coupled with TRBP (trans-activation response RNA-binding protein). This cleavage originates a double stranded and imperfect miRNA, with approximately 21-24 *nt*. One of the strands (the "guide strand") from the duplex is transferred into Argonaute 2 (Ago2), which belongs to the complex Dicer/TRBP. The mature single stranded miRNA is incorporated into miRISC (miRNA-induced silencing complex). The miRNA-loaded miRISC regulates its target mRNAs through binding of complementary base pairs, therefore affecting translation and/or stability (33).
It has been shown that each tissue exhibits a particular miRNA expression profile, which implies that the same miRNA in a different tissue may have a different and specific function (35). It is important to refer that the expression of a one gene can be regulated by multiple miRNAs and a single miRNA can regulate the expression of hundreds of genes (36). And because a single miRNA can regulate the expression of several genes that are involved in a specific cellular mechanism makes miRNAs potent biological regulators.

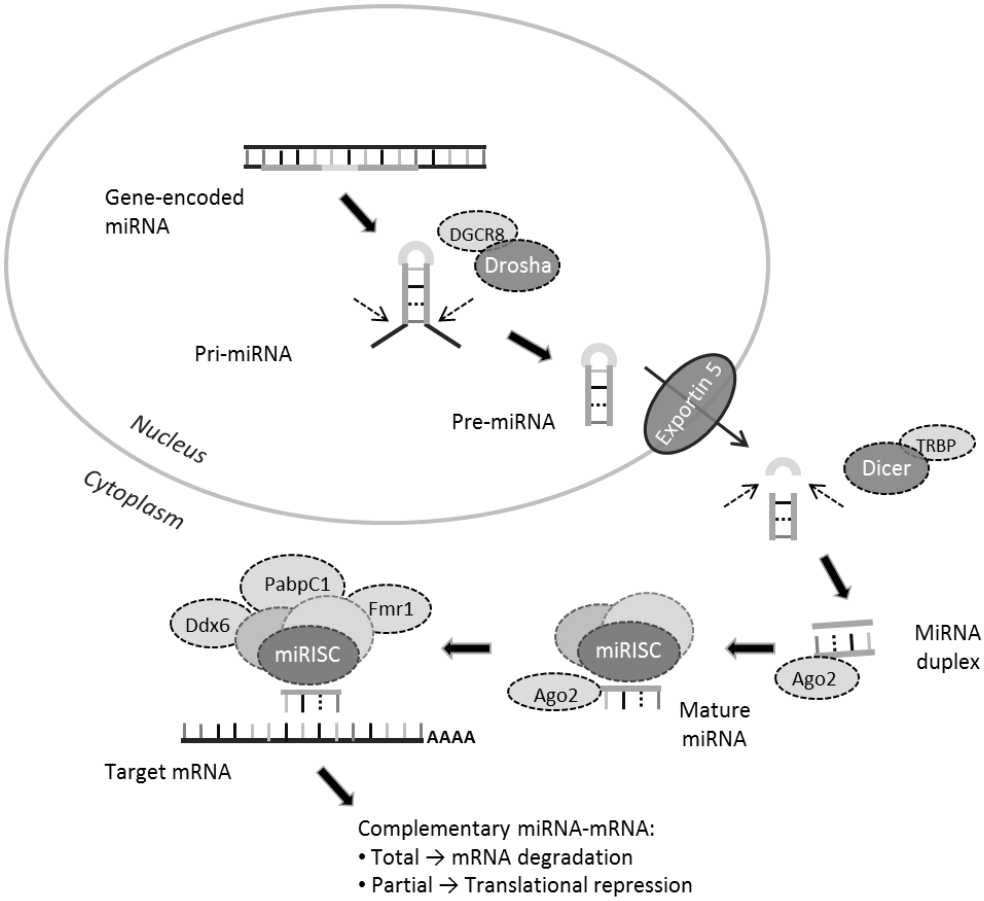


Figure 1.5 - MiRNA biogenesis process. Transcription and processing begin in the nucleus by Drosha coupled to DGCR8, leading to a pre-miRNA that is transported to the cytoplasm by XPO5. In the cytoplasm, pre-miRNA is processed by Dicer coupled to TRBP into a double stranded mature miRNA. Ago2 integrates the mature miRNA in the miRISC, where various proteins are present, such as Ddx6, PabpC1 and Fmr1. In the miRISC, miRNA binds to the target mRNA, by complementarity, and inhibits its processing. Adapted from Leal, 2015 (19).

3.2 - MiRNA & Metabolism

Since miRNA are molecules highly regulated, any dysfunction on the process of biogenesis can lead to cellular and physiological modifications, such as reduced protein synthesis and decrease mRNA levels. And also, miRNA regulates several and distinct cellular functions, therefore up or downregulation of miRNA expression alters a cell function. For example, miRNAs regulate metabolic processes, such like adipocyte differentiation and insulin, lipids and glucose metabolism (37-39).

3.2.1 - MiRNA & adipogenesis

Adipogenesis has many signalling pathways and miRNAs activating or inhibiting any of these signalling pathways are likely to affect this process. It was showed by others that stable overexpression of some miRNAs increases adipocyte differentiation *in vitro* (39).
Let-7 is one of the miRNAs related to clonal expansion. This microRNA is well known to regulate cell proliferation and differentiation processes. It is known that the levels of let-7 expression decrease from day 0 to day 1 and then increase during terminal adipogenesis (40).

3.2.2 - MiRNA and glucose & insulin metabolism

Diabetes *mellitus* is the most common metabolic disorder worldwide being characterized by elevated blood glucose levels, which can be attributable to one of the two hypothesis: a lack of insulin-producing pancreatic-cells (type 1 diabetes *mellitus*) or insulin resistance (type 2 diabetes *mellitus*). Circulating glucose modulates insulin production, therefore any change in the levels of circulating glucose can lead to an increase in glucose uptake in peripheral tissues, for example adipose tissue and muscle.
There are miRNAs present in pancreas responsible for regulation of insulin sensitivity in the peripheral tissues, through regulation of expression of many components of the insulin signaling pathway (36). Two scientific groups have recently shown that the let-7 family of miRNAs regulates glucose homeostasis and insulin sensitivity (41, 42). It was shown that global and pancreas-specific overexpression of let-7 in mice results in impaired glucose tolerance and reduced glucose-induced pancreatic insulin secretion. Let-7 directly targets components of the insulin-signaling pathway, such as Igf1r, Insr, Irs2, Pik3ip1, Akt2, Tsc1, and Rictor, therefore reducing insulin sensitivity (41, 42).

3.2.3 - MiRNA & lipids metabolism

Dyslipidemia is associated with metabolic syndrome and obesity. Recent studies showed that miRNAs are involved in the regulation of levels of lipoproteins in the plasma, such as mir-122, miR-27a/27b, miR-33/33, miR-378/378, miR-34a and miR-21. These miRNAs have significant impact on lipid homeostasis (43).

The miR-122 was the first miRNA to be correlated with the regulation of lipid metabolism and liver-enriched and liver-specific miRNA, being the most abundant miRNA in the liver (36). This miRNA has approximately 135 000 copies per human hepatocyte and its was demonstrated that the deletion of miR-122 leads to a decrease in total serum cholesterol and triglyceride (TG) levels, but increased hepatic steatosis and hepatic cancer (43).

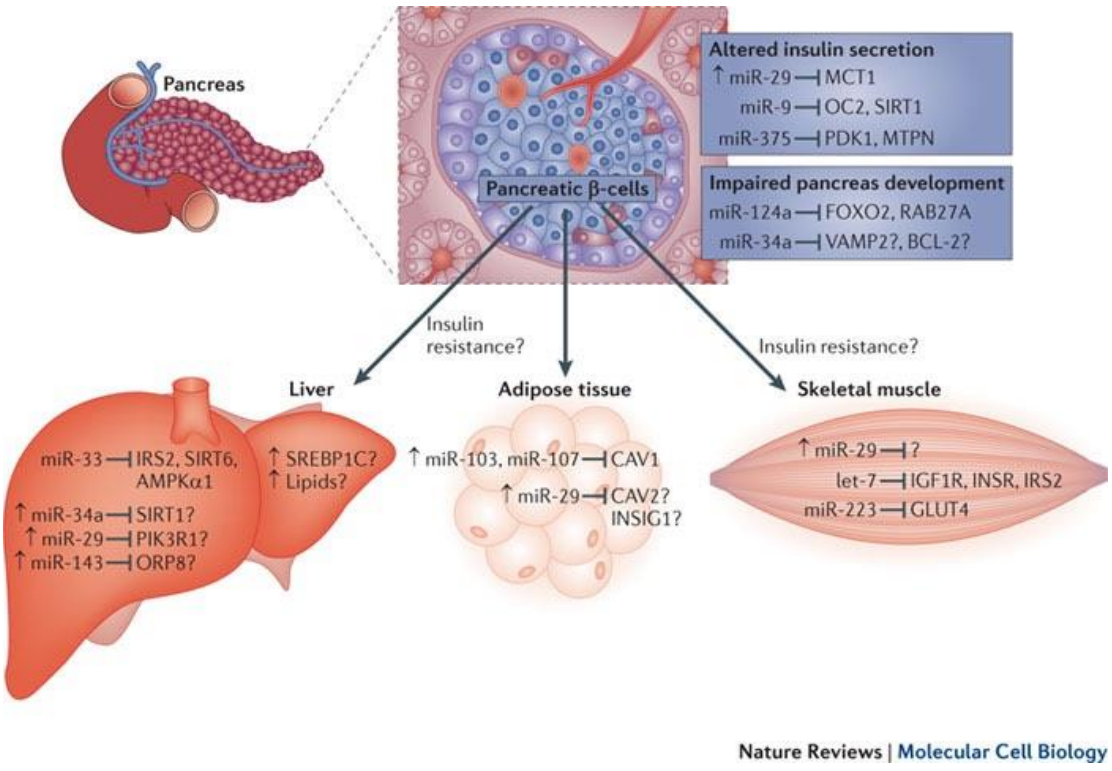


Figure 1.6 - MicroRNAs in metabolic disorders. After a meal, pancreatic β-cells release insulin to several tissues, such as liver, muscle, adipose tissue to cause uptake of glucose. FOXO2 - forehead box protein O2; RAB27A, VAMP2 - vesicle-associated membrane protein 2; BCL-2 - B cell lymphoma 2; MCT1 - monocarboxylate transporter I; OC2 - one cut homeobox 2; SIRT1 - sirtuin 1; PDK1 - phosphoinositide-dependent kinase 1; MTPN - myotrophin; AMPKα1 - AMP-activated protein kinase-α subunit 1; PIK3R1 - phosphatidylinositol 3-kinase subunit-α; ORP8 - oxysterol-binding protein-related protein 8; CAV1 - caveolin 1; INSIG1 - insulin-induced gene 1; IGF1R-(insulin-like growth factor receptor 1; INSR - insulin receptor; GLUT4 - glucose transporter type 4. Adapted from Rottiers and Näär, 2013 (44).

3.3 - MiRNA & Hypothalamus

Several components of miRNA biogenesis are correlated to the energy homeostasis and it is well documented that each tissue have certain miRNA expression. This suggests that miRNA shave specific functions depending on the tissue (35).

It is described that the anorexia mouse model *anx/anx* exhibit a tissue specific de-regulation of genes targeted by miRNAs (45). Also, conditional Dicer knock-out (KO) mice showed the importance of miRNA in the central nervous system, in detail, in neuronal development, differentiation and survival (46).

In the hypothalamus, the nutrient availability regulates the Dicer transcript expression, therefore, fasting up-regulates Dicer. Likewise, the deletion of Dicer in adult mice in the ARC caused hyperphagia and obesity (47). Several studies reported that the rodent hypothalamus have several miRNAs present, for example miR-124a, miR-125a, miR-136 miR-138, miR-338, miR-451, let-7c genes, miR7a and miR-7b. And because of Farh *et al*. findings, through high-throughput sequencing, miRNA expression profiling of the ARC of the rat hypothalamus revealed similar expression patterns.

4 - MicroRNA let-7 family

The miRNA lethal-7 (let-7) family was one of the first miRNA to be discovered and are characterized by being highly conserved though species. This miRNA family have 9 members.

The let-7 gene was discovered in *C. elegans*, having an essential role as a development gene because its main function is to regulate development timing and stage-specific neuromuscular tissue development (34). This microRNA has orthologs in several species, for example *C. elegans*, *D. melanogaster, D. rerio, G. gallus, H. sapiens, M. muscles* and *X. tropicalis.* And in these species, let-7 was found to have several copies in a genome. To distinguish the diversified isoforms of the miRNA, a letter is placed after let-7. This indicates that between different letters, there are differences in the sequence, and a number in the end shows that the same sequence is present in multiple genomic locations (33).

The size of miRNA let-7 family can vary between organisms in total number of genes; more complex animals will exhibit higher numbers. It is known that the miRNA let-7 family plays a role in mammals, despite not being completed understood. In the mouse, it is described that let-7 is pledge to neural lineage specificity of embryonic stem cells, brain development and mammary epithelial progenitor cell maintenance by induction of loss of self-renewal. For the humans, 12

genomic *loci* encode the let-7 family members and it was described that is unregulated in several cancers (34).

4.1 - Let-7 Biogenesis, Mechanism & Regulation

The biogenesis of let-7 is similar to other miRNAs. The first step occurs in the nucleus and it is mostly the transcription of pri-miRNA by Drosha coupled to DGCR8, a RNA polymerase type II, which originates a dsRNA. The second step of the miRNAs process happens in the cytoplasm when pre-miRNA is transported by XPO5 from the nucleus, followed by cleavage by Dicer. This cleavage originates a double stranded and imperfect miRNA.

One of the strands is transferred into Ago2, which belongs to the complex Dicer/TRBP and the mature single stranded miRNA is incorporated into miRISC. The miRNA-loaded miRISC regulates its target mRNAs through binding of complementary base pairs, therefore affecting translation and/or stability (33).

MiRISC is guided by miRNAs to perceive messenger RNA (mRNA) and to downregulate gene expression by one of the two mechanisms: translational repression or mRNA cleavage. The degree of miRNA-mRNA complementarity is the primary factor of the regulatory mechanism process. If the complementary degree is high, it enables the Ago-catalyzed degradation of target mRNA sequence through the mRNA cleavage mechanism process. If the complementary degree is low, it omits degradation and facilitates the translational repression mechanism (48).

When it comes to regulation of miRNA let-7, it is regulated in several stages of its biogenesis and it depends on cell type. In the same way, let-7 regulates various transcription factors with extensive roles in regulation of cell cycle, cell differentiation and apoptosis. There are factors that control let-7 expression and factors that are regulated by let-7 expression, lead to the formation of circuits. This circuits became networks patterns in development and differentiation (34).

4.2 - Let-7 & Metabolism

Let-7 miRNAs have been shown to have an extensive role in metabolism. It is recognized its expression in adipose tissue (38, 49) and glucose homeostasis (41).

During adipogenesis, several let-7 isoforms appear to be unregulated, as shown *in vitro* study by Tingwan Sun's team, using adipocyte cell line 3T3-L1. The differentiation of pre-adipocytes into mature fat cells involves several changes and steps well organized in gene expression. This scientific group found that let-7a, let-7b and let-7d are present in adipogenesis, but in specific let-7b was highly expressed in pre-adipocytes. In regard to let-7a, its expression regulates the transition from clonal expansion to terminal differentiation, as observed in *C. elegans (49)*. The authors also demonstrated that let-7 targets HMGA2 (high-mobility group AT-hook 2), a factor that modifies chromatin structure, related to low adipose tissue and oncogene expression when downregulated (34, 49).

MiRNAs have been shown to have important roles in several development processes but also in disease. For example, regulation of pancreatic insulin secretion and influence of insulin sensitivity in peripheral tissues. It has been described that mice overexpressing let-7 showed impaired glucose tolerance and also glucose-induced pancreatic insulin secretion. Therefore, miRNA let-7 controls peripheral glucose homeostasis.

4.3 - Let-7 & Hypothalamus

From a neurobiological perspective, the hypothalamus in the onset of puberty requires the upregulation of let-7 miRNAs for the maturation of development features, such as sexual differentiation of the brain.

Let-7 miRNAs are regulated by Lin28 protein present in the nucleus and cytoplasm. The regulation happens when Lin28 binds to the terminal loop region in pri-miR and pre-miR, therefore restricting the biogenesis of the miRNA.

It has been described that with the progress of puberty, the levels of Lin28b, an isoform of Lin28, decline, remaining low. Moreover, alteration in the let-7/Lin28 system in the hypothalamus leads to delayed puberty (18).

4.3.1 - Let-7 & Neurogenesis

Being neurogenesis the process by which neurons or nerve cells are generated in the brain, it is important to refer that neural stem cell self-renewal and differentiation give rise to neuronal and glial linages (50). This capacity of proliferation and self-renewal is regulated by a precise control of gene expression that involves nuclear receptor TLX (NR2E1 - Nuclear receptor subfamily 2 group E member 1). Studies done in the past came to the conclusion that let-7 promotes neuronal differentiation of stem cells by inhibiting TLX expression through its 3' UTR.
MiRNA let-7 regulates neural stem cell fate decision through reducing cell cycle progression, repressing TLX expression and its downstream effector, cyclin D1. Therefore, let-7 plays a crucial role in neural stem cell proliferation and differentiation across a range of development phases through targeting two component in one pathway (50).

4.3.2 - Let-7 & Inflammation

After triggering inflammation, NF-kB levels increased with IL6, which is a known target of let-7 through a direct binding.
Li *et al.* suggested that TLR4 signaling-triggered, tumor cell-released microparticles that somehow transfer let-7b to macrophages, leading to the down regulation of IL-6 expression (51). Therefore, these studies show that there is a negative feedback loop between let-7 and inflammatory factors (51).

4.4 - Let-7 and Cancer

Let-7 acts as a tumor suppressor by targeting oncogenes and other key components of cell cycle. It was described that let-7 is underexpressed in several cancers, for example lung and breast cancer, and that it may inhibit cancer growth (34). Also, in more differentiated tumor cells, let-7 expression is higher and its target oncogenes (HMGA2 and ras) are downregulated (34).
Because of its low expression levels in several cancers, let-7 is an attractive molecule for therapeutic purposes. It may be applied to preventing tumorigenesis and angiogenesis. Precisely, in breast cancer, let-7 when induced targets HMGA2 and H-ras inhibiting cell proliferation, mammosphere formation and the population of undifferentiated cells by downregulating both of the foregoing oncogenes (34).

Although the restoration of expression levels of let-7 was proven beneficial, the possible therapeutic application has certain limitations. The preeminent limitation is the narrow knowledge regarding transcriptional and processing control during biogenesis and also its explicit role in tumorigenesis. Another determining component is the delivery method. The strategy to overcome this, biological vectors, such as lentivirus, may be used for targeted delivery (34).

5. Previous Work

Previous work (19) performed in our laboratory concerning this experiment is summarized in Table 1. First, let-7 expression levels in the mouse hypothalamus were measured and it was observed that let-7 levels decrease in the hypothalamus of mice fed with HFD (HFD+miR-neg group) but its expression increased in HFD+let-7 mice when compared to HFD+miR-neg mice, showing that the lentiviral injection was successful.

Several metabolic aspects were evaluated in order to assess whether hypothalamic let-7 overexpression could prevent obesity in mice. In HFD+let-7 mice, body weight gain decreased in about 29%, epididymal white adipose tissue (as % of body weight) decreased in about 40% and brown adipose tissue (as % of body weight) was not changed when compared to HFD+miR-neg mice. Moreover, total food intake and glucose tolerance test decreased in HFD+let-7 group when compared to HFD+miR-neg group. For the fasting glucose levels and total activity, there were no differences between HFD+let-7 mice when compared to HFD+miR-neg mice.

Lastly, mRNA levels of inflammation markers IL1β and IL6 were measured in the mice hypothalamus. HFD+miR-neg mice showed increased levels for both makers when compared to mice fed with chow diet; and with the microRNA let-7 overexpression, their levels decreased in 36% and 37%, respectively, when compared to HFD+miR-neg.

Table 1 – Resume of the results performed in our laboratory concerning this experiment. (19)

Parameter Evaluated	**HFD+miR-neg (compared to Chow)**	**HFD+let-7 (compared to HFD+miR-neg)**
Let-7 Expression Levels *(in the hypothalamus)*	↓ (23%)	↑ (19%)
Body Weight Gain (% of initial weight)	↑↑ (151%)	↓ (29%)
Epididymal White Adipose Tissue (% of body weight)	↑ (138%)	↓ (40%)
Brown Adipose Tissue (% of body weight)	=	=
Total Food Intake (Kcal/mice)	↑ (85%)	↓ (9%)
Fasting glucose (mg/dL)	↑ (20%)	=
Glucose Tolerance Test (AUC)	↑ (46%)	↓ (14%)
Total activity (control units)	=	=
Inflammation markers mRNA levels *(in the hypothalamus)*	↑ IL1β (56%) ↑ IL6 (44%)	↓ IL1β (36%) ↓ IL6 (37%)(

6. Objectives

Obesity causes cellular injury in the hypothalamus, a region of the brain responsible for maintenance of body homeostasis and feeding behaviour.

MicroRNAs are molecules involved in several different biological processes and their expression levels are modified in distinct pathologies, making them an interesting target to study. But, however, miRNAs function in the hypothalamus is not yet fully understood and obesity-induced metabolic and neuropathological variations have not been investigated thoroughly.

Previous studies in our laboratory affirmed that let-7 is involved in metabolism, neurogenesis and inflammation, given that there are alterations in let-7 microRNAs expression levels in the hypothalamus of rats in HFD regime.

Taking into account these data, the major aim of this project was to investigate the putative protective effect of let-7 in obesity associated metabolic alterations and hypothalamic dysfunction. To achieve the above mentioned goals, this work was divided into two segments: investigate if let-7 microRNA can prevent peripheral alterations induced by obesity in mice and explore the possibility of let-7 microRNA prevent hypothalamic injury induced by obesity in mice.

To achieve our goal, let-7 or control microRNA (miR-neg) were over-expressed in the mouse hypothalamus using lentiviral vectors and the mice were exposed to high-fat diet (HFD) for 7 weeks. And to assess the putative beneficial role of let-7 in peripheral metabolic alterations, certain parameters were evaluated, including body weight, food consumption, glucose tolerance, serum cholesterol, triglycerides and hormones levels, adipose tissue weight and adipocytes diameter and liver steatosis. Concerning hypothalamic injury, immunohistochemistry technique was used to assess neuro-inflammation, impairment of neurogenesis and alterations of neuropeptides levels.

Chapter II:
Materials and Methods

2. *In vivo* experiments

All experiments procedures were performed in accordance with the European Union Directive 86/609/EEC for the care and use of laboratory animals. Animals were housed in a licensed animal facility and the Centre Of Neuroscience and Cell Aging animal experimentation board approved the use of animals for this project. People coordinating the animals studies have received appropriate education (FELASA course) as required by the Portuguese authorities.

2.1 Experimental animals

Male C57BL/6 mice was purchased from Charles River Laboratories and randomly divided into three groups. Control mice were fed normal chow diet (8% fat) and the other two groups were fed a 40% fat diet (LabDiet - Western diet for rodents) for 7 weeks. The mice were housed in a temperature/humidity controlled room with *ad libitum* access to water and food.

2.2 Layout of the *in vivo* experiment

Plasmids with a precursor microRNA let-7 and a control microRNA (miR-neg) were constructed for this experiment and cloned into a lentiviral backbone. The control microRNA has a random sequence validated to not target any rat, mouse or human gene. The let-7 precursor or the miR-neg were inserted downstream of H1 promoter, along with a reporter gene GFP (Green Fluorescent Protein) expressed under the control of PGK (Phosphoglycerate Kinase 1) promoter (Figure 2.1A).
Lentiviral particles were produced in HEK 293T cells using a four plasmid combination (52). Afterwards, lentiviral particles to overexpress the microRNAs were delivered bilaterally to the mouse mediobasal hypothalamus by means of stereotaxic injection (Figure 2.1B).
Three distinct mice groups were constituted: chow group - control mice fed chow diet with no stereotaxic injection; HFD+miR-neg group - mice fed with HFD with control microRNA overexpression in the hypothalamus; HFD+let-7 group - mice fed HFD with let-7 microRNA overexpression in the hypothalamus. HFD+miR-neg and HFD+let-7 were submitted to HFD for 7 weeks, starting 1 week after surgery, and then sacrificed (Figure 2.1C). At the time of sacrifice, tissues were collected for further experimentation.

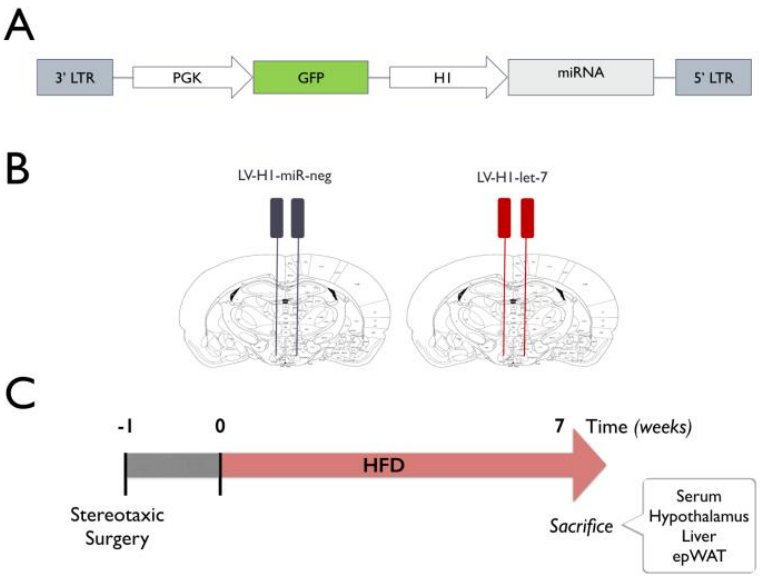


Figure 2.1 - Overexpression of microRNA let-7 in the mouse hypothalamus. Schematic representation of the plasmids used for overexpression of let-7 microRNA (H1-microRNA let-7) and control microRNA (H1-microRNA miR-neg) (A). Plasmids were delivered bilaterally to the mouse mediobasal hypothalamus by means of stereotaxic injection (B). Layout of the *in vivo* experiment indicating the tissues that were collected at the time of sacrifice (C).

2.4 Delivery of lentiviral vectors

Lentiviral particles were produced in HEK 293T cells using a four plasmid combination, as described before (52). Male C57BL/6 mice were anesthetized, placed on a stereotaxic frame and injected with 2.5 μL of lentiviral suspension in each hemisphere. Lentiviral particles were prior diluted in 1% BSA in PBS to comprise 400 ng of p-24 antigen. Injection was performed bilaterally into the medial hypothalamus following the coordinates:1.65 mm posterior to bregma; -0.35 mm/+0.35 mm lateral to bregma and 6.00 mm ventral to the brain surface.

2.5 Animal sacrifice

Ad libitum fed mice were anesthetized with a lethal dose of anesthetic (Pentobarbital). Blood samples were collected and mice were perfused transcardially with 4% paraformaldehyde (PFA). After that, the brain, liver and epididymal white adipose tissue samples were collected and processed as described in the following sections.

2.6 Tissue Analysis

2.6.1 Serum analysis

Blood samples were centrifuged for 15 minutes, 100 rpm, at 4ºC. The supernatant (serum) was collected and stored at -20ºC. ELISA kits were used to measure serum levels of leptin (Mouse Leptin, 96-Well Plate Assay, Cat. # EZML-82K) and insulin (Rat/Mouse Insulin, 96 Well Plate Assay, Cat. # EZRMI-13K), according to the manufacturer instructions. Triglycerides and total cholesterol were assessed in an external clinical laboratory.

2.6.2 Liver and WAT Coloration

For evaluation of liver and epWAT histology, PFA-fixed samples were paraffin-embedded sectioned 3 µm sections and stained by regular hematoxylin-eosin (H&E) methodology. With the protocol used, hematoxylin has a deep blue-purple color and stains nuclei acids by complex, incompletely understood reaction; eosin is pink and stains proteins nonspecifically. In a typical tissue, nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining.

2.6.3 Quantification Criteria for epWAT

Two fields per animal were randomly selected and the area of every adipocyte was measure by outlining the adipocyte cytoplasmic membrane using AxioVision software. The values were organized in two distinct graphics: a graphic of area distribution, where adipocytes area are organized by animal group, and a second graphic with the number of adipocytes per area interval (five different intervals: <2500 μm^2; 2500-5000 μm^2; 5000-7500 μm^2; 7500-10000 μm^2 and >10000 μm^2).

2.7 Immunohistochemical (IHC) analysis

Mice brains were post-fixed in PFA overnight, at 4ºC, infiltrated with 30% sucrose in PBS at 4ºC overnight and stored at -80ºC. Brains were sectioned at 30 µm in the coronal plane using a cryostat (Leica), collected to 0.1% azide in PBS and stored at 4ºC.
Antigen unmasking was performed by boiling the sections in antigen retrieval solution [Tris-EDTA solution, 302.5 mg Tris-Base (LA1_Trizma #8) and 92.5 mg EDTA (LA1_Sigma-Fluka (03620)] for 15 minutes at 80ºC, followed by cooling at room temperature for, at least, 1hour. Immunohistochemistry was performed on brain sections using the following primary antibodies: rabbit anti-Iba1 (1:1000), rabbit anti-GFAP (1:1000; Dako), rabbit anti-AgRP (1:1000; Phoenix Pharmaceuticals), mouse anti-SOX2 (1:100; R&D Systems), chicken anti-POMC (1:1000; Abcam), mouse anti-HSP70 (1:100; Enzo) and rabbit anti-NPY (1:6000; Sigma-Aldrich). Brain coronal sections were blocked in PBS with 10% newborn goat serum (NGS; Gibco), 0,1% Triton and 3% bovine serum albumin (BSA) and then incubated in primary antibody overnight at 4ºC. For immunofluorescence antibody detection, sections were the incubated with secondary antibodies Alexa Fluor 568, Alexa Fluor 594 or Alexa Fluor 647 labeled anti-rabbit, anti-goat or anti-mouse at a 1:500 dilution (Invitrogen) and DAPI (nuclear marker), for 2 hours at room temperature.
Brain sections were observed on Imager Z2 Microscope (Zeiss).

2.7.1 Hypothalamic immunohistochemical analysis

Using the Paxino's Mouse Brain Atlas, the ARC was identified in the sections to use for all the IHCs. Three sections of approximately 240 µm spacing were chosen between Bregma -0.58 mm to -2.65 mm. For each marker evaluated, sections of all the mice groups were subjected to immunohistochemistry procedure in the same day and image acquisition was performed using the same exposures.
The observer was blinded to the identity of the sections at the time of counting. Quantification was performed bilaterally for every section belonging to each animal.
For the microglial cells marker Iba1, cell number was counted manually having in consideration the presence of nuclear marker DAPI, and by visually counting Iba1 positive cell bodies within specific regions of interest (ROIs) (shown in the respective figures) using Image J software. Still for this marker, microglial cell size was also determined for the 10 biggest cells of each field, by manually delimiting the cell body, as described before (1). This was achieved through the use of Image J software.

Regarding neuropeptide POMC, cell number was counted manually having in consideration the presence of nuclear marker DAPI, and by visually counting POMC positive cell bodies within specific ROIs (shown in the respective figures) using Image J software.

As for SOX2, two sections *per* animal were selected and quantified. Cell number was counted manually having in consideration the presence of nuclear marker DAPI, and by visually counting SOX2 positive nuclei within specific ROIs (shown in the respective figures) using Image J software. The immunoreactivity levels were also evaluated through densitometric quantification using Image J software.

Concerning AGRP, GFAP and NPY markers, quantification of immunoreactivity levels was performed through densitometric quantification using Image J software. Average immunoreactivity for each group is shown as percentage of HFD+miR-neg immunoreactivity.

3. Statistical analysis

All results are expressed in mean ±SEM. Statistical analysis as performed using GraphPad PRISM software. Unpaired student's T-test was used for comparison of mean values between 2 groups and One-way ANOVA was used for comparison of mean values of more than 2 groups. For the analysis of number of adipocytes *per* area interval, group differences were assessed by one-way ANOVA; for the remaining analysis, Unpaired student's T-test was used. All P-values less than 0.05 were considered significant.

Chapter III:
Results

3.1.Previous Experiments and Results

Previous work was performed in our laboratory concerning this study. Three distinct mice groups were constituted: chow group, HFD+miR-neg group and HFD+let-7 group. HFD+miR-neg and HFD+let-7 groups were submitted to HFD for 7 weeks, starting 1 week after surgery, and then sacrificed. At the time of sacrifice, tissues were collected for further experimentation.

The first results obtained by our group, and not included in this thesis, focused on the evaluation of the impact of let-7 overexpression on metabolic characterization and, comparing to HFD with a scramble microRNA (HFD+miR-neg), we observed the following results: body weight gain, epididymal adipose tissue content increase, total food intake increase, glucose tolerance test (GTT) decrease, with no change on brown adipose tissue content and fasting glucose levels. Moreover, we evaluated the mRNA levels of inflammatory markers IL-1β and IL-6 in the mice hypothalamus. We observed an increase on the levels of IL-1β and IL-6 in the hypothalamus of HFD+miR-neg mice when compared to mice fed with chow diet.

In this thesis we further characterized the impact of let-7 overexpression in the hypothalamus of mice fed with HFD.

3.2. Peripheral metabolic alterations

3.2.1. Biochemical parameters

To further evaluate the effect of let-7 overexpression in the mouse hypothalamus, other peripheral metabolic alterations were assessed including determination of hormones levels and other biochemical parameters in the serum (Figure 3.1). Histological differences in epididymal white adipose tissue (epWAT) (Figure 3.2) and in the liver (Figure 3.3) were also determined.

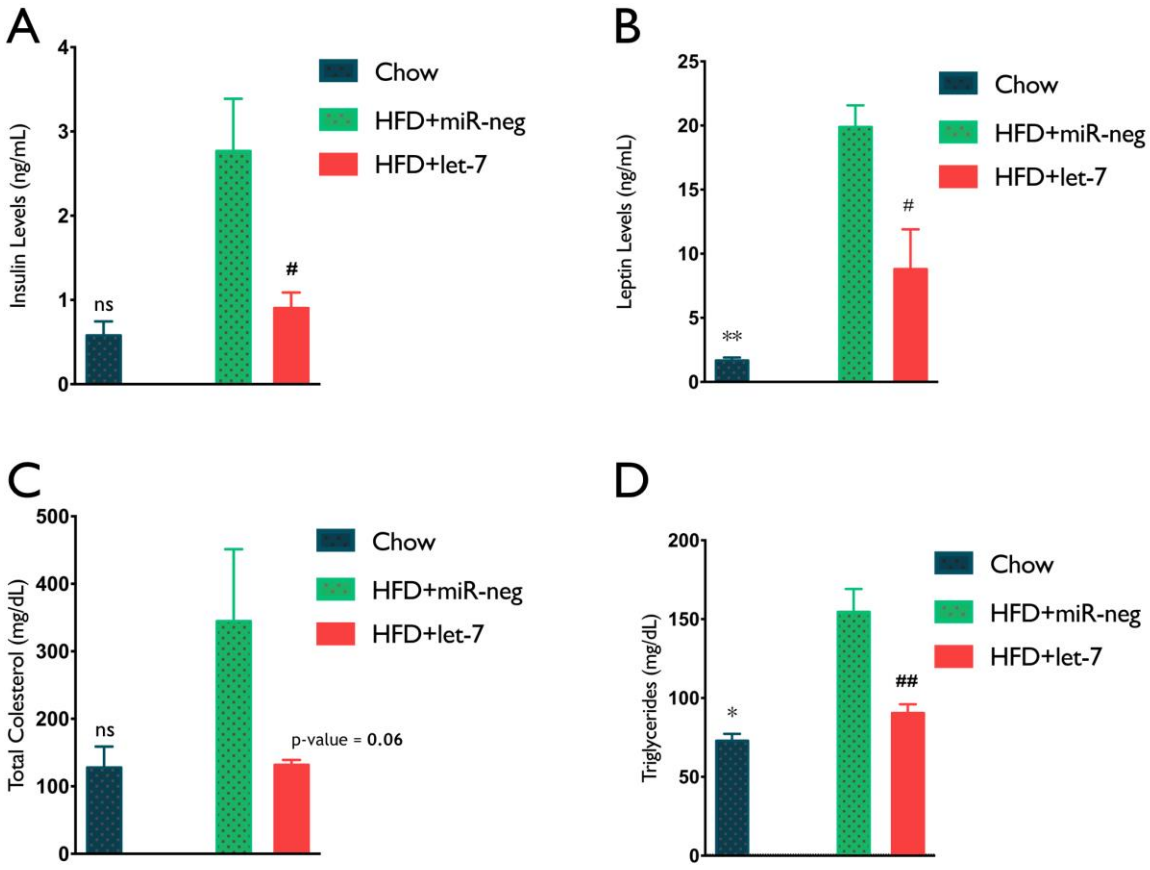


Figure 3.1 - Alterations of biochemical parameters in mice overexpressing let-7 in the hypothalamus. Serum levels of (A) insulin, (B) leptin, (C) total cholesterol and (D) triglycerides of mice fed with chow diet or HFD and with control microRNA overexpression in the hypothalamus (HFD+miR-neg) or HFD with let-7 microRNA overexpression in the hypothalamus (HFD+let-7). Unpaired T-test; Chow compared with HFD-let-7; ns $p>0.05$; * $p<0.05$; ** $p<0.01$; HFD+let-7 compared with HFD+miR-neg; # $p<0.05$; ## $p<0.01$.

As expected, the levels of insulin and leptin increased in mice fed HFD with control microRNA overexpression in the hypothalamus (HFD+miR-neg) (Figure 3.1A and 3.1B). In the mice group HFD+let-7, insulin and leptin levels were 50%, 67% and 55% lower, respectively, when compared to HFD+miR-neg. Insulin hormone levels almost returned to the levels that mice fed chow diet presented, as opposed to leptin hormone levels.

As expected, the levels of total cholesterol and triglycerides increased in mice fed high fat diet with control microRNA overexpression in the hypothalamus (HFD+miR-neg) (Figure 3.1C and 3.1D). Overexpression of microRNA let-7 decreased the total cholesterol and triglycerides levels, 61.0% and 41.5%, respectively, almost to chow group levels when it comes to total cholesterol.

3.2.2. Adipocytes Area

As individuals become obese, their adipocytes enlarge in area (11) and the volume of white adipose tissue increase (49). epWAT samples were analysed and the adipocytes area were determined using using (H&E) staining (Figure 3.2, A-C).
Concerning the area of adipocytes (Figure 3.2D), there was a significantly decrease in adipocyte area average in the mice fed with HFD with Let-7 microRNA overexpression in the hypothalamus (HFD+let-7) when compared to HFD+miR-neg. Regarding the number of adipocytes with different areas (Figure 3.2E), there was significant difference between mice fed with HFD with control microRNA overexpression in the hypothalamus (HFD+miR-neg) or let-7 microRNA overexpression in the hypothalamus (HFD+let-7).

3.2.3. Liver Histology

Accumulation of lipids in the cytoplasm of hepatocytes is a consequence of obesity and HFD consumption (8). In this experiment, liver tissue slices were stained with H&E. Lipidic droplets using H&E staining in paraffin slices appear as non-stained droplets.
Comparing liver slices obtained from mice fed with HFD with control microRNA overexpression in the hypothalamus (HFD+miR-neg) (Figure 3.3B) with liver slices obtained from mice fed with HFD with Let-7 microRNA overexpression in the hypothalamus (HFD+let-7) (Figure 3.3C), we observed lower levels of lipid droplets in one animal (of a total 3 animals) of the HFD+let-7 group (Figure 3.3E) when compared to HFD+miR-neg (Figure 3.3D).

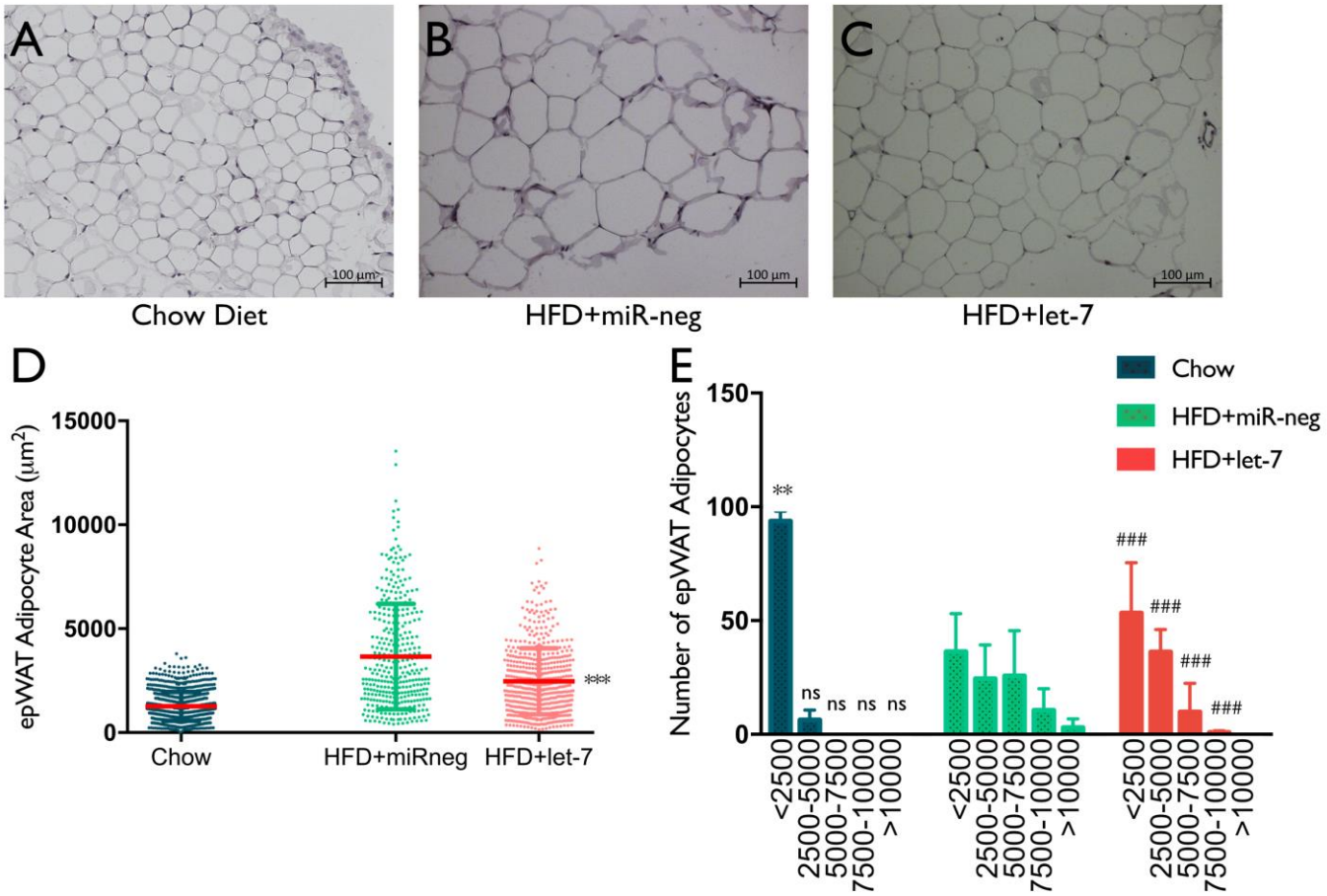


Figure 3.2 - Histological analysis of the mouse white adipose tissue (WAT). (A-C) Representative images of histological analysis of epWAT stained with H&E staining of mice fed with chow diet (A) or HFD with control microRNA overexpression in the hypothalamus (HFD+miR-neg) (B) or HFD with let-7 microRNA overexpression in the hypothalamus (HFD+let-7) (C). (D) Epididymal WAT adipocyte area distribution and (E) number of adipocytes per area interval. (n=3 mice per group). (D) Unpaired t-test; HFD+let-7 compared to HFD+miR-neg; *** $p<0.001$. (E) Unpaired t-test; Chow compared to HFD+miR-neg; ** $p<0.0001$; One-way ANOVA; HFD+let-7 compared to HFD+miR-neg; ### $p<0.0006$.

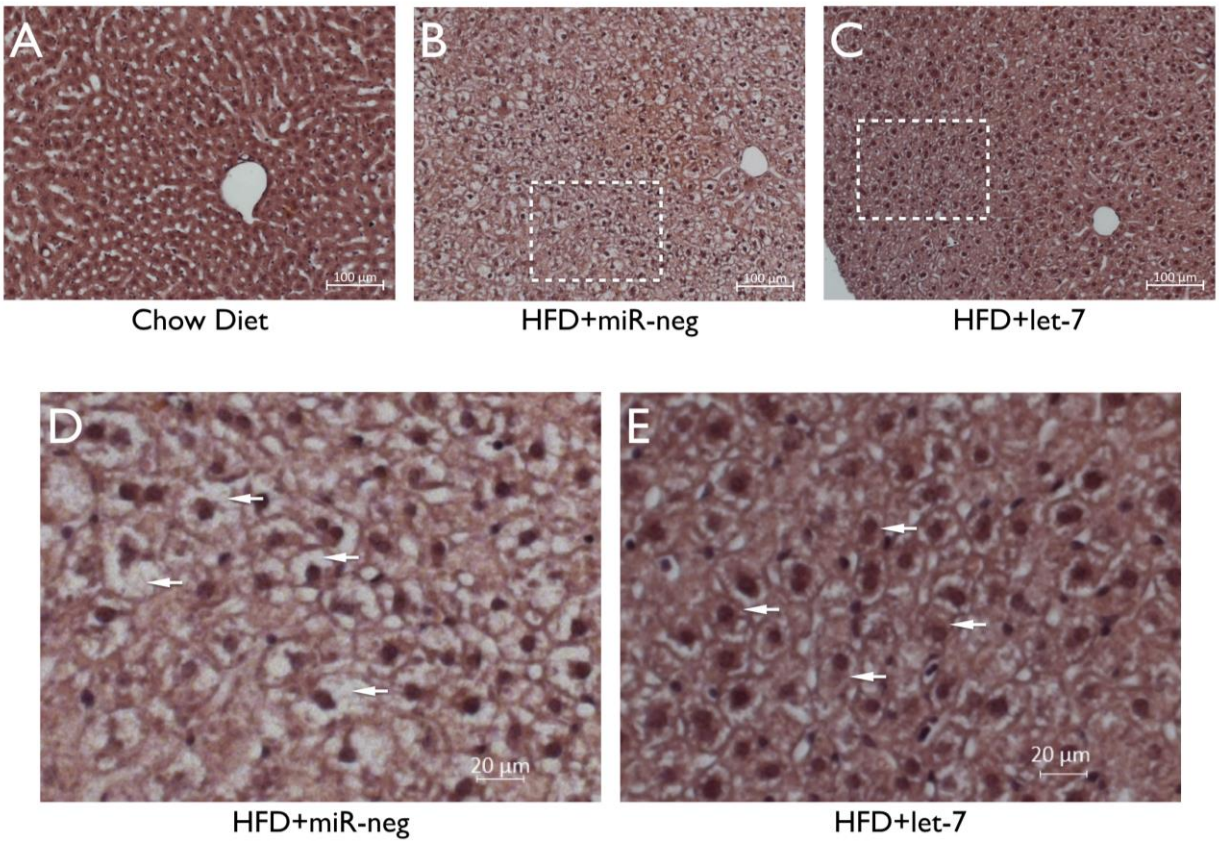


Figure 3.3 - Histological analysis of the mouse liver. Representative images of histological analysis of liver samples stained with H&E of mice fed mice fed with chow diet (A) or HFD with control microRNA overexpression in the hypothalamus (HFD+miR-neg) (B) or HFD with Let-7 microRNA overexpression in the hypothalamus (HFD+let-7) (C). The dashed box indicates the region where the higher magnification view was made. Higher magnification view of liver histology from (D) HFD+miR-neg and (E) HFD+let-7. White arrows indicate liver cells that had lipid drops accumulation in their cytoplasm. Lower levels of lipid droplets were observed in some mice of the group HFD+let-7.

3.3. Alterations in the hypothalamus

HFD causes a rapid onset of inflammation and reactive gliosis in the hypothalamus of mice (1). In order to evaluate the possible beneficial effects of let-7 overexpression in this process, we determined the levels of glial fibrillary acidic protein (GFAP; gliosis marker) (Figure 3.4) and Iba1 (microglia marker) (Figure 3.5) in the mice hypothalamus.

3.3.1 Astrocyte changes in the hypothalamus

It was previously reported an increase of intensity of GFAP reactivity and the formation of a dense fibrous network of astrocytic processes when mice were fed with HFD (1).
Therefore, we used GFAP immunohistochemistry as a marker of astrocytes in the mouse ARC and evaluate whether let-7 overexpression could prevent astrocytes activation in mice fed with HFD (Figure 3.4). We observed that the immunoreactivity levels of GFAP (Figure 3.4G) were similar in the three group of animals.

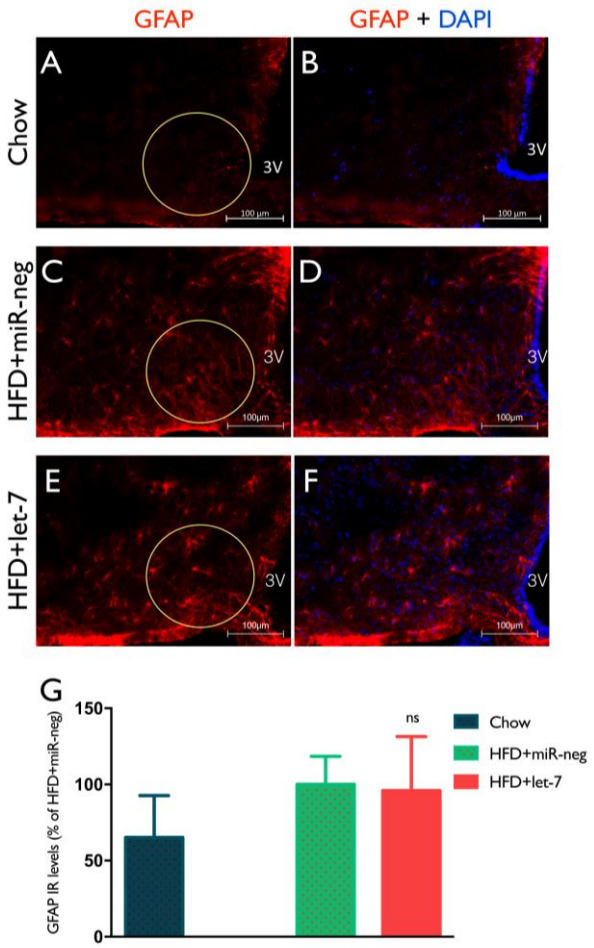


Figure 3.4 - Immunohistochemistry analysis of glial fibrillary acidic protein (GFAP) in mouse hypothalamus. (A-F) Representative images of astrocytes detected by GFAP protein staining in coronal sections of mice arcuate nucleus from animals fed with chow diet or HFD with control microRNA overexpression in the hypothalamus (HFD+miR-neg) (C-D) or HFD with let-7 microRNA overexpression in the hypothalamus (HFD+let-7) (E-F). The yellow line circles indicate the area used for GFAP immunoreactivity quantification. (G) Quantification of GFAP immunoreactivity levels (n=4-5 per group). Unpaired T-test; HFD+let-7 compared HFD+miR-neg; ns $p>0.05$. 3V, third ventricle. DAPI, nuclear staining. IR, immunoreactivity.

3.3.2. Microglia changes in the hypothalamus

It was previously described that, microglial cell number increased in the ARC of mice exposed to HFD when compared to mice fed with chow diet and also that microglial cells enlarged (increased body cell size) and adopted a more activated morphology (1).

We used immunohistochemistry to evaluate microglia-specific cytoplasmic marker Iba1 (Figure 3.5). We observed a decrease of 21% in the number of $Iba1^+$ cells (microglial cells; Figure 3.5J) in the hypothalamus of animals fed with HFD with Let-7 overexpression in the hypothalamus (HFD+let-7) (Figure 3.5G and 3.5H) and animals fed with HFD with control microRNA overexpression in the hypothalamus (HFD+miR-neg) (Figure 3.5D and 3.5E). Likewise, we observed a lower microglial cell size (Figure 3.5K) in HFD+let-7 group compared to HFD+miR-neg group.

As for microglia morphology, comparing mice fed chow diet (Figure 3.5C) with mice fed HFD+miR-neg (Figure 3.5F), we observed that microglia from HFD+miR-neg mice have larger cell bodies with thickened and shortened processes, when compared to comparing to mice fed chow group (Figure 3.5, C and F). However, in HFD+let-7 mice microglia cells became more rounded and with fewer processes (Figure 3.5I).

Together, this data suggest that overexpression of microRNA let-7 is favorable in diminishing the activation of microglial cells in the hypothalamus.

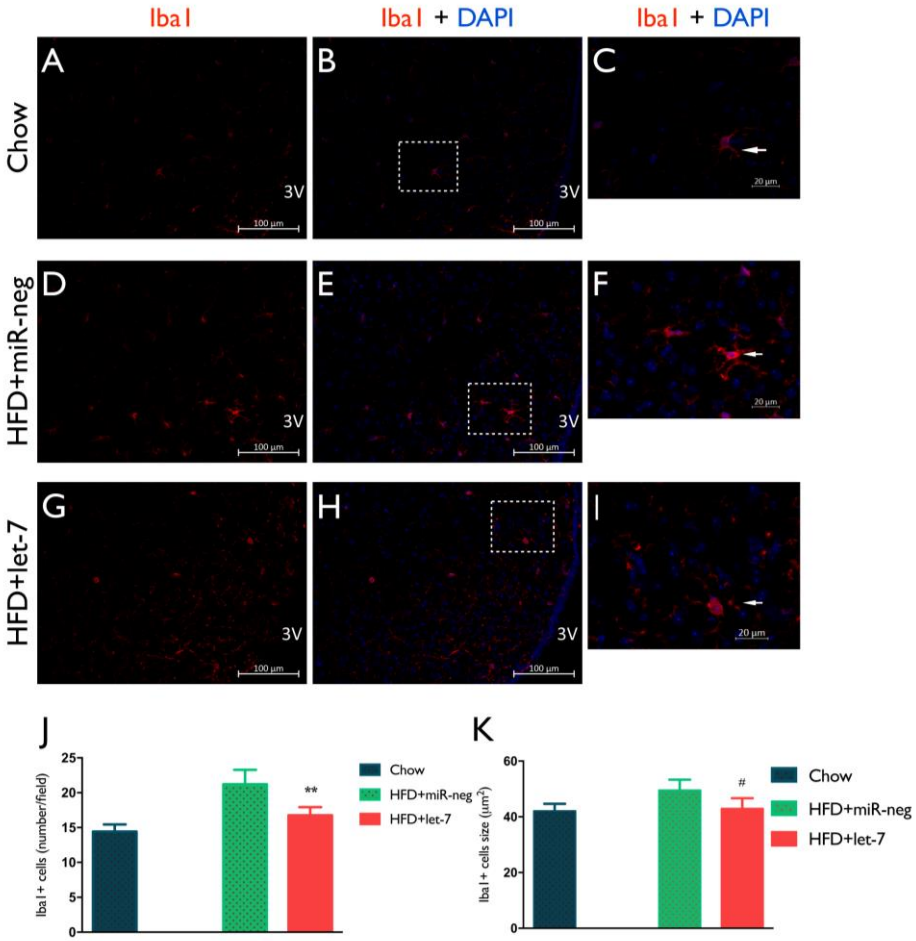


Figure 3.5 - Immunohistochemical analysis of microglia marker Iba1 in the mouse hypothalamus. (A-B; D-E; G-H) Representative images of Iba1 immunohistochemistry staining in coronal sections of mice arcuate nucleus from animals fed with chow diet or HFD with control microRNA overexpression in the hypothalamus (HFD+miR-neg) (D-E) or HFD with let-7 microRNA overexpression in the hypothalamus (HFD+let-7) (G-H). The dashed box indicates the region showed in high magnification. (C; F; I) Higher magnification of ARC (C) chow mice, (F) HFD+miR-neg mice and (I) HFD+let-7 mice. (J) Quantification Iba1 positive cells number and (K) cell size from the same group of animals (n=4-5 per group). Unpaired T-test; HFD+let-7 compared with HFD+miR-neg; # $p<0.05$; ** $p<0.01$. 3V, third ventricle. DAPI, nuclear staining.

3.3.3. Alterations of neuropeptides levels

To determine if alterations on hypothalamic neuropeptides levels were responsible for the metabolic alterations in mice overexpressing let-7 microRNA in the hypothalamus, we evaluated the cell number or immunoreactivity of neuropeptides AGRP (Figure 3.6), NPY (Figure 3.7) and POMC (Figure 3.8).

3.3.3.1 AGRP levels in the hypothalamus

The Agouti-Related Protein (AGRP) is an orexigenic peptide that increases food intake, being an important modulator of energy balance (21). To determine possible alterations of AGRP levels upon overexpression of the microRNA let-7, we used immunohistochemistry to evaluate AGRP in the ARC of mice (Figure 3.6) We observed that there was no significant difference on the levels of AGRP immunoreactivity levels in the hypothalamus of mice from the three groups (Figure 3.6).

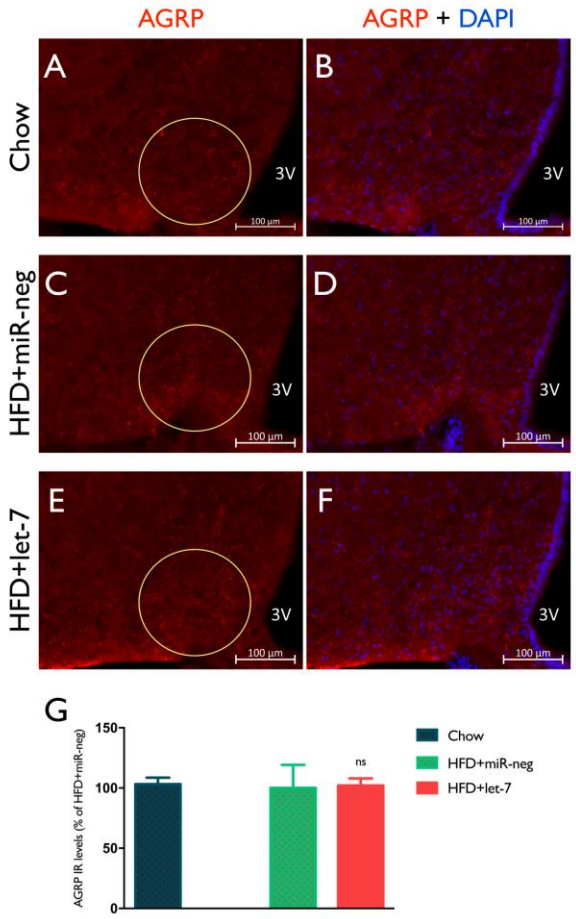


Figure 3.6 - Immunohistochemical analysis of neuropeptide agouti-related protein (AGRP) in the mouse hypothalamus. (A-F) Representative images of neuropeptide AGRP in coronal sections of mice arcuate nucleus from animals fed with chow diet or HFD with control microRNA overexpression in the hypothalamus (HFD+miR-neg) (C-D) or HFD with let-7 microRNA overexpression in the hypothalamus (HFD+let-7) (E-F). The yellow line circles indicate the area used for quantification of AGRP immunoreactivity. (G) Quantification of AGRP immunoreactivity levels from the same group of animals (n=4-5 per group). Unpaired T-test; HFD+let-7 compared HFD+miR-neg; ns $p>0.05$. 3V, third ventricle. DAPI, nuclear staining. IR, immunoreactivity.

3.3.3.2 NPY levels in the hypothalamus

The Neuropeptide Y (NPY) is an orexigenic peptide that plays a role in regulating energy homeostasis. To determine possible alterations of NPY levels upon overexpression of let-7 microRNA, in this experiment, we used immunohistochemistry to detect NPY in the ARC of mice (Figure 3.7.) We observed that there was no significant difference on the levels of AGRP immunoreactivity levels in the hypothalamus of mice from the three groups (Figure 3.7G).

3.3.3.3 POMC and neuronal injury marker HSP70 analysis in the hypothalamus

It was previously known that HFD causes a decrease in the number of POMC neurons in mouse hypothalamus (1). We observed that HFD+let-7 animals showed an increase of POMC neurons number (Figure 3.8J) roughly of 18%, as compared to HFD+miR-neg group animals. This increase of POMC neurons number suggests that let-7 microRNA could prevent the loss of these neurons.
Moreover, using immunohistochemistry, we evaluated the levels of Heat Shock Protein 70 (HSP70) immunoreactivity. HSP70 is a component of the neuroprotective response to neuron injury which was previously reported to be increased in HFD-fed mice comparing to chow-fed mice (1). In the hypothalamus of mice submitted to HFD for 7 weeks (HFD+miR-neg) we observed staining of HSP70. This is in accordance to previous work showing HSP70 immunoreactivity in the hypothalamus of mice fed with HFD for 7 days but not in the chow group (1). It suggests that neurons in the ARC are undergoing neuronal injury. In the HFD+let-7 group, there was no staining for HSP70 (Figure 3.8E), suggesting a protective role of let overexpression against neuronal injury in the hypothalamic neurons of these mice.
Moreover, in the HFD+miR-neg group there were POMC-positive cells expressing HSP70 protein (Figure 3.8, C and D), suggesting that POMC neurons may undergo neuronal injury due to HFD. Interestingly, there were no POMC-positive cells expressing HSP70 protein in the HFD+let-7 group, suggesting that let-7 overexpression protect POMC neurons from neuronal injury induced by HFD.

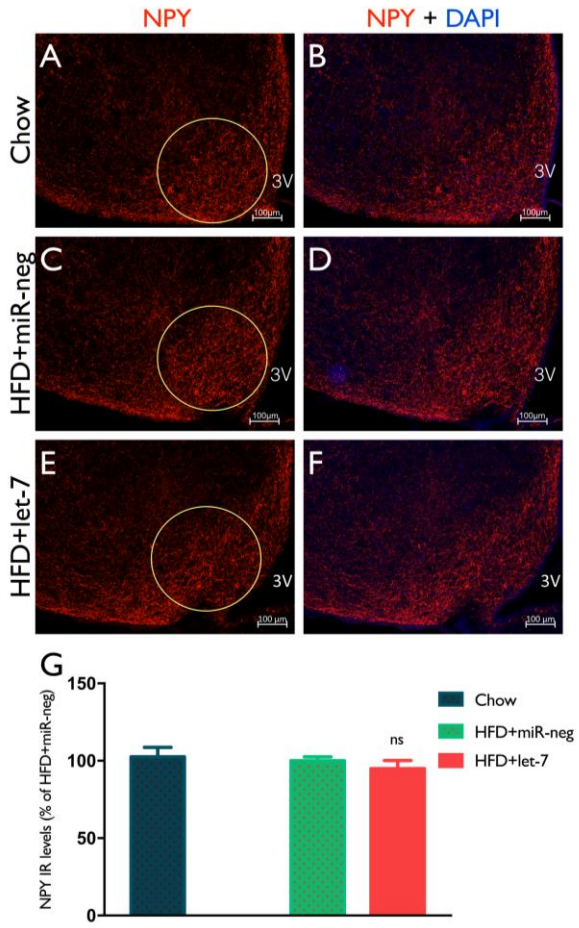


Figure 3.7 - Immunohistochemical analysis of neuropeptide Y (NPY) in the mouse hypothalamus. (A-F) Representative images of NPY (red) in coronal sections of mice arcuate nucleus from animals fed with chow diet or HFD with control microRNA overexpression in the hypothalamus (HFD+miR-neg) (C-D) or HFD with let-7 microRNA overexpression in the hypothalamus (HFD+let-7) (E-F). The yellow line circles indicate the area used for quantification of NPY immunoreactivity. (G) Quantification of NPY immunoreactivity from the same group of animals (n=4-5 per group). Unpaired T-test; HFD+let-7 compared HFD+miR-neg; ns $p>0.05$. 3V, third ventricle. DAPI, nuclear staining. IR, immunoreactivity.

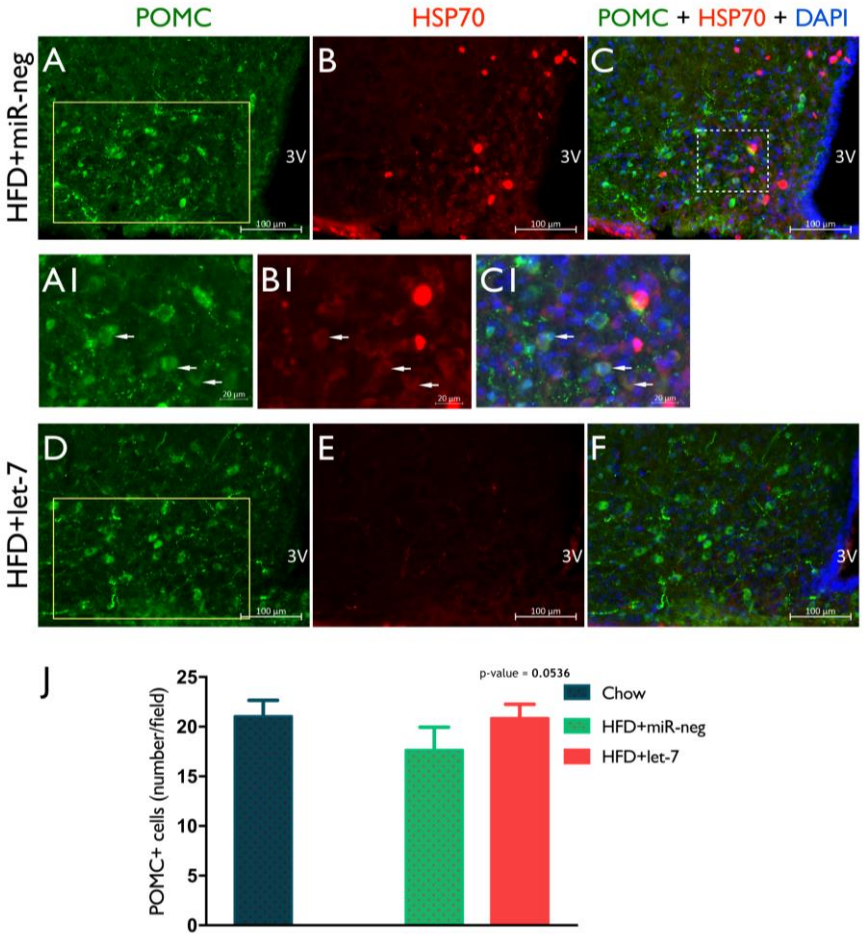


Figure 3.8 - Immunohistochemical analysis of neuropeptide pro-opiomelanocortin (POMC) and neuronal injury marker heat shock protein 70 (HSP70) in the mouse hypothalamus. Representative images of POMC (A and D; green), HSP70 (B and E; red) and merged images (C and F) in coronal sections of mice arcuate nucleus from animals fed with high fat diet with control microRNA overexpression in the hypothalamus (HFD+miR-neg) (A-C) or high fat diet with let-7 microRNA overexpression in the hypothalamus (HFD+let-7) (D-F). The dashed box indicates the region where the higher magnification view was made. The yellow line box indicates the area used for quantification of POMC cell number. (G-I) Higher magnification view of POMC and HSP70 positive cells (white arrows) in HFD+miR-neg mice. (J) Quantification of POMC neurons number in the same group of animals (n=4-5 per group). Unpaired T-test; HFD+let-7 compared HFD+miR-neg; ns $p>0.05$. 3V, third ventricle. DAPI, nuclear staining.

3.3.3.4 Alteration on Neurogenesis markers

Hypothalamic NSCs are important in maintaining body homeostasis and in regulation of appetite (24). It has been shown that NSCs are present in the mediobasal hypothalamus and in the adjacent third ventricle wall of adult hypothalamus of mice, through the use of SOX2, a marker of NSCs. HFD can lead to impaired hypothalamic neurogenesis in mice, hence the decrease of number of SOX2-positive cells in the MBH (51). So, we determine if, overexpressing let-7 microRNA in hypothalamus of mice fed with HFD could prevent the loss of SOX2-positive cells (Figure 3.9). We observed that there was no significant difference in the number of SOX2-positive cells in the ARC of mice from the three groups of animals (Figure 3.9G).

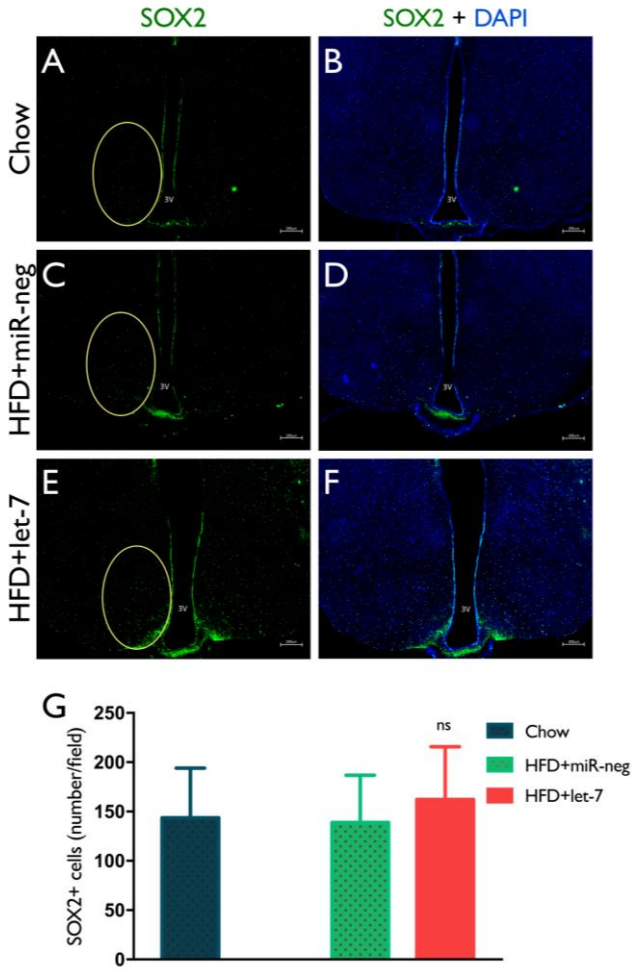


Figure 3.9 - Immunohistochemical analysis of neural cells marker SOX2 in the mouse hypothalamus. (A-F) Representative images of SOX2 in coronal sections of mice mediobasal hypothalamus from animals fed with chow diet or HFD with control microRNA overexpression in the hypothalamus (HFD+miR-neg) (C-D) or HFD with let-7 microRNA overexpression in the hypothalamus (HFD+let-7) (E-F). The yellow line elipses indicates the area used for quantification of SOX2 cell number (bilaterally). (G) Quantification of SOX2-positive cells in the MBH for the same group of animals (n=4-5 per group). Unpaired T-test; HFD+let-7 compared HFD+miR-neg; ns $p>0.05$. 3V, third ventricle. DAPI, nuclear staining.

Chapter IV:
General Discussion and Conclusions

The prevalence of obesity continues to increase around the globe, becoming a health problem with a major impact in our society (53). Obesity is mainly characterized by the increase of body weight, being an individual considered obese when BMI (kg/m^2) is 30 or higher (7).

In our study, we used C57BL/6 mice fed with HFD since these rodents mimic the most prevalent cause of obesity in humans being obesity prone when fed HFD for a certain period of time (54). And because of their susceptibility to obesity, body weight increased higher than 25% after 7 weeks of 40% fat diet regime, being considered obese (19). Furthermore, in obesity mouse models it can be observed hyperphagia, hyperglycemia and hyperinsulinemia (54). In a previous study, we were able to see hyperglycemia and the increase of fat accumulation, and other characteristics of obesity (19). Overall, the induction of obesity by HFD consumption in this rodent specie were successful.

MicroRNAs are non-coding RNAs that regulate the cell cycle and development. In specific, they are known to regulate stem-cell differentiation, insulin secretion, apoptosis, aging, among other processes (34). But, their putative role in hypothalamic physiology and obesity has not been completed unravelled.

Previous studies have shown that the body energy state can change miRNA biogenesis in the hypothalamus (45). It has also been shown that there is an up-regulation of miRISC genes in anorexia animal model mice (45) and up-regulation of miR-200a in obesity rodent models (*ob/ob* mice and *db/db* mice) (55). Also, Dicer KO POMC neurons (56) and Dicer KO ARC nucleus (47) can lead to development of obesity. In our investigation group has been elucidated that let-7 microRNA family are present in the hypothalamus of obese rats.

Here we show that overexpression of microRNA let-7 in the hypothalamus has a beneficial impact in peripheral parameters in mice fed with HFD. We evaluated the following peripheral metabolic parameters: insulin, leptin, total cholesterol and triglycerides serum levels; adipocyte area and number of adipocytes in epididymal white adipose tissue, and liver histology.

In the literature it is described that HFD-fed mice exhibit impaired glucose homeostasis through insulin resistance (7), implicating higher insulin serum levels, as we obtained in HFD+miR-neg mice group. Being leptin an adipocyte-derived cytokine, its levels are correlated with adipose mass (21). Therefore, it was expected the leptin levels to rise in HFD+miR-neg mice group, as seen in Figure 3.1B. Despite not all obese individuals being hypertriglyceridemic, hypertriglyceridemia is a common lipid abnormality in persons with visceral obesity (57), being the average serum cholesterol level significantly higher in overweight subjects than in lean ones. Therefore, there is a significant correlation between obesity and plasma triglycerides. Thus, the serum levels of triglycerides were expected to be higher in HFD+miR-neg mice group when compared to HFD+let-

7 mice group, what was in according to what we showed. Obesity patients have an impaired plasma lipoprotein–lipid profile, which includes elevated triglyceride (TG) and apolipoprotein (apo) B concentrations and reduced HDL cholesterol levels (58). Furthermore, the levels of low-density lipoprotein (LDL)-cholesterol in obese patients are usually higher than lean ones.

To summarize, we observed that the serum levels of leptin, insulin, cholesterol and triglycerides were significant lower in HFD+let-7 mice, when compared to HFD+miR-neg. These results show that overexpression of microRNA let-7 in the hypothalamus has a beneficial role in mice fed HFD.

We also analyzed two relevant tissues: white adipose tissue and liver. With obesity, adipocytes enlarge in area and, consequently, decrease the number of adipocyte *per* area. Also, in this parameter we observed a significantly decrease in the average of area of adipocyte in the HFD+let-7 mice when compared to HFD+miR-neg. These results suggest that the overexpression of let-7 in the hypothalamus has impact in adipose tissue. Being the adipose tissue considered an important organ with a role in energy balance, it is relevant to know if it has a connection with the hypothalamus. It has been described that the NPY system represents a form of communication between the hypothalamus and adipose tissue (59). NPY is responsible for regulating adiposity though the promotion of energy storage in white adipose tissue. Adipose tissue, besides accumulating and realizing lipids, also has endocrine function, producing hormones and appetite-regulating factors according to whole-body energy homeostasis. Therefore, this cross-talk between NPY, orexigenic neuropeptide, and adipose tissue is relevant in maintaining whole-body energy homeostasis. In this work, we showed a possible intercommunication between WAT and the hypothalamus through the effect of the overexpression of let-7.

Regarding liver histology, it is described that with obesity there is accumulation of cytoplasmic lipidic droplets in the cytoplasm of hepatocytes (8). In the present study, we were able to observe an amelioration in liver pathology in some animals of the HFD+let-7 group. Inhibition of diet-induced hypothalamic inflammation has been proven to correct liver steatosis (13). It has been described that the inhibition of hypothalamic TLR4 and TNF-α leads to a restoration of liver macroscopic aspect (13). In our histological evaluation, we were able to observe the reduction of liver fat deposition.

It was already described by others that HFD induces relevant changes in the hypothalamus: hypothalamic inflammation, gliosis (increase of astrocytes and reactive microglia), loss of POMC neurons, and compromising the whole-body energy balance (1). The gliosis induced by HFD is characterized by an increase of intensity of GFAP, the formation of a dense fibrous network of astrocytic processes, and increase number of activated microglia (1). In our study, we were able to

observe a higher intensity of GFAP and microglia cells in hypothalamus of HFD+miR-neg mice, compared with chow diet group. Let-7 overexpression (HFD+let-7 mice group) was not able to decrease GFAP immunoreactivity, but significantly decrease the number of activated microglial cells, comparing with HFD+miR-neg mice.

It has been described an hypothesis for microglia activation in the presence of HFD regime. It suggests that microglia cells have a neuroprotective effect due to their enlargement when the inflammation is already established (1). It is important to note that we used a mouse model prone to obesity, therefore it would be relevant to determine if genetic and ambient factors influence hypothalamic responses to HFD feeding. In spite of this, there was a decrease of he number of $Iba1^{+}$ cells in the hypothalamus of HFD+let-7 mice group, and also observed a lower microglial cell size in HFD+let-7 group compared to HFD+miR-neg group. Together, our data suggest that overexpression of microRNA let-7 in the hypothalamus has a beneficial role on gliosis induced by HFD.

The ARC is the main feeding control area which has several neuropeptides with exclusive functions. With HFD, fatty acids cause primary inflammation in the ARC, leading to possible alterations on hypothalamic neuropeptides levels. In the present study, we wanted to evaluate if let7 overexpression in the hypothalamus was able to change neuropeptides levels that could have impact on metabolic alterations in mice. We observed that HFD neither let7 overexpression, changed the levels of the orexigenic neuropeptides (AGRP and NPY). Others described that HFD decreases the number of anorexigenic neurons (POMC neurons) in the mouse hypothalamus (1). In our study we observed that let-7 overexpression in the hypothalamus (HFD+let-7 animals) increased the number of POMC neurons but decrease the number of neurons with the neuronal injury marker (HSP70), which suggests that let-7 microRNA could prevent the loss of these neurons. Being HSP a component of the neuroprotective response to neuron injury, when overexpressed activates and increases autophagy (60).

Autophagy is a lysosomal degradative pathway responsible for maintaining cellular homeostasis (1). It has been shown that the increase of autophagy is a response to HFD regime and its neuronal inflammation and injury. In HFD+miR-neg mice group, we are able to observe a higher presence of HSP, therefore more autophagy and less cells. As opposed to in HFD+let-7, we cannot see the same level of HSP, therefore there is less autophagy and more POMC neurons. This data suggests that increased numbers of autophagosomes reflects ongoing cell injury and the inability to prevent cell loss with HFD exposure (1).

Hypothalamic neurogenesis is a highly regulated and transient process that can be influenced by the energy availability and whole-body energy balance (1). NSCs are present in the mediobasal hypothalamus and in the adjacent third ventricle wall of adult hypothalamus of mice and others described that HFD decreases number of NSCs (SOX2-positive cells) in the median-basal hypothalamus (51). However, in our study we were not able to observe differences in the number of Sox2-positive cells in the groups of animals in HFD compared to chow diet, or even between HFD+miR-neg mice and HFD+ let-7.

Table 2 - Metabolic parameters change

Tissue/Sample Analyzed	**Parameter Evaluated**	**HFD+miR-neg (compared to Chow)**	**HFD+let-7 (compared to HFD+miR-neg)**
Serum	**Insulin**	↑↑	↓↓
	Leptin	↑↑	↓↓
	Total Cholesterol	↑↑	↓
	Triglycerides	↑↑	↓↓
Epididymal White Adipose Tissue	**Adipocyte Expansion**	↑↑	↓
Liver	**Lipid droplet**	↑	↓

In conclusion, the results of this thesis suggest that the overexpression of let-7 microRNA in the hypothalamus may have a protective role against metabolic changes that occurs in obesity. Consequently, strategies that increase let-7 microRNA in the hypothalamus could be new therapeutic strategy for prevent obesity.

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