Renata Sandres de Souza Araujo

Controlling the effect of injection in the hypothalamus of diet induced obese mice

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Controlling the effect of injection in the hypothalamus of diet induced obese mice

Master in Oncobiology – Molecular Mechanism of Cancer

This work was done under the supervision of:

Clévio Nóbrega, Ph.D

Department of Biomedical Sciences and Medicine

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Authorship Statement

I hereby declare to be the author of this work, which is original and unpublished. Authors and papers consulted are duly cited in the text and are listed in the included references.

"A coisa mais bela que o homem pode experimentar é o mistério. É esta emoção fundamental que está na raiz de toda a ciência e toda a arte".

Albert Einstein (1879 – 1955)

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24-OHC – 24-hydroxycholesterol

25-OHC – 25-hydroxycholesterol

27-OHC - 27-hydroxycholesterol

7α-OHC - 7α-hydroxycholesterol

7β-OHC - 7β-hydroxycholesterol

7-KC – 7-Ketocholesterol

25-OHC – 25-hydroxycholesterol

α-EPOX - 5α, 6α-epoxycholesterol

β-EPOX - 5β, 6β-epoxycholesterol

ABCA1/ABCG1 - ATP-binding cassette transporters

AAV- adeno-associated virus

AAV5 - adeno-associated viral vector serotype 5

ABC - ATP binding cassete

ABCA1- ABC transportes A1

Acetyl-CoA - acetyl coenzyme A

AdipoR1 - adiponectin receptor 1

AdipoR2 - adiponectin receptor 2

AT - Adipose tissue

AMPK - adenosine monophosphate-activated protein kinase

ANH - anterior hypothalamic nucleus

APPL1 - adapter protein

Apo-E - apolipoprotein E

AC - anterior commissure

ACAT1 - acyl-coenzyme A cholesterol acyltransferase 1

ACVD – atherosclerotic cardiovascular disease

ACC - acetyl-CoA carboxylase

AD – Alzheimer's disease

AgRP - agouti-related peptide

AGNE - non-esterified fatty acids

AQP4 - aquaporin 4

AHA - anterior hypothalamic area

ALS - Amyotrophic lateral sclerosis

AkT - protein kinase B

AMPK – adenosine monophosphate-activated protein kinase

ANS - autonomic nervous system

Apo – apolipoprotein

ApoER2 – apolipoprotein E recptor

APP – amyloid precursor protein

APPL1 - adapter protein

ARC – arcuate nucleus

asWATs - anterior subcutaneous WAT

ASCs - primary adipose-derived stromal cells

ATGL - adipose triglyceride lipase

ATP – Adenosine triphosphate

ATMs - adipose tissue macrophages

AB – amyloide B-peptide

AVP - vasopressin arginine

BAT – Brown adipose tissue

β cells - Beta cells

BBB – blood- brain barrier

BMI – body mass index

BW – body weight

°C - degree Celsius

C- peptide - connecting peptide

CCL2 - chemokine (C-C motif) ligand 2

cAMP – cyclic adenosine monophosphate

CART - cocaine and amphetamine-regulated transcript

CBMR - Centre for Biomedical Research

cDNA - complementary DNA

Chow – low-fat control diet

CO2 – Carbon dioxide

CRH - corticotropin releasing hormone

Cyt-c – cytochroma C

CSF - cerebrospinal fluid

CP - choroid plexus

cm – centimeter

CNS – central nervous system

CRP – C - reactive protein

CRH - corticotropin releasing hormone

CSF – cerebrospinal fluid

CDV – cardiovascular diseases

CYPs – Cytochrome P450

CYP46A1 – cytochrome P450 family 46 subfamily A member 1

CYP7A1 - cytochrome P450 family 7 subfamily A member 1

CYP3A4 - cytochrome P450 family 3 subfamily A member 4

CYP27A1 - cytochrome P450 family 27 subfamily A member 1

DAG – diacylglycerol

DCs – dendritic cells

DIO - diet-induced obese

DGAV - Direção Geral de Alimentação e Veterinária

DMH - dorsomedial hypothalamic nucleus

DMN - dorsomedial nucleus

DNA – Deoxyribonucleic acid

DHA – dorsal hypothalamic area

dL – deciliter

DM – diabetes mellitus

DMN – dorsomedial nucleus

EBP – enhancer-binding protein

EChP - choroid plexus epithelial cells

EECs - endocardial endothelial cells

ERK – Extracellular signal-regulated kinases

ER – endoplasmatic reticulum

ER - estrogen receptor

ET – Endothelins

FA – fatty acid

FAO - fatty acid oxidation

FAS - Fatty acid synthase

FELASA - Federation of Laboratory Animal Science Associations

FoxO1 – Forkhead box O1

FFA – free faty acids

FTO - *Fat mass and obesity associated*

FXR - farnesoid X receptor

g – gram

GLP-1- glucagon-like peptide-1

GABA – gamma-aminobutyric acid

GLP-1 – Glucagon-like peptide 1

GLUT – glucose transportes

GLUT 4 - glucose transporter 4

GTT – glucose tolerance test

GTP – Guanosine triphosphate

GWAS - Genome wide association studies

GPR183 - G 183 protein

h – Hours

20-HETE - 20-hydroxyeicosatetraenoic acid vasoactive

HD – Huntington's disease

HDL – Chol – high-density lipoprotein cholesterol

HEK – human embryonic kidney

HER – Human epidermal growth factor receptor

HFD – high-fat diet

HIF – Hypoxia-inducible factors

HIF-1α - hypoxia-inducible factor 1 α

HIF2α - hypoxia-inducible factor 2 α

H2O2 - hydrogen peroxide

HP - hypothalamic-pituitary

HDL - high-density lipoprotein

HMG – CoA – 3-hidroxi- 3- methyl-glutaril-CoA

HPT - hypothalamic-pituitary-thyroid

HPA - hypothalamic-pituitary-adrenal

HTT – huntingtin gene

IGT – impaired glucose tolerance

IL – interleukin

IL-2 - interleukin 2

IL-4 - interleukin 4

IL -6 – interleukin 6

IL-1β – interleukin 1β

IL-10 – interleukin 10

IL-12 – interleukin 12

IL-13 – interleukin 13

IGFs - Insulin-like Growth Factors

iWAT - subcutaneous

ICV - intracerebral ventricle

INRS – insulin recptor

INRS – insulin receptor substrate

IKKβ - κB-kinase-β inhibito

IR – insulin resistance

IRS – insulin resistance syndrome

IGFBP- 2 IGF Binding Proteins

ITT – insulin tolerance test

IFN-γ - interferon-γ

IVA - visceral WAT

ingWAT - inguinal WAT

JAK2 – janus kinase 2

Jnk - c-Jun N - terminal kinase

KATP - ATP-sensitive potassium channel

LDL – low density lipoprotein

LDL- R – low density lipoprotein receptor

Lep – leptin

LepR – leptin receptor

LHA – lateral hypothalamic area

LL – lipoprotein lipase

LPOA – medial preoptic area

LXR – live X receptors

M2 – square meter

M1 - M1 macrophages

M2 - M2 macrophages

MAPK – mitogen-activated protein kinase

MB – mammillary body

MBH - medio basal hypothalamus

MCP-1 - monocyte chemoattractant protein-1

mRNA – messenger RNA

miRNAs - microRNA

α-MSH - α-melanocyte stimulating hormone

mTOR – mammalian target of rapamycin

MC3/4R – melanocortin receptor ¾

MCH - melanin-concentrating hormone

MCP- 1 – monocyte chemoattract protein-1

ME – median eminence

MMe - metabolically activated phenotype

mg - milligram

min – minutes

MJD – Machado – Joseph disease

mm Hg – millimeter of mercury

mmol – millimole

M – Molar

mL – Milliliter

mM – Millimolar

MPN – median preoptic nucleus

MPOA – lateral preoptic area melanocortin-4

mWAT - mesenteric WAT

MSH – melanocyte stimulating hormone

NAFLD – non- alcoholic fatty liver disease

NASH – non- alcoholic steatohepatitis

NADPH - nicotinamide adenine dinucleotide phosphate

NEFA – non- esterified acids

NF-κB - nuclear factor-κB

NOD - non-obese diabetic mice

nm – nanometer

nM – Nanomolar

NPC1/2 – Niemann- Pick type C1/2

NPY – neuropeptide Y

NK - natural killer cells

OC – optic chiasm

OFT - open field test

OSA – obstructive sleep apnea

OS - oxidative stress

OXY – oxytocin

oxLDL - oxidized LDL

V2 O_2 - oxygen volume

V2 CO_2 - carbon dioxide volume

VLDL - very low-density lipoprotein

VMN - ventromedial nucleus

P450s - Cytochrome P450 enzymes

PAHs - polycyclic aromatic hydrocarbon

PBS – phosphate buffered saline

PCR - polymerase chain reaction

PGK1 - phosphoglycerate kinase

PNS - peripheral nervous system

POA – preoptic area

POMC – pro-opiomelanocortin

PPARγ - peroxisome proliferator-activated receptor gamma

PD – Parkinson's disease protein

PDGF – Platelet-derived growth factor

pgWAT - perigonadal WAT

PGC - 1α Peroxisome proliferator-activated receptor-γ coactivator

PGI2 - prostacyclin

PGK1 – phosphoglycerate kinase 1

PHN – posterior hypothalamic nucleus

PI3K – phosphatidylinositol – 3 – kinase

PIP 2 – Phosphatidylinositol 4,5-bisphosphate

PIP 3 – Phosphatidylinositol 3,4,5-triphosphate

PRDM16 - PR domain containing 16

PSA - polysialic acid

PTP1B – protein tyrosine phosphatase 1B

PTEN – Phosphatase and tensin homolog gene

PVN – paraventricular nucleus

RER – rough endoplasmic reticulum

RNA - Ribonucleic acid

ROS - reactive oxygen species

s – seconds

SAT - subcutaneous WAT

SCA – spinocerebellar ataxia

SCN – suprachiasmatic nucleus

SEM – standard error of mean

ShRNA – short hairpin RNA

SHP2 - protein tyrosine phosphatase 2

SERM - estrogen receptor modulator

sWAT – subcutaneous white adipose tissue

sBAT – subcutaneous brown adipose tissue

SLOS – Smith- Lemli- Optizy

SOCS3 – suppressor of cytokine signaling 3

SCAs - Spinocerebellar ataxias

SMN – supramammilary nucleus

SNS – sympathetic nervous system

SNP - single nucleotide polymorphisms

SNVs - unique nucleotide independent variants

SON – supraoptic nucleus

SERM - estrogen receptor modulator

SREBP – sterol regulatory element-binding proteins

STAT – signal transducer and activator of transcription

TG – triglyceride

Thr – Threonine

TLRs - Toll-like receptors

TZD - thiazolidinedione

TH - tyrosine hydroxylase

Tyr – Tyrosine

TH2 - T helper 2

TMN – tuberomammillary nucleus

TNF-α - tumor necrosis factor- α

Tregs - regulatory T cells

TRH - thyrotropin releasing hormone

TxA2 - thromboxane A2

TSEs - epoxyieicosatrienoic acids

UCP-1 – uncoupling protein-1

UCP-2 - uncouple protein-2

USA – United States of America

v. g – viral genomes

AVP - vasopressin arginine

VEGF – vascular endothelial growth factor

VLDL – very- low- density lipoprotein

VLDLR – very-low-density lipoprotein receptor

VMN – ventromedial nucleus

VMH – ventromedial hypothalamus

WAT – white adipose tissue

WHO – Word Health Organization

V2 O_2 - oxygen volume

V2 CO_2 - carbon dioxide volume

y- gamma

µg – microgram

µl – microliter

µm – micromete

µM – Micromolar

α – Alpha

β – Beta

WHO – World Health Organization

Abstract

Diet-induced obesity causes a central inflammatory process in the brain, more specifically in the arcuate nucleus of the hypothalamus; in addition to substantially altering cholesterol homeostasis in the brain. The brain is one of the richest organs in cholesterol and cholesterol homeostasis has proved to be very important not only to maintain healthy brain physiology, but also directly influencing the whole-body homeostasis. In addition, several metabolic disorders are correlated with different neurodegenerative diseases. Cholesterol in the brain is converted primarily to 24-hydroxycholesterol (24-OHC) by *CYP46A1*. Changes in oxysterol metabolism have been correlated with obesity. Previous studies from our laboratory have identified *CYP46A1* as a relevant therapeutic target, not only for Machado de Joseph disease, but also for other neurodegenerative diseases. One of these studies revealed that the silencing of the expression of the *Cyp46a1* gene (using AAV5-*shCyp46A1*), in the hypothalamus of C57BL/6J mice fed a low fat diet (Chow) (control diet) and a high fat diet (HFD), had a profound impact on the dysregulation of the entire physiological process of body homeostasis.

In this sense, additional studies became necessary to demonstrate that the effect of silencing the *Cyp46a1* gene in the arcuate nucleus was specific, discarding the effect of the surgical procedure or viral vectors administration. Thus, in this study, our main objective was to control the effect of *Cyp46a1* silencing on the hypothalamus (arcuate nucleus). For this, through stereotaxic surgery, a control gene (GFP) was delivered by AAV vectors in the arcuate nucleus; both in animals fed with Chow ration and those fed with HFD ration. The project's main hypothesis is that the GFP protein does not interfere in the physiology of the hypothalamus and consequently in the metabolism of the whole-body, regardless of diet. Our data confirm that stereotaxic surgery and GFP expression did not alter the homeostasis of the hypothalamus and, consequently, there was no change in the whole-body metabolism of the mice.

Keywords: hypothalamus; cholesterol metabolism; *Cyp46a1*; GFP; stereotaxic surgery, oxysterols; whole-body energy homeostasis

Resumo

A epidemia do excesso de peso e obesidade representa um dos mais importantes problemas de saúde pública do século XXI. A obesidade induzida por dieta causa um processo inflamatório central no cérebro, mais especificamente no núcleo arqueado do hipotálamo. O núcleo arqueado é uma região especial do cérebro responsável pela regulação da homeostasia energética corporal, possuindo uma importância crucial na manutenção do equilíbrio entre consumo e gasto energético. Na obesidade há disfunção hipotalâmica e esta disfunção pode alterar substancialmente a homeostasia do colesterol no cérebro. O cérebro é um dos órgãos mais ricos em colesterol e a sua homeostasia do colesterol é muito importante para manter a fisiologia cerebral saudável e também influencia diretamente a homeostasia do resto do organismo. Além disso, o facto de os distúrbios metabólicos estarem correlacionados com diferentes doenças neurodegenerativas, como: doença de alzheimer, doença de Parkinson e doença de Huntington. O colesterol no cérebro é convertido principalmente em 24S-hidroxicolesterol pela enzima Cyp46a1. Alterações no metabolismo do oxisterol foram correlacionadas com a obesidade. Estudos do nosso laboratório identificaram o *CYP46A1* como um alvo terapêutico relevante, não apenas para a doença Machado de Joseph, mas também para outras doenças neurodegenerativas. Um desses estudos, revelou que o silenciamento da expressão do gene *Cyp46a1* (utilizando-se *AAV5-shCyp46A1*), no hipotálamo (núcleo arqueado), de morganhos C57BL/6J alimentados com dieta com baixo teor de gordura (Chow – *low fat control diet*) (dieta controlo) e com uma dieta com alto teor de gordura (HFD – *high fat diet*), tem impacto profundo na desregulação de todo o processo fisiológico da homeostasia corporal destes ratinhos. Tem ainda grande impacto no perfil fisiológico dos órgãos metabólicos e alterações na morfologia estrutural de cada um deles, como: WAT, BAT, fígado, pâncreas, entre outros. Desta forma, os resultados deste estudo sugerem que o silenciamento do *Cyp46a1* nos animais Chow AAV5-*shCyp46a1* e HFD AAV-*shCyp46a*1 resulta em um fenótipo de obesidade, alterações nos níveis de oxisteróis cerebrais e diabetes mellitus tipo 2; além de modificações significativas no comportamento. Os resultados sugerem o papel importante do gene *Cyp46a1* na manutenção do metabolismo do colesterol no cérebro e no controlo da homeostasia energética corporal. Diante destas observações, estudos adicionais tornaram-

se necessários para comprovar o efeito do silenciamento do gene *Cyp46a1* no núcleo arqueado, descartando o efeito do procedimento cirúrgico.

Desta forma, neste estudo, o nosso principal objetivo foi controlar o efeito do silenciamento de *Cyp46a1* no hipotálamo (núcleo arqueado). Para tal, por meio de cirurgia estereotáxica foi realizada a injeção de um gene controlo (GFP) mediado por vetores *AAV* no núcleo arqueado; tanto em morganhos alimentados com dieta Chow, como em morganhos alimentados com dieta HFD. A hipótese do projeto é que a proteína GFP não interfere na fisiologia do hipotálamo e consequentemente no metabolismo de todo o corpo, independentemente da dieta.

Este estudo foi conduzido durante um período de 12 semanas; no início do estudo os morganhos (C57BL/6J) foram divididos aleatoriamente em dois grupos: Chow (controlo) e HFD. A partir deste momento, cada grupo foi alimentado com a dieta específica por 12 semanas. Na quarta semana do estudo, os dois grupos foram submetidos a injeção estereotáxica bilateral no núcleo arqueado. Os resultados demonstraram que a proteína GFP não interferiu na fisiologia do hipotálamo (núcleo arqueado) e que as injeções estereotáxicas (AAVGFP) não causaram inflamação comprometedora que tenha alterado a fisiologia e o fenótipo alcançado através exclusivamente da dieta administrada (Chow e HFD) aos grupos dos morganhos utilizados. O fenótipo obeso foi previsivelmente alcançado pela indução de dieta gordurosa apenas nos morganhos HFD, assim como, a alteração da morfologia de vários tecidos metabólicos (WAT, BAT, pâncreas e fígado) e houve uma grande acumulação de lípidos nos órgãos. Os morganhos controlo (dieta Chow) não desenvolveram obesidade, não apresentaram alterações na morfologia dos tecidos metabólicos (WAT, BAT, pâncreas e fígado) e também não apresentaram modificações no acúmulo lipídico dos órgãos. Como este estudo é um controlo importante para o projeto de silenciamento do gene *Cyp46a1* realizado anteriormente, os resultados aqui apresentados confirmam que a cirurgia estereotáxica e a expressão de GFP não alteram a homeostasia do hipotálamo e consequentemente não há alteração no metabolismo corporal dos morganhos.

Palavras chave: obesidade; hipotálamo; metabolismo do colesterol; *Cyp46a1*, GFP, cirurgia estereotáxica, oxisteróis, homeostasia energética corporal

CHAPTER 1 - Introduction

1.1 Obesity

The epidemic of overweight and obesity represent one of most important problems of public health in the 21st century. The numbers are alarming; in 2016 an estimated 1.9 billion adults were overweight and 650 million were obese. Children are also of high concern, reaching 380 million children with overweight or obesity in 2016. The latest projections from World Health Organization (WHO) indicate that the proportion of overweight and obese people will continue to increase and reach 3.3 billion in 2030 (WHO, 2018).

Also, according to WHO, Europe had the second highest proportion of overweight or obese people in 2014, behind the Americas. Globally, in 2014, 39% men and 40% of women aged 18 or over were overweight. These numbers raise above 58% in Europe and America. By contrast, it was considerably lower in Africa and south East Asia (Eurostat, 2019). Recent report indicates that in Portugal, 67.6% of the population over 15 years of age is overweight and 29% obese, analyzing a period from 2006 to 2016 (OECD/EU, 2018).

To these impressive numbers, it is also important to consider that the prevalence of obesity is increasing in developed countries, due to a number of behavioral and environmental factors, which contributed to the long-term rise in obesity in western countries, including widespread availability of energy-dense foods and an increasingly sedentary lifestyle. These factors have created obesogenic environments (Gloria González, 2017). Importantly, obesity increases the risk of developing other pathologies such as: myocardial infarction, fatty liver disease, type 2 diabetes, hypertension, metabolic syndrome, stroke, neurodegenerative diseases and various types of cancers, thus, increasing the public health problem and contributing to a decline in life quality and life expectancy (Blüher, 2019). Therefore, it is of utmost relevance and a major challenge to understand and elucidate the causes of obesity and of course to investigate forms to prevent and treat obesity.

The rampant economic growth, over-mechanized transportation, growing urbanization, stressful and sedentary lifestyle, a nutritional transition to processed food, highly calorie and high fat provide a fertile scenario for obesity development (Hruby and Hu, 2015). However, even though unhealthy environmental factors and lifestyle are crucial for

obesity development, it is also known that this condition has an important genetic and polygenic etiology (Khera *et al.*, 2019). There is a great individual variability in the involvement of obesity, which is due to the complex and heterogeneous pattern of inheritance of specific genes, resulting from the combination of genetic, epigenetic, metagenomics susceptibility and environmental factors (Thaker, 2017). The first genetic studies involved monogenetic syndromes and extreme obesity, in which the researchers focused their attention on the dysfunction of the leptin-hypothalamus pathway (Ranadive and Vaisse, 2008). There are several obesity monogenic syndromic forms, such as Alstrom syndrome, Bardet-Biedl syndrome and Cohen syndrome, caused by functional mutations in the *ALMS1*, *BBS1* or *BBS10* and *COH1* genes, respectively, although they have a very low frequency in the general population (Kaur *et al.*, 2017). Different types of monogenic non-syndromic obesity have also been described in patients with homozygous and heterozygous loss of function in genes that are part of the melanocortin leptin pathway: *LEP*, *LEPR*, *POMC*, *PCSK1* and *MC4R* (Huvenne *et al.*, 2016) (Albuquerque *et al.*, 2017). Despite the existence of these forms of obesity, polygenic obesity, in which several polymorphisms in several genes contribute to the phenotype, is the most common form of obesity in modern societies (Albuquerque *et al.*, 2017). Genome wide association studies (GWAS) allowed to identify several single nucleotide polymorphisms (SNP) involved in the inter-individual weight variation, being associated with the obese phenotype, fat distribution and risk of metabolic syndrome (Albuquerque *et al.*, 2015; Yengo *et al.*, 2018; Li and Qi, 2019). A recent GWAS led to the discovery of more than 940 unique nucleotide independent variants (SNVs), associated with body mass index (BMI) (Yengo *et al.*, 2018). Based on the outcome of all GWAs, the *FTO* gene (*Fat mass and obesity associated*) is currently seen as the main contributor to polygenic obesity in the European population (Stryjecki, Alyass and Meyre, 2018).

Although a large number of obesity-related genes have been identified by association studies across the genome, these genetic studies have been able to explain a limited proportion of the variation in obesity; therefore, a deeper understanding of the factors associated with the development of obesity is needed (Cheung and Mao, 2012). In the last decade, epigenetic regulation of gene expression has emerged as a new mechanism that can fill the gap in the etiology of this complex disease (Relton and Smith, 2010). Recent studies suggest that the epigenetic regulation of gene expression, through (DNA methylation and histone modification) may be a major contributor to the variation observed in disease in disease susceptibility (Sun *et al.*, 2017). Therefore, it is now

recognized that epigenetic changes (DNA methylation) can contribute substantially to the development of chronic diseases such as obesity, cancer and cardiovascular diseases (He *et al.*, 2019) Soubry et al., 2013).

From a physiological point of view, obesity is a complex multifactorial condition that is characterized by an excessive fat accumulation, which is often considered to result from excessive calorie consumption (food intake) and/or insufficient or inadequate calorie expenditure (metabolic and physical activity). In this sense, excess fat accumulated in obesity alters the entire constitution and functionality of the adipose tissue and its interplay with other metabolic organs (Gómez-Hernández *et al.*, 2016). Therefore, the adipose tissue is a central regulator of whole-body energy homeostasis. When the nutritional state changes, it will undergo a dynamic remodeling including adipose tissue expansion by a combination of an increase in adipocyte size (hypertrophy) and number (hyperplasia) (Michele Longo *et al.*, 2019). Changes in adipocyte number and size affect the expanded adipose tissue microenvironment in obesity, altering adipokine secretion, causing adipocyte death, local hypoxia and fatty acids flow. At the same time, stromal vascular cells in adipose tissue, including immune cells are involved in various adaptive processes such as adipogenesis and angiogenesis, all of which are dysfunctional in the remodeling of obese adipose tissue (Laforest *et al.*, 2015).

It is also known that obesity resulting from chronic over nutrition causes uncontrolled inflammatory responses, leading to chronic inflammation and insulin-resistant metabolic disorders and changes the peripheral organs of the whole body (Choe *et al.*, 2016). In fact, diet-induced obesity is associated with an important chronic inflammation in the central nervous system, more specifically in the hypothalamus, which is a crucial regulator of body energy homeostasis. Several studies have revealed the involvement of different neuronal cell types, as well as the molecular mechanisms contributing for hypothalamic inflammation due to diet (Timper and Brüning, 2017). Diet-induced hypothalamic inflammation results in the onset and development of obesity and related metabolic diseases. It has been shown that feeding mice with high-fat diet (HFD, 60% fat) directly cause brain damage (Samodien *et al.*, 2019). This pro-inflammatory response is considered one of the first causal steps involved in onset and maintenance of the obese phenotype (Guillemot-Legris and Muccioli, 2017).

1.2 Inflammation in Obesity

Inflammation is a classic tissue defense response to infection and injury that has many beneficial effects. However, low-grade inflammation associated with obesity can contribute to other comorbidities, including an increased incidence of acute pancreatitis, cardiovascular disease, diabetes, neurodegenerative diseases, and cancer (Ye and McGuinness, 2013). This chronic inflammation is initiated by a local hypoxia, which increases angiogenesis and improve the blood supply of adipose tissue, in which macrophages and pro-inflammatory cytokines play an essential role in adipose tissue remodeling and adipocyte differentiation (Thomas and Apovian, 2017). The expansion of adipose tissue in obesity can lead to local hypoxia and induction of HIF-α (Hosogai *et al.*, 2007). The activation of the HIF-α signaling cascade has been implicated in adipocyte dysfunction, increased adipose tissue inflammation and insulin resistance in rodent models with HFD-induced obesity (Michailidou, 2019).

Obesity-related inflammation was first described in the adipose tissue (Cawthorn and Sethi, 2008), although following studies reported this inflammation in other organs, including the hypothalamus (De Souza *et al.*, 2005). In fact, in obesity there are two types of inflammation: i) peripheral inflammation, which occurs in metabolic tissues (adipose tissue, muscle tissue, bone tissue, among others) and peripheral organs (liver, pancreas, kidneys, blood vessels, among others); ii) and central inflammation (CNS), more specifically, in the hypothalamus (Crispino *et al.*, 2020).

Here is evidence that a state of low-grade chronic inflammation is a key link between obesity and the associated metabolic syndrome (Lumeng and Saltiel, 2011). In general, obesity is a state of hyperinsulinemia, hyperlipidemia, hyperleptinemia and chronic inflammation that accounts for the highest incidence of metabolic syndrome in the world (Buettner, Schölmerich and Bollheimer, 2007; Lainez and Coss, 2019).

1.2.1 Peripheral Inflammation in Obesity

In peripheral tissues, the deleterious metabolic consequences of obesity arise in part from cellular inflammation triggered by the excess of nutrients (Hotamisligil and Erbay, 2008). Excessive visceral adiposity triggers a slow and gradual inflammatory process. Macrophages that infiltrate adipose tissue are an important source of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), such as IL-12 and interleukin-6 (IL-6) (Thomas and Apovian, 2017). Fat infiltration by immune cells occurs gradually in

multiple organs, including epididymis, heart, liver, skeletal muscle, vasculature, kidney and pancreas (Tchkonia *et al.*, 2010). Recent studies in rodents fed with HFD found that obesity is associated with the accumulation of macrophages in adipose tissue and liver, as well as an increase in TNF-α in plasma serum (Sidles *et al.*, 2019) (Jeon *et al.*, 2012). This complex chronic inflammatory process and pathological remodeling in the adipose tissue deregulate the physiological responses that maintain sensitivity to leptin and insulin, in addition to activating immune system responses. Excessive intake of HFD induces chronic low-grade inflammation in many metabolic organs, including the pancreas, adipose tissue, skeletal muscle, vasculature, kidney and liver, and others **(Figure 1.1)** (Lumeng and Saltiel, 2011; Laurentius *et al.*, 2019).

Although this inflammatory process is initiated by autonomous cell mechanisms; the subsequent infiltration of immune cells generates an inflammatory environment, which further interrupts the signal transduction of the insulin receptor (Xu *et al.*, 2003). In addition, signals from Toll-like receptors (TLRs), are amplified by signaling intermediates such as MyD88, activate the κB-kinase-β inhibitor (IKKβ) / nuclear factor-κB (NF-κB), c-Jun N-terminal kinase (Jnk) and other intracellular inflammatory signals in response to stimulation by the circulation of saturated fatty acids (Fessler, Rudel and Brown, 2009). Thus, a vicious cycle of inflammation and impaired use of nutrients follows, which produces progressive systemic metabolic impairment, contributing to reduced insulin sensitivity and metabolic impairment, predisposing to diabetes, steatohepatitis and atherosclerosis (Qatanani and Lazar, 2007).

The cytokines released by inflammatory tissue affect the metabolic functions of various organs, including liver, heart, muscle and brain (Hotamisligil, 2006). Pro-inflammatory cytokines released by activated immune cells can impair signaling of insulin in the insulin-responsive organs and cause systemic insulin resistance, which increases the risk of developing hyperglycemia and type 2 diabetes. A reduction in interstitial oxygen was observed first in the adipose tissue of obese rodents (Ye *et al.*, 2007) and later confirmed in models of obesity in humans (Pasarica *et al.*, 2009; Ye, 2009).

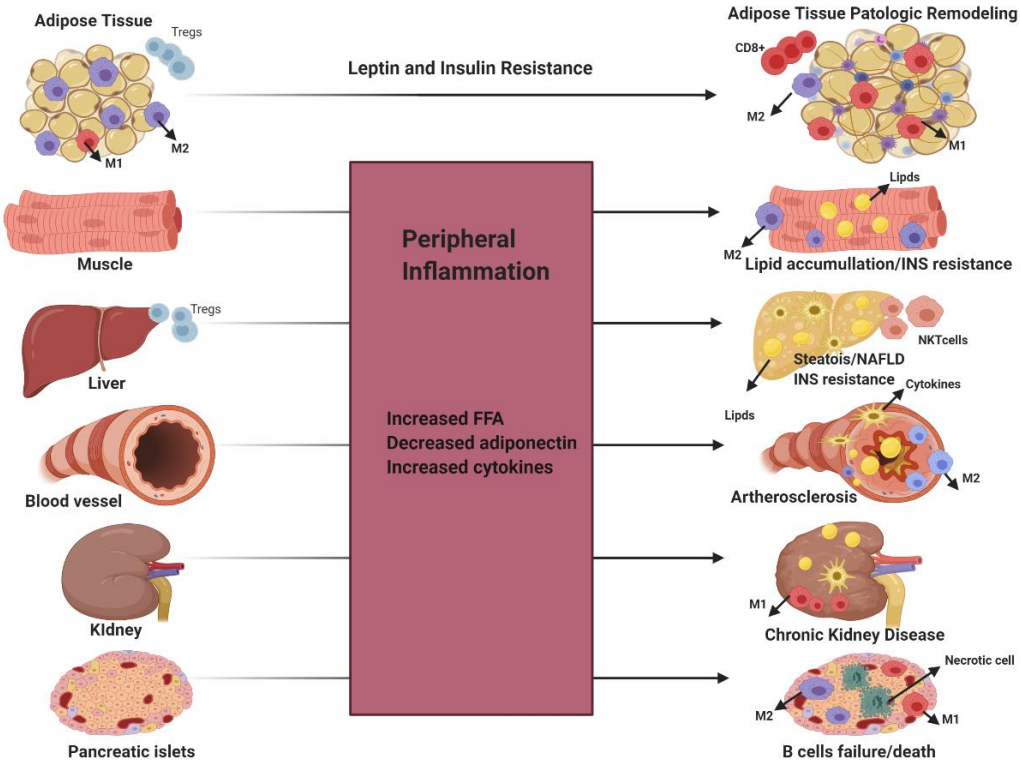


Figure 1.1 Peripheral Inflammation. Obesity triggers a chronic inflammatory process and a pathological remodeling in the adipose tissue that dysregulate physiological responses that maintain sensitivity to leptin and insulin. Over time, ectopic lipid accumulation occurs in muscles, liver, blood vessels, kidneys, pancreas and other organs. In this way, it activates via leukocytes, lymphocytes and macrophages (M1 and M2), releasing cytokines widely into the circulation, contributing to the development of specific diseases and exacerbating systemic insulin resistance.

1.2.2 Central Inflammation in Obesity

In addition to peripheral inflammation, obesity is associated with neuroinflammation (Miller and Spencer, 2014; Guillemot-Legris and Muccioli, 2017; Lainez and Coss, 2019). Brain inflammation has been implicated in some of the mechanisms that lead to obesity, which has also been recognized as an important player in inducing some degree of immune dysfunction (Palavra *et al.*, 2016), being affected by the peripheral inflammation associated with obesity (Uranga and Keller, 2019). The neuroinflammation occurs in multiple brain structures, including the hypothalamus, hippocampus, amygdala, neocortex and cerebellum (Miller and Spencer, 2014; Guillemot-Legris and Muccioli, 2017; Lainez and Coss, 2019). In these brain regions, diet-induced obesity is associated with increased levels of pro-inflammatory cytokines (IL-6, IL-1β and TNF-α), in addition

to greater expression of NF-κB and TLR4, which are two important molecular mediators of innate immune responses **(Figure1.2)** (Biessels *et al.*, 2014).

Periodic neuroinflammation is a necessary defense for the brain, which is mediated by inflammatory cells, such as microglia and astrocytes, plays key roles in regulating synaptic structure and function (Chen *et al.*, 2018). However, when neuroinflammation becomes prolonged or uncontrolled (chronic neuroinflammation), normal protective barriers break and lead to poorly adaptive synaptic plasticity and the development of different neuronal dysfunctions (Purkayastha and Cai, 2013). In fact, it has been shown that chronic neuroinflammation impairs the neurogenesis of the adult hippocampus and its blockade has been shown to restore it (Ekdahl *et al.*, 2003). In addition, impaired neurogenesis was found in the hypothalamus of rodents fed with HFD, probably due to chronic neuroinflammatory (Dorfman and Thaler, 2015). Moreover, chronic brain inflammation has also been associated with neurodegenerative disorders such as: Alzheimer's disease (AD), Amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), Huntington's disease (HD) (Amor *et al.*, 2014; Rocha *et al.*, 2016; Joly-Amado *et al.*, 2020; Kloske and Wilcock, 2020; Nóbrega *et al.*, 2020). Obesity and metabolic syndrome have been associated with impaired cognitive function. In addition, clinical data showed that obesity and diabetes mellitus are related not only to cognitive decline, but also to other brain disorders, such as dementia, anxiety and depression (Simon *et al.*, 2006; Riederer *et al.*, 2017; Sanderlin, Todem and Bozoki, 2017).

Several studies have reported that neuroinflammation derived from obesity in middle age is associated with an increased occurrence of central disorders, such as depression and impaired cognitive function, altered executive functioning, short-term memory and dementia (Cournot *et al.*, 2006; Kivipelto and Solomon, 2006; Whitmer *et al.*, 2008; Sabia *et al.*, 2009; Nguyen, Killcross and Jenkins, 2014; Guillemot-Legris and Muccioli, 2017). Similar results have been demonstrated in studies performed on animal models of obesity induced by HFD (Murray *et al.*, 2009; McNeilly *et al.*, 2011; Nguyen, Killcross and Jenkins, 2014).

HFD feeding induces inflammatory signaling in the hypothalamus, causing local resistance to both insulin and leptin (Thaler and Schwartz, 2010). In addition, inflammation-induced changes in the mediobasal hypothalamus (MBH) directly affect the integrity of homeostatic processes, such as hepatic glucose storage and blood delivery

(Mravec, Horvathova and Cernackova, 2019). Moreover, there are significant differences in the mRNA levels of IL-6, IL-1β or TNF-α in the temporal tissues of rodents fed by HFD. There is also a change in the expression of TNF-α in the hippocampus and microglia compared to rodents fed with control diet (Moroz *et al.*, 2008; Berkseth *et al.*, 2014). Accordingly, other studies demonstrated that pro-inflammatory cytokines, such as TNF-α and IL-1β, were released in the hypothalamus and activated apoptotic signaling in fed with HFD (De Souza *et al.*, 2005; Jeon *et al.*, 2012; Valdearcos *et al.*, 2017).

These recent studies demonstrate that peripheral inflammation and obesity-specific neuroinflammation orchestrate a vicious and complex cycle related to neurological impairment and consequently dysfunction of body homeostasis, more specifically by directly affecting the hypothalamus and its special neuron circuits, particularly in the arcuate nucleus – ARC (Jeon *et al.*, 2012; Ellulu *et al.*, 2017; Maldonado-Ruiz, Fuentes-Mera and Camacho, 2017; Valdearcos *et al.*, 2017; Chan, Cathomas and Russo, 2019; Macedo *et al.*, 2019). Therefore, a better understanding of the relationship between these elements in obesity could provide a solid basis for the design of new therapeutic approaches.

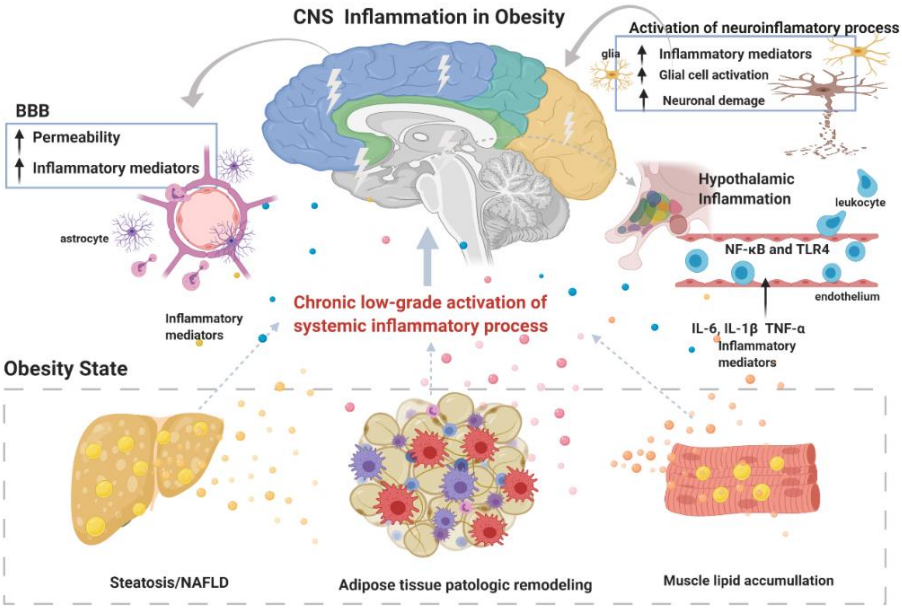


Figure 1.2 – Central Inflammation triggered by obesity. HFD feeding induces inflammatory processes in peripheral tissues and neuroinflammation occurs in multiple brain structures, including the hypothalamus. Peripheral organs release inflammatory mediators into the circulation such as IL-6, IL-1β or TNF-α that activate TLR4 and NF-κB. The NF-κB pathway regulates pro-inflammatory genes that recruit leukocytes to endothelium; at the same time the brain-blood barrier (BBB) is more permeable, allowing the passage of inflammatory mediators that will cause inflammatory process in the brain, where there is activation of microglia and consequent degradation and neuronal damage

1.3 Obesity and Adipose Tissue

The adipose tissue is a critical regulator of systemic energy homeostasis, acting not only as a caloric reservoir and thermal protector, but also as an endocrine organ secreting cytokines, hormones, proteins and metabolites (called adipokines), affecting the functionality of cells and tissues throughout the body. In addition to the regulation of the systemic energy balance along with the Central Nervous System (CNS), it also has great importance as an immunologically active organ (Choe *et al.*, 2016; Parimisetty *et al.*, 2016). Anatomically, it consists of different fat deposits with unique characteristics. Histologically, it is a loose, heterogeneous connective tissue, composed mainly of adipocytes, but it also contains the stromal-vascular fraction of cells (vascular endothelial cells, pre-adipocytes and fibroblasts), and immune cells, such as macrophages and T cells (Thomou *et al.*, 2017; Arhire, Mihalache and Covasa, 2019; Liu and Nikolajczyk, 2019). Compared to other metabolic organs, such as liver and muscle; several inflammatory responses are dynamically regulated in the adipose tissue. In fact, the immune cells present in the adipose tissue are involved in metabolic complications mediated by obesity, including insulin resistance and metabolic syndrome (Mathieu, Lemieux and Després, 2010; Huh *et al.*, 2014). An increase in adiposity associated with obesity is among the most common factors of adipose deposit dysfunction and metabolic communications (Guilherme *et al.*, 2017; Zoico *et al.*, 2019).

The adipose tissue is distributed throughout the body and is able to expand, accommodating an excess of energy in the form of accumulated lipids, a feature that distinguish the adipose tissue from other organs and tissues (Gesta *et al.*, 2006). It is known that each anatomical fat deposit has a specific physiological role, implying also particular metabolic and hormonal features. Moreover, there is strong evidence that some fat deposits are more clearly associated with the development of different types of diseases (Uranga and Keller, 2019).

In mammals, the adipose tissue forms in the uterus, just before birth, and throughout life. A continuous generation of new adipocytes in adult humans has been demonstrated (Spalding *et al.*, 2008). The adipose tissue is traditionally classified into two main types: white adipose tissue (WAT) and brown adipose tissue (BAT). However, more recently, a third type is being considered, the beige adipose tissue. The different types of adipocytes (white adipocytes, brown adipocytes and beige adipocytes) have different morphology, distribution, gene expression and functions **(Figure 1.3)**. In a single adipose deposit,

different subpopulations of adipocytes can be found; highlighting a complex relationship between the different adipocytes (Esteve Ràfols, 2014; Ikeda, Maretich and Kajimura, 2018; Zhang *et al.*, 2018).

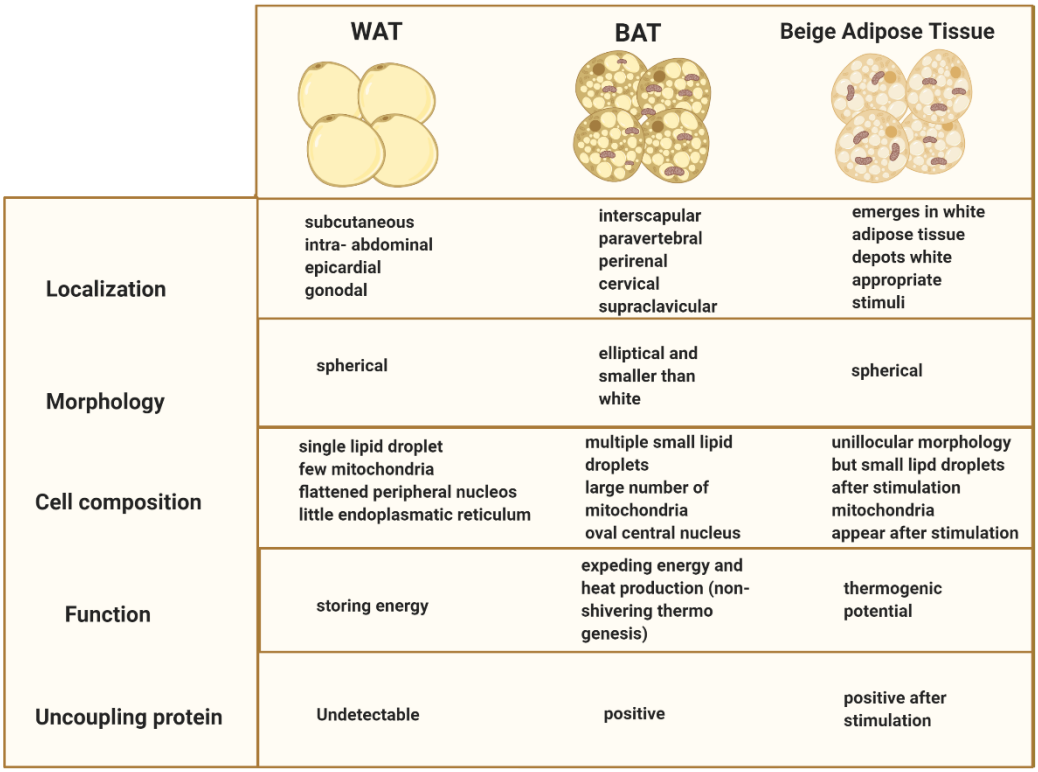


Figure 1.3 Types of Adipose Tissue and adipocytes. Main characteristics of white, brown and beige adipocytes.

1.3.1 White Adipose Tissue (WAT)

The precise origin of WAT during fetal development is still unclear; however, lineage tracking studies in mice indicate that subcutaneous and intra-abdominal deposits arise from different lineages (Chau *et al.*, 2014). Human WAT expands from birth to adolescence, by expanding the size and the number of cells. In adulthood, adipocytes change at a rate of 10% per year, however, the number of adipocytes remains relatively stable, regardless of BMI or weight loss (Spalding *et al.*, 2008). The importance of WAT is very clear in individuals or animal models without functional adipose tissue (called

lipodystrophy). Lipodystrophy results from impaired adipocyte development or from the inability to synthesize triglycerides (Hussain and Garg, 2016).

The main functions of WAT are energy isolation and storage; being mainly composed of adipocytes with a large unilocular lipid drop, which stores triacylglycerol (TAG) in white adipocytes (lipogenesis), being mobilized as fatty acids (lipolysis) when necessary. Importantly, it secretes many hormones and cytokines that regulate metabolism and insulin resistance. WAT can expand when necessary, in order to store excess energy through fat cell hyperplasia and hypertrophy. WAT is found throughout the body, with multiple deposits of subcutaneous and white visceral fat, however, the distribution of mass between each deposit varies in the population depending on age, genetics and, for some deposits, sensitivity to hormones and glucocorticoids (Berryman and List, 2017). Anatomically, WAT comprises two main deposits, the subcutaneous (SAT) and the visceral (IVA), around the internal organs. IVA is concentrated in the abdominal cavity, and subdivided into mesenteric, omental, peri-renal and peritoneal deposits **(Figure 1.4)**. In obesity, the excess WAT is closely linked to metabolic complications, such as insulin resistance and type 2 diabetes. Mesenteric and omental fat tissues are particularly important for insulin resistance and fatty liver because the liver is directly exposed to factors released from these adipose tissues through the porta hepatis region (Item and Konrad, 2012; Choe *et al.*, 2016).

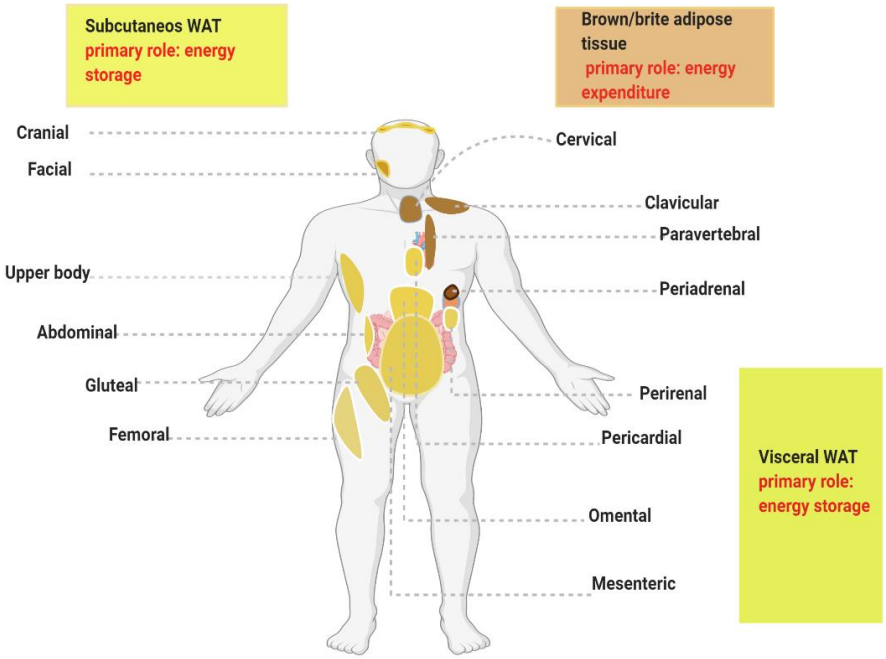


Figure 1.4 Human adipose tissue. Human adipose tissue illustrating the multiple depots of WAT and BAT.

In rodents, on the other hand, there is a great heterogeneity in the adipose tissue, characterized by different deposits of WAT, each deposit containing a mixture of white adipocytes of different subtypes (Kahn, Wang and Lee, 2019). WAT deposits in rodents includes anterior subcutaneous WAT (asWATs), comprehending the interscapular and axillary WAT (located in the scapular region), inguinal WAT (ingWAT; fixed dorsally along the pelvis to the thigh of the hind limb), perigonadal WAT (pgWAT, surrounding uterus and ovaries in females and epididymis and male testicles), and mesenteric WAT (mWAT, intestinal surface lining) **(Figure 1.5)** (Zhang *et al.*, 2018). In rodents, subcutaneous WAT transplantation improves glucose metabolism, indicating that these deposition effects are mediated, at least in part, by autonomous cell differences, and not just by anatomical position (Tran and Kahn, 2010; Stanford *et al.*, 2015; Choe *et al.*, 2016).

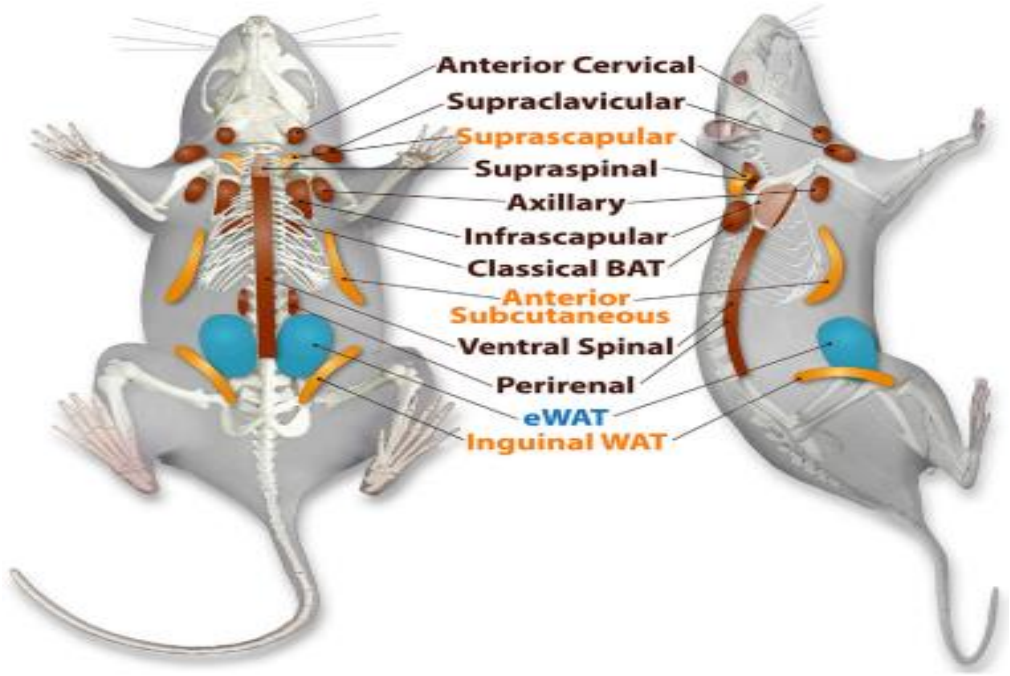


Figure 1.5 Heterogeneity and location of adipose tissue in rodents. Heterogeneity and location of adipose tissue in rodents. WAT is indicated on blue fat pads, with the ability to beige in yellow and the fatty tissues type BAT in brown (Source: Zhang *et al.*, 2018).

1.3.1.2 WAT and pathologic remodeling in obesity

Obesity is characterized by an increase in WAT associated with hypertrophy of white adipocytes, and sometimes even hyperplastic phenomena. The way in which white adipose tissue (WAT) expands and remodel directly affects the risk of developing metabolic syndrome (Choe *et al.*, 2016). The abnormally increased WAT in obesity is associated with systemic metabolic changes, mainly hyperglycemia, insulin resistance and dyslipidemia. The preferential accumulation of visceral WAT is associated with an increased risk of insulin resistance, while subcutaneous expansion of WAT appears to be protective. In addition, the pathological remodeling of WAT, typically characterized by adipocyte hypertrophy, chronic inflammation and fibrosis, is associated with insulin resistance (Vishvanath and Gupta, 2019).

Several of the comorbidities of obesity, including type 2 diabetes and, cardiovascular disease, neurodegenerative diseases and cancer seems to be related to chronic, low-grade

inflammation of the WAT. During the initial stages of excessive caloric intake, there is a physiological expansion of WAT in order to accommodate more triglycerides and prevent ectopic lipid deposition. However, if the positive energy balance is maintained, the development of obesity occurs gradually through the pathological expansion of adipose tissue, which is characterized by a combination of hypertrophy of mature adipocytes (increase in size) and hyperplasia of newly recruited adipocytes (increase in number). The dysfunction of adipose tissue is characterized by adipocyte hypertrophy, mild chronic inflammation, oxidative stress, resulting in less ability to generate new adipocytes from undifferentiated cells (pre-adipocytes) (Michele Longo *et al.*, 2019). In this sense, compromised adipogenesis triggers chronic systemic inflammation, with increased cytokine production by the expanded adipose tissue, resulting in elevated serum levels of inflammatory cytokines, such as TNF-α and several interleukins (Xu *et al.*, 2003). These cytokines lead to the recruitment of immune cells, particularly macrophages to assist in tissue remodeling, which in turn promote an increase in the production of reactive oxygen species (ROS), resulting eventually in local and systemic oxidative stress (OS) **(Figure 1.6)** (Fonseca-Alaniz *et al.*, 2007; Chait and den Hartigh, 2020).

In an obesity context, adipose tissue macrophages (ATMs) adopt a 'metabolically-activated' (MMe) phenotype, although the functions of these macrophages are poorly understood (Coats *et al.*, 2017). White adipocytes under stress suffer local infiltration of immune cells, such as Mme macrophages, which exhibit an increased expression of lipid metabolism transcripts and potentiate inflammation. This promotes the release of adipocytes killed by lysosomal exocytosis, in addition of helping to recruit other cells of the innate immune system, such as neutrophils, NK cells, TNK type 2 cells, mast cells and dendritic cells (Lumeng, 2013). Moreover, it also promotes the local increase in TCD4 and TCD8 cells, which secrete pro-inflammatory cytokines. Later, the elevated inflammatory stimuli, induce activation of IKKB, NFKB and JNK pathways, which negatively regulate the action of insulin in adipocytes and hepatocytes (Shoelson, Lee and Goldfine, 2006; Chait and den Hartigh, 2020).

Systemic inflammation influences signaling and insulin sensitivity and negatively regulates adiponectin production, which leads to insulin resistance and diabetes (Nishimura, Manabe and Nagai, 2009). All these events help to reduce metabolic flexibility and lead to the progression of obesity. The low-grade inflammatory state established in white adipose tissue generates several pathogenic results, such as insulin

resistance and possible pro-oncogenic events associated with obesity (Villarroya *et al.*, 2018).

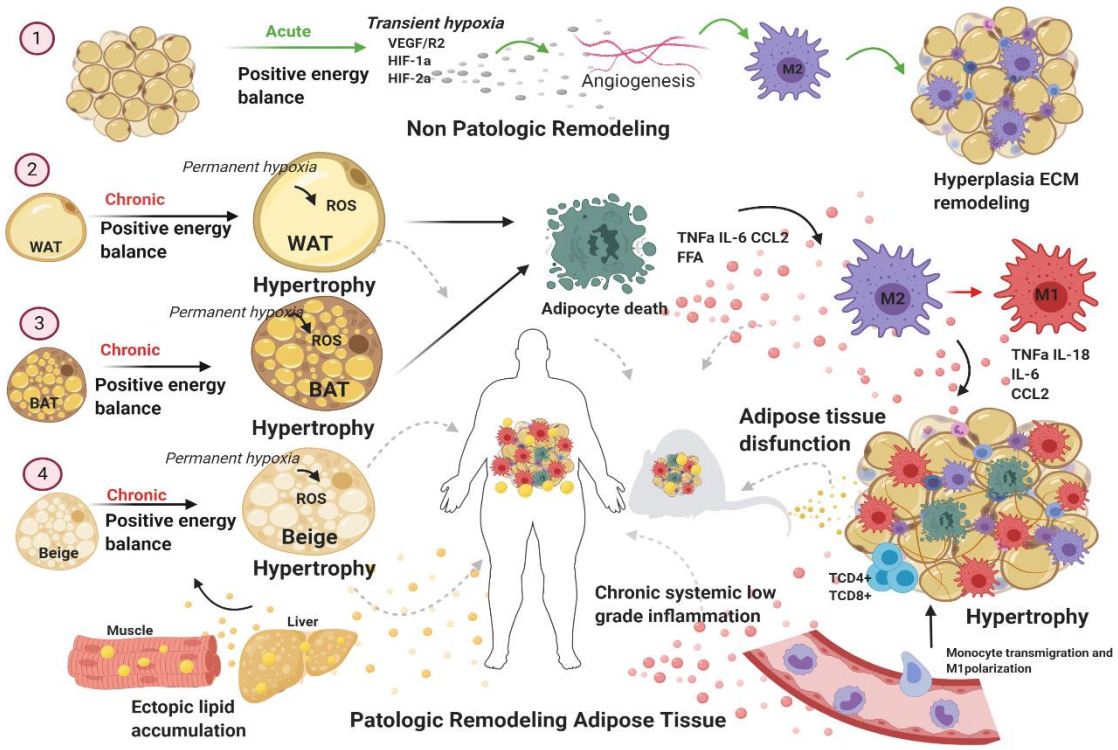


Figure 1.6 Patologic Remodeling Adipose Tissue (WAT, BAT and Beige Adipose Tissue). **(1)** Initially, the positive energy balance induces a transient hypoxia in white adipose tissue (WAT). The factors VEGF / R2, HIF-1a and HIF-2a are secreted, however the body is able to stabilize the acute process and there is only a remodeling of the non-pathological adipose tissue to reorganize the accumulation of fat. In the lean state, TH2 T cells from adipose tissue, Treg cells, eosinophils and M2-like resident macrophages predominate. Treg cells secrete IL-10 and also stimulate IL-10 secretion from resident M2-like macrophages. Eosinophils secrete IL-4 and IL-13 and still contribute to the insulin-sensitive anti-inflammatory phenotype. **(2, 3 and 4)** The positive energy balance is chronic and frequent, again inducing hypoxia in WAT, BAT and Beige tissue adipose, however a permanent hypoxia, leading to the death of adipocytes and the consequent release of cytokines such as TNF-α, IL- 6, CCL2 and FFA that stimulates the recruitment of macrophages. Immune cells mediate inflammation in adipose tissue. In obesity-induced inflammation, immune cells are recruited and contribute to the inflammation of adipose tissue. Monocytes respond to chemotactic signals and transmigrate to adipose tissues and become polarized to the highly pro-inflammatory state of type M1. Once recruited, these M1-type macrophages secrete pro-inflammatory cytokines that function in a paracrine manner. The eosinophil content decreases in obese adipose tissue. Obesity also induces a change in T cell populations in adipose tissue with a decrease in Treg content and an increase in CD4 + TH1 and CD8 + effector T cells, which secrete pro-inflammatory cytokines. The number of B cells also increases and activates T cells, which enhance the polarization of type M1 macrophages, inflammation and insulin resistance. The cytokines and chemokines of adipose tissue can also be released into the circulation and act in an endocrine manner to promote inflammation in other tissues. The set of this AT dysfunction and its harmful metabolic clinical repercussions is what we call the pathological remodeling of adipose tissue.

1.3.2 Brown Adipose Tissue (BAT)

Unlike WAT, which has the main function of storing energy, BAT is specialized in dissipating energy to produce heat, thus regulating body temperature by thermogenesis. In the last decade, BAT is in the spotlight due to its rediscovery in adult humans. This is evidenced by many clinical trials currently registered to target BAT as a therapeutic tool in the treatment of metabolic diseases, such as obesity or diabetes (Alcalá *et al.*, 2019).

BAT is a highly oxidative organ, containing multilocular adipose cells with abundant density of mitochondria that oxidize fatty acids and generate heat via decoupling of the UCP1 protein, which can also affect the metabolism of the whole body and alter the sensitivity to develop obesity (Lee *et al.*, 2014; Alcalá *et al.*, 2019). Although BAT is easily observed in infant and adult rodents, it was proposed that BAT in humans was limited to newborns, being gradually replaced by WAT with aging. However, recent studies of positron emission tomography/computed tomography demonstrated that BAT is viable and functional in human adults (Cypess *et al.*, 2009; Virtanen *et al.*, 2009; Choe *et al.*, 2016).

BAT activity is constantly related to the resting metabolic rate after cold stimulation (Van Marken Lichtenbelt *et al.*, 2009; Vijgen *et al.*, 2011; Yoneshiro *et al.*, 2011; Chen *et al.*, 2013). Other studies have reported that high BAT activity is associated with low body mass index (Cypess *et al.*, 2009; Saito *et al.*, 2009; Van Marken Lichtenbelt *et al.*, 2009; Vijgen *et al.*, 2011; Yoneshiro *et al.*, 2011) and low fat mass (Saito *et al.*, 2009; Vijgen *et al.*, 2011; Yoneshiro *et al.*, 2011). BAT mass and activity also change with age. In newborns, an increase in BAT mass at birth has been linked to a decrease in body fat accumulation during the first 6 months of life (Entringer *et al.*, 2017). In adulthood, a decline in mass and BAT activity has been observed in men and women as they age (Pfannenberg *et al.*, 2010; Yoneshiro *et al.*, 2011). A recent study examined the relationship between parameters related to adiposity and the incidence of cold activated BAT in 162 healthy volunteers, aged between 20 and 73 years, focusing, particularly on aging. The results of this study suggest the protective role of BAT against aging-related body fat accumulation (Fuse *et al.*, 2020).

1.3.2.2 BAT and pathologic remodeling in obesity

Recently, it was demonstrated in a rat model with HFD-induced obesity, that BAT shows higher levels of inflammation (macrophages and T-cell infiltration), stress in the endoplasmic reticulum, oxidative damage and worsened mitochondrial respiratory activity (Alcalá *et al.*, 2017). Compared to WAT, the BAT of mice fed with HFD tends to show an enriched gene expression in immune cells such as chemokines (*Ccl5 and Ccl8*), T-cell receptors (*Cd3g, Cd3d)* and macrophage markers (*Cd68, Emr1)* and markedly lower macrophage infiltration (CD11b + / CD11c +) (marker of classical M1 macrophage differentiation) significantly lower, suggesting that BAT "resists" obesity-induced inflammation (Fitzgibbons *et al.*, 2011). However, similarly to WAT, the BAT of mice fed with HFD (60%) exhibits high levels of mRNA for inflammation markers, including TNFα and interleukins, among other cytokines that can also be secreted by brown adipocytes (Sakamoto *et al.*, 2016). Inflammatory signs can also promote cellular apoptosis, which prevents the expansion of BAT. For example, induction of apoptosis by TNFα is traditionally described in white adipocytes (Furuoka *et al.*, 2016; Zoller *et al.*, 2016) and brown adipocytes (Miranda *et al.*, 2010). Subsequently, inflammation can indirectly inhibit the proliferation of brown adipocytes, inhibiting catecholamine signaling (Villarroya *et al.*, 2018). In addition to their metabolic effects, pro-inflammatory cytokines appear to both alter the specific thermogenic activity and impair the insulin sensitivity of BAT. Glucose uptake is essential for BAT function, as glucose supports thermogenesis both directly as fuel and indirectly, replenishing the tricarboxylic acid cycle intermediates or providing fatty acid synthase (FAs) for thermogenesis via anterior lipogenesis (Cannon and Nedergaard, 2004; Carpentier *et al.*, 2018). In fact, BAT activity in humans is most often measured based on its ability to actively absorb positron-releasing glucose derivatives, as assessed by positron emission tomography procedure (K. Y. Chen *et al.*, 2016). BAT is among the most insulin-sensitive tissues in experimental rodent models (Ferre *et al.*, 1986) and insulin-induced glucose uptake has been shown to be impaired in the BAT of obese rodent models and also in humans (Penicaud *et al.*, 1987; Orava *et al.*, 2011, 2013; Carpentier *et al.*, 2018).

After exposure to cold, it appears that large amounts of iWAT (subcutaneous) lipids flow to be transformed in BAT (Bai *et al.*, 2017; Xu *et al.*, 2019). At the same time, β-adrenergic signaling occurs in BAT, which activates the expression of the co-activator

peroxisome proliferator-activated receptor gamma (PPARγ) coactivator 1α (PGC-1α). PGC-1α is the only protein that can powerfully activate the UCP-1 in BAT cell lines (Cannon *et al.*, 1996) and mitochondrial genes (Cheol *et al.*, 2008; Hatazawa *et al.*, 2015; Cheng, Ku and Lin, 2018). In addition to the expression of UCP-1, the cold-induced BAT increases the capture of lipids for efficient heat production, accompanied by mitochondrial biogenesis (Yu *et al.*, 2015). Cold exposure is a ubiquitous environmental stress that stimulates BAT activity and the formation of beige adipocytes (also known as browning by WAT) and induces the expression of thermogenic UCP1 (Lim *et al.*, 2012; Van Der Lans *et al.*, 2013). Comprehensive studies have demonstrated distinct lipidomic or transcriptome profiles in brown and white adipose of mice and humans during activated thermogenesis (Marcher *et al.*, 2015; Lu *et al.*, 2017; Lynes *et al.*, 2018).

Diet-induced obesity is also associated with decreased expression of UCP1 (Kopecký *et al.*, 1996; Seale *et al.*, 2011; Lee and Cowan, 2013). In some cases, however, some degree of obesity-related inflammation is observed, even in the presence of increased levels of UCP1 expression and BAT thermogenesis. Cold-induced thermogenesis is severely impaired in the BAT of diet-induced obese mice and in obese humans (Penicaud *et al.*, 1987; Sakamoto *et al.*, 2016). In this sense, there is a relationship between the pathological basis of obesity and the involvement of the BAT in the pathological remodeling of the adipose tissue (Alcalá *et al.*, 2019).

1.3.3 Beige Adipose Tissue

The beige adipose tissue is found intercalated in WAT, particularly sWAT, and has a multilocular morphology like brown adipocytes, which is why they are also called brite adipocytes (brown-in-white) (Sanchez-Gurmaches, Hung and Guertin, 2016). Beige adipocytes express distinct markers of the cell surface, such as Tmem26 and Cd137 (Garcia, Roemmich and Claycombe, 2016).

In rodents, beige adipocytes are induced in response to several stimuli, including exposure to cold, β-adrenergic stimulation and exercise. After stimulation, beige adipocytes have

an increased capacity for fuel oxidation and thermogenesis. Although beige adipocytes appear similar to brown adipocytes, they both express brown and beige fat cells express PRDM16 and UCP1, in fact, they are derived from different lineages: brown adipocytes originate from a muscle-like Myf5 cell line, while beige adipocytes are derived from a Myf5 cell line (Kajimura, Spiegelman and Seale, 2015). It is important to note that the deposits of "brown fat" in humans share more molecular properties with beige rodent fat than with classic BAT (Wu *et al.*, 2012). Agents that selectively affect the browning of white fat, including the transgenic expression of PRDM16, cause metabolic benefits, suggesting that browning of white tissues may be important (Seale *et al.*, 2011). On the other hand, it is argued that there are insufficient beige cells to affect the physiology of the entire body under environmental conditions (Nedergaard and Cannon, 2013).

It is now demonstrated that brown and beige adipose tissues play a role in glucose homeostasis, insulin sensitivity and lipid metabolism, all factors related to the pathogenesis of type 2 diabetes. Several rodent deposits with topological similarities to human BAT and beige deposits have recently been identified (Zhang *et al.*, 2018). Another study showed that the pattern of gene expression in sBAT mice was like that of human sBAT (Mo *et al.*, 2017). Under prolonged cold conditions, the size and activity of brown fat increases, a term called BAT "recruitment". MTD recruitment is associated with increased proliferation and differentiation of MTD precursor cells. Cold exposure also increases the volume and BAT activity in humans (Blondin *et al.*, 2014) and in individuals with obesity and type 2 diabetes (Hanssen *et al.*, 2016). The adrenergic stress response to prolonged exposure to cold induces the darkening of WAT in rodents (Cousin *et al.*, 1992). However, in humans, ten days of exposure to cold, which resulted in the activation of pre-existing BAT deposits and a modest increase in catecholamine levels, did not result in darkening of sWAT (Van Der Lans *et al.*, 2013). These results suggest that the possibility of a trans-differentiation of adipocytes from white to beige in human sWAT depending on the adrenergic stress duration (Sidossis *et al.*, 2015). Currently, some studies are investigating how to start the darkening of white adipose tissue, these studies have been tested in animal models and in humans (Yoneshiro *et al.*, 2013; Porter, Chondronikola and Sidossis, 2015; Sidossis *et al.*, 2015; Hoppela *et al.*, 2018). These findings on the recruitment of beige fat, has modified its substantial importance in the etiology and treatment of type 2 diabetes (Kaisanlahti and Glumoff, 2019).

1.3.3.2 Beige adipose tissue pathologic remodeling in obesity

Changes in beige adipocytes are also observed during obesity induced by HFD, like the changes that occur in BAT. It includes the presence of inflammatory cytokines, immune cells, especially M1 and M2 macrophages, and a recurrent aggravating pro-inflammatory state, broadly modifying the homeostatic and physiological balance of adipose tissue, and finally leading to its dysfunction. Dysfunction of beige adipose tissue is now also recognized as one of the main pillars in the development of the obese phenotype and metabolic syndrome, as well as insulin resistance and type 2 diabetes (Zoico *et al.*, 2019). Recent studies demonstrated that beige and brown adipocytes could be important to fight obesity and metabolic diseases (Lizcano, 2019).

1.4.4 Pathologic remodeling of rodent adipose tissue with HFD

The metabolomics remodeling of white adipose tissue in obesity has a great impact on the composition of adipose tissue and on the energy expenditure of the whole body. Several studies have shown that in wild-type mice (C57BL/6J) fed with HFD for 4, 6 and 12 continuous weeks showed a significant increase in body adiposity, a decreased oxygen volume (V2 O_2) and a decreased carbon dioxide volume (V2 CO_2). In addition, they also presented changes in mitochondria functionality (loss of mitochondrial biogenetic capacity), inflammation of adipose tissue (presence cytokines and inflammatory cells), an increase in insulin resistance and pre-disposition to metabolic syndrome. In the metabolic profile, these rodents showed changes in several metabolic pathways, with dysregulation of the metabolism of carbohydrates, lipids, nucleotides and amino acids. In this sense, these metabolic changes suggest substantial changes in energy expenditure and fat storage in these animals (Sun, Kusminski and Scherer, 2011). Other studies have shown that increased hypertrophic adipocytes due to the administration of HFD feeding are less responsive to insulin and that the size of the adipocytes could serve as a predictor for the development of type 2 diabetes (Gao, Ma and Liu, 2015). Recently, new studies have shown that, changes in the size of the adipocytes after HFD feeding in rodents

correlate with a dramatic remodeling of actin cytoskeleton. This contributes to the deterioration of adipocyte function and expansion of primary adipocytes, after 2 weeks of feeding with HFD diet in C57BL6/J mice and at the same time decreasing the insulin response (Hansson *et al.*, 2019). Importantly, it has been proposed that the inflammatory responses accumulated in adipose tissue may play a causal role in obesity-induced insulin resistance. Likewise, adipose tissues appear to act as primary tissues that respond to an HFD and initiate inflammation in obesity (Longo *et al.*, 2019). Currently, many studies have been directed to explore and develop strategies to combat inflammation in adipose tissue aiming to control obesity and related diseases. In fact, this approach has been effective in several studies on animal models of obesity (Goran and Alderete, 2012; Alsaggar *et al.*, 2020)**.**

1.5 Adipose Tissue as an endocrine organ

The adipose tissue is considered one of the largest endocrine organs in the body, as well as an active tissue for cellular reactions and metabolic homeostasis. The multifunctionality of adipose tissue depends on its ability to synthesize and release many hormones, cytokines, extracellular matrix proteins and vasoactive growth factors, collectively called: adipokines (Kajimura, 2017). These influence a variety of physiological and pathophysiological processes. Adipokines are essential in the balance between apatite and satiety, regulation of body fat stores and energy expenditure, glucose tolerance, insulin release and sensitivity, cell growth, inflammation and angiogenesis and reproduction (Blüher, 2013). The dysfunctionality of the adipose tissue is clearly associated with the appearance of important pathologies, including obesity, type 2 diabetes, dyslipidemia, cancer, non-alcoholic fatty liver, heart disease, among others (Unamuno *et al.*, 2018).

The complex composition of adipose tissue: adipocytes, pre-adipocytes, mesenchymal stem cells, fibroblasts, vascular endothelial cells, macrophages and other immune cells, including T cells, seems relevant in the modulation of different endocrine activities, promoting insulin sensitivity and healthy metabolism (functional adipose tissue), insulin resistance, diabetes and systemic inflammation (adipose tissue undergoes pathological remodeling) (Choe *et al.*, 2016). The target organs / systems of adipose tissue include the

brain, liver, muscle, heart, pancreas, thymus, spleen and lymph nodes. The cytokines released in the adipose deposit also influence the local and systemic metabolism, but are slightly different from the adipokines, since the cytokines can be secreted directly from the adipose tissue, however they can also be released in other cell populations (stromal vascular cells of the adipose deposit as pre-adipocytes, fibroblasts) and immune cells (macrophages, dendritic cells and T cells) (Booth *et al.*, 2016; Scheja and Heeren, 2019).

1.6 Metabolic Mediators

1.6.1 Leptin

Leptin was the first adipokine described was leptin (Zhang *et al.*, 1994), constituting one of the first links between adipose tissue and the neuroendocrine control of energy homeostasis (Coleman, 1978). Leptin is an essential adipokine in the control of energy balance and satiety through its signaling in the central nervous system (Amitani *et al.*, 2013). Nevertheless, several effects of leptin on the systemic control of metabolism in various organs and systems are well documented and still widely explored by many groups (Varela and Horvath, 2012).

Leptin is a 16 kDa hormone that is synthesized in WAT and released into the circulation in a direct proportion to the amount of body fat (Considine *et al.*, 1996; Karvonen *et al.*, 1998). Leptin serves as an adipostat, that is, it can "inform" the rest of the body about the available fat stores. The importance of leptin in controlling systemic energy homeostasis is demonstrated by clinical observations in people with genetic leptin loss, who develop hyperphagia and morbid obesity in early childhood, which is reversed by leptin replacement therapy (Farooqi *et al.*, 1999). Leptin binds to the long leptin receptor (LepRb) isoform, leading to activation of the JAK2/STAT3 and PI3K/AKT/mTOR pathways (Cota, Barrera and Seeley, 2006; Xu *et al.*, 2018). In the hypothalamus, activation of the mTOR pathway by leptin is important for inducing energy expenditure and satiety and, consequently, a long-term effect in preventing excessive WAT accumulation (Cota *et al.*, 2006). On the other hand, mTOR has proved to be essential for adipogenesis (Shan *et al.*, 2016). Studies reported that leptin induces the formation of lipid droplets in a mTOR-dependent manner, in different types of cells (Maya-Monteiro and Bozza, 2008; Amorim *et al.*, 2018). In peripheral tissues, leptin participates in reproduction, activation of immune cells (Souza-Almeida *et al.*, 2018), cell proliferation

(Fazolini *et al.*, 2015), osteogenesis (Yue *et al.*, 2016), among many other functions. Leptin levels increase during obesity, but leptin signaling in the hypothalamus is impaired, a phenomenon called central leptin resistance (Myers *et al.*, 2010; Amitani *et al.*, 2013; Balland and Cowley, 2015). Recent studies have tested leptin's ability to modulate adipogenesis in a murine strain of NIH3T3-L1 pre-adipocytes and primary adipose-derived stromal cells from subcutaneous and visceral deposits of WAT. This study demonstrated that leptin is able to accelerate the differentiation of pre-adipocytes and that leptin induces adipogenesis even in the absence of insulin that is considered an essential hormone for the induction of adipogenesis (Palhinha *et al.*, 2019). In rodents, leptin is known to improve insulin response in skeletal muscle (Kahn and Flier, 2000) and insulin-stimulated glucose disposal (Yaspelkis *et al.*, 2004), an effect widely attributed to increased oxidation of FA and decreased intramuscular content of triacylglycerol (Minokoshi *et al.*, 2002; Stefanyk *et al.*, 2011). Recently, it was shown that the administration of leptin to obese rodents induced by diet reverses impaired glucose uptake, via improved insulin signaling. In the muscle of these obese rats, treatment with leptin increases the phosphorylation of AS160 and AKT and simultaneously reduces the concentration of harmful lipid intermediates, DAG and ceramide (Booth *et al.*, 2016). Leptin has a direct and indirect effect on increasing the expression of IGFBP-2 mRNA and the concentration of proteins that subsequently mediates the enhanced glucose uptake by increasing phosphorylated AKT and decreasing PTEN levels in the insulin signaling pathway. Leptin exerts insulin sensitization effects centrally through the NHS, as well as directly through the STAT-3 and PI3K/AKT (Yau *et al.*, 2014). In fact, leptin is a hormone that modulates glucose homeostasis through central and peripheral mechanisms. Central leptin plays a role in integrating metabolic signals that balance energy intake and expenditure. Peripherally, this adipokine regulates lipid and glucose homeostasis in various tissues. Dysfunctions in the leptin endocrine system can cause several serious pathologies, mainly obesity (leptin resistance) diabetes and related metabolic diseases (Friedman and Halaas, 1998; Friedman, 2014).

In addition to regulating food intake, leptin increases energy expenditure through sympathetic nerve activity. In rodents, leptin stimulates the thermogenesis of BAT by increasing the expression of UCP-1 (Haynes *et al.*, 1997; Scarpace *et al.*, 1997; Park and Ahima, 2015). The thermogenic effect of leptin is mediated in part by suppression of MCH and the Forkhead box O1 transcription factor (FoxO1) (Cheng and White, 2011).

1.6.2 Adiponectin

Adiponectin is a 30 KDa adipokine encoded by the *AdipoQ* gene, produced and secreted mainly by adipocytes and highly abundant in human plasma. Adiponectin is known to increase insulin sensitivity of target organs, such as liver and muscle, finally regulating peripheral glucose and fatty acid metabolism (Hotta *et al.*, 2001; Yamauchi *et al.*, 2002).

The adiponectin hormone is unique in that it correlates positively with lean body types and is linked to insulin sensitivity and high-density lipoprotein (HDL-C) levels (Duncan *et al.*, 2004; Schneider *et al.*, 2005). Consequently, adiponectin and its receptors decrease in obese or non-obese pre-diabetic individuals (Díez and Iglesias, 2003). Through its AMPK-stimulating activities, adiponectin has similar effects on exercise in relation to increased glucose uptake and suppression of hepatic glucose production (Booth *et al.*, 2016). In addition of being a metabolic regulator, adiponectin is also known for its anti-inflammatory and antioxidant activity (Takemura *et al.*, 2007; Liu *et al.*, 2015). These characteristics make adiponectin a protective factor in conditions such as obesity, type 2 diabetes and cardiovascular diseases (Yamauchi *et al.*, 2003; Spranger, 2006; Antoniades *et al.*, 2009).

Adiponectin acts by binding to three different receptors: adiponectin receptor 1 (AdipoR1), adiponectin receptor 2 (AdipoR2) and T-cadherin. AdipoR1 and AdipoR2 are the most abundant sites for binding to adiponectin and mediate most actions of adiponectin (Yamauchi *et al.*, 2003, 2007). The activation of AdipoRs by adiponectin leads to the recruitment of the APPL1 adapter protein (adapter protein, phosphotyrosine interacting with the PH domain and leucine zipper 1) (Mao et al., 2006) which, in turn, mediates adiponectin signaling downstream through various pathways including AMPK, PI3K-Akt, MAPK-Erk1 / 2, PPARα, p38-MAPK, PTEN and JNK (Chandrasekar *et al.*, 2008; Coope *et al.*, 2008; Kim and Park, 2019).

In ob/ob and non-obese (NOD) diabetic mice, a single physiological injection of purified adiponectin decreases circulating fasting glucose levels by approximately 30% and is associated with a reduction in hepatic glucose production (Berg *et al.*, 2001). Adiponectin is needed to improve insulin sensitivity via the AMPK pathway. Supporting this hypothesis is the fact that rodents fed with HFD develop rapid glucose intolerance and inhibit the response to the PPARy agonist, a robust stimulator of AMPK activity

(Nawrocki *et al.*, 2006). Moreover, it was showed that low adiponectin concentration is associated with increased levels of circulating inflammatory markers such as: TNF alpha, C-reactive proteins, IL-6 and reactive oxygen species (Ouchi and Walsh, 2007). In this sense, its inverse relationship with insulin resistance, adiposity and inflammatory markers support the role of protective adiponectin for diseases related to obesity, such as type 2 diabetes; indicating glucose tolerance and metabolic homeostasis (Achari and Jain, 2017).

1.7 The blood-brain barrier and CNS

The brain plays an important role in the orchestration of energetic homeostasis; more precisely this function is performed by the hypothalamus; receiving information from peripheral tissues, responding and regulating various sensations, such as reward and satiety. In addition, direct innervation and indirect signaling via hormones allow for a feedback system back to peripheral organs (Clemmensen *et al.*, 2017).

The transport of these peripheral signals to the brain and target regions is regulated in a controlled manner by the blood-brain barrier. In essence, the BBB is a layer of endothelial cells that divides the microvasculature of the brain compartment. These cells differ from other endothelial cells in their almost impermeable narrow junctions and in that they have various membrane structures, such as receptors, transporters and metabolically active molecules, guaranteeing their selection function (Hampl, Bičíková and Sosvorová, 2015). Additionally, several types of cells, such as pericytes and astrocytes, contribute to the architecture and function of the BBB, which protects the brain against neurotoxins, while governing the passive diffusion of hydrophobic gases and molecules, as well as the active transport of hydrophilic nutrients, large-scale peptide amino acids and hormones (Obermeier, Daneman and Ransohoff, 2013; García-Cáceres *et al.*, 2016).

The BBB acts as a secretory and targets endocrine tissue. The main part concerns the transport of large classes of hormones: steroids, including neurosteroids, thyroid hormones, insulin, leptin and other peptide hormones that regulate energy homeostasis, as well as growth hormone and various cytokines. Moreover, BBB is itself a target for hormones, such as leptin and insulin, which affect many of its functions. The BBB is also a secretory body, releasing substances into the blood or interstitial fluid of the brain. The BBB selectively allows classic and non-classic hormones to enter and exit the CNC, allowing it to be an endocrine target and a secretory tissue. The BBB can also be affected by endocrine disorders, such as diabetes mellitus, and can cause or participate in

endocrine disorders, including those related to thyroid hormones and obesity. BBB's endocrine mechanisms may extend the definition of endocrine disease to include neurodegenerative conditions, including Alzheimer's disease, and hormones to include cytokines, triglycerides and fatty acids (Banks, 2019).

1.8 CNS and Leptin

One of the peptide hormones that requires active BBB transport is leptin, to reach its neuronal targets in the medio basal hypothalamus (MBH) and other areas of the brain. How leptin is transported through the BBB and its response neurons, defined by the expression of the leptin receptor (LepR), is still a matter of debate (Rodriguez et al., 2010). Previous work suggest that the transport of BBB leptin is facilitated by tanycytes, that is, ependymal cells specialized in the median eminence (ME), a circumventricular organ at the bottom of the MBH involved in the secretion of signals derived from the brain to the pituitary and peripheral organs by circulation (Scott and Knigge, 1970; Henry *et al.*, 2011).

Some adipokines such as leptin and TNFα can cross the BBB and act directly on the brain, while other adipokines act on the endothelial brain cells, regulating the permeability of the BBB and the access of other circulating mediators to the brain. It is important to note that in pathological conditions such as inflammation, the integrity of the BBB is compromised, allowing the penetration of adipokines and other substances to which the brain is normally inaccessible (Thundyil *et al.*, 2012).

In the ME, leptin can diffuse freely in the parenchyma of the MS due to the fenestration of the capillaries and the lack of a functional BBB. Subsequently, leptin is transported via tanycytes to the cerebrospinal fluid (CSF) of the ventricular space, from where it diffuses laterally in the MBH (Balland *et al.*, 2014). More recent results points to a crucial direct involvement of BBB endothelial cells as the main leptin transport mechanism (Di Spiezio *et al.*, 2018). The knockdown of LepR specifically in BBB endothelial cells was functionally linked to impaired leptin transport to CSF and LepR positive brain regions and aggravated obesity when mice were exposed to HFD (Harrison *et al.*, 2019).

The expression of LepR is found especially in the choroid plexus (CP) (Bjørbæk, Elmquist, Michl, *et al.*, 1998), an important component of the BBB anchored in the walls of the lateral, central and fourth ventricles. Although CSF production is the most described role of the CP, it also acts as an important selective portal for CSF. The molecules that enter the CSF can diffuse freely in many regions of the brain that line the ventricles (Whish *et al.*, 2015). Consequently, CP is strongly linked to leptin transport to the brain (Zlokovic *et al.*, 2000). There is substantial evidence indicating that leptin regulates the hippocampal synaptic plasticity and memory. LepRs in hippocampal neurons are closely associated with the somato-dendritic and synaptic regions, indicating the potential of leptin to modulate synaptic function (Harvey, 2013). On the other hand, leptin treatment has been shown to improve learning performance and memory in different models (Oomura *et al.*, 2006; Irving and Harvey, 2014).

At the intracellular level, leptin signaling is downregulated by the SOCS3 suppressor and PTP1B. SOCS3 binds to LepR and JAK2 to inhibit its activities, while PTP1B dephosphorylates tyrosine residues that deactivate LepR and JAK2. PTP1B has been associated with central resistance to leptin in humans (Myers *et al.*, 2010), as well as in a variety of animal models of obesity (Cheng *et al.*, 2002; Zabolotny *et al.*, 2002; Ye, 2009) and aging (Morrison *et al.*, 2007). SOCS3 and PTP1B were also found isolated in the brain of mouse models with Alzheirmer's disease (Iwahara *et al.*, 2017; King *et al.*, 2018) and patients with AD (Walker *et al.*, 2015). Therefore, targeting PTP1B and SOCS3 can be valuable in overcoming central leptin resistance in obesity, aging and AD (Vieira *et al.*, 2017).

Interestingly, most obese individuals, however, are not deficient in leptin, but rather resistant to leptin. Despite the high circulating levels of leptin, individuals resistant to leptin usually experience a desire to eat extra calories, which prevents sustainable weight loss (Woods *et al.*, 2000). The molecular reasons for leptin resistance are still not entirely clear, but they can lead to impaired signaling, linked to SOCS3 expression (Bjørbæk, Elmquist, Frantz, *et al.*, 1998), elevated levels of circulating c-reactive protein (Chen *et al.*, 2006) or impaired histone deacetylase 5 activity in the hypothalamus (Kabra *et al.*, 2016). In addition, impaired leptin transport to the brain is seen as an important contributor to leptin resistance (Banks and Farrell, 2003). This is supported mainly by leptin transport studies that used radiolabeled leptin and showed a decrease in blood and

CSF leptin rates, suggesting blunted leptin transport kinetics and therefore a cause of leptin resistance (Banks, 2001). Likewise, other studies carried out a series of leptin injection experiments in the intracerebral ventricle (ICV), which also indicated a decrease in BBB leptin transport (El-Haschimi *et al.*, 2000). Moreover, current studies have addressed the issue that leptin transport to CP, ME and MBH (medial basal hypothalamus) is altered in obese mice induced by the HFD diet and therefore resistant to leptin, compared to lean and leptin-sensitive mice (Harrison *et al.*, 2019). In addition, leptin levels were compared in ME and MBH of HFD mice subjected to weight loss by modest diet, deep caloric restriction or repeated treatment with exendin-4, to clarify whether altered leptin transport to MBH can explain the superior leptin restoration. Leptin re-sensitization requires pharmacotherapy, but it does not appear to be restricted to a single signaling pathway (Müller *et al.*, 2012). Recently, the understanding of the transport of leptin through the BBB was analyzed in an innovative way, using 3D images of the entire brain of mice. A comparable accumulation of leptin was shown in the circumventricular organs of lean and obese mice, predominantly in the CP from fluorescence microscopy in light sheets. The results suggest a crucial role for CP in controlling leptin transport to the cerebrospinal fluid and from there to target areas such as MBH, potentially mediated by the leptin receptor. Similar levels of leptin in the circumventricular organs and the MBH of lean and obese mice further suggest the intact BBB transport of leptin in leptin-resistant mice (Harrison *et al.*, 2019).

1.9 CNS and Insulin

Insulin, a pleiotropic hormone, has several important metabolic effects on the body. A fundamental metabolic action of insulin is to control the concentration of glucose in the blood, stimulating the transport of glucose to muscle and fat tissue and inhibiting the production of hepatic glucose (Samuel and Shulman, 2012). Recent studies have highlighted the important role of insulin action in the CNS, in glucose and energy homeostasis, in memory and mood, in addition to aging and neurodegenerative diseases (Brown, Lockwood and Sonawane, 2005; Hölscher, 2020). Currently, it is clear that the brain is recognized as an insulin-sensitive organ, responsible for physiological changes in altered metabolic disorders, such as obesity and type 2 diabetes (Sripetchwandee, Chattipakorn and Chattipakorn, 2018; Zierath, 2019). In the brain, the insulin receptor is

widely expressed in regions such as the hypothalamus, hippocampus and cerebral cortex, all involved in the metabolic control of insulin action, including eating behavior, body weight homeostasis, neuronal development and cognitive function (Timper and Brüning, 2017). Insulin also plays important roles in the formation of neuronal circuits, synaptic maintenance, neuronal survival, dendritic afforestation, in addition to learning and memory (Banks, Owen and Erickson, 2012). Currently, it is known that there is no cell type in the CNS that does not express the insulin receptor (Rhea, Salameh and Banks, 2019).

A recent study has shown that the insulin receptor is expressed as two functionally distinct isoforms, differentiated by a single exon encoding for 12 amino acids. The two receptor isoforms, called IR/A and IR/B, are expressed in a highly specific way for tissues and cells and the relative proportions of the different isoforms vary during the stages of development, aging and disease. This study showed for the first time that IR/A and IR/B are both expressed in neurons in the adult human brain (Spencer *et al.*, 2018).

In rodents, the expression of the IR is more abundant in endothelial cells, around twice as high as astrocytes, with neurons lagging behind in terms of RNA expression levels (Zhang *et al.*, 2014). However, this expression pattern was not observed in human tissue samples (Zhang *et al.*, 2016). In human astrocytes, in vitro and in vivo; a characterization, subcellular localization and modulation of the IR/A and IR/B receptors was demonstrated while glial cells demonstrated to express IR/A and IR/B in vitro, in neurons, only IR/A demonstrated to be expressed in vitro (Garwood *et al.*, 2015). Subsequent analyzes of the IR expression of the neuronal IR expression appear to show that IR/A is predominantly expressed in populations of immature/progenitor cells, with little or no IR/B detected (Ziegler *et al.*, 2014).

An important experimental study showed that the specific brain deletion of the IR in rodents leads to obesity, hyperphagia and systemic insulin resistance, clearly demonstrating the important role of cerebral insulin signaling in the regulation of metabolic homeostasis (Bruning *et al.*, 2000). Emerging data also reveal that cerebral insulin signaling plays a central role in regulating peripheral metabolism by modulating the output from the autonomic nervous system to peripheral tissues (Scherer *et al.*, 2011). For example, activation of hypothalamic insulin signaling inhibited lipolysis and stimulated lipogenesis. However, it was observed that rodents without the neuronal IR demonstrated unrestricted lipolysis and decreased lipogenesis in adipose tissue (Scherer

et al., 2011).In addition to these metabolic roles of the insulin receptor in the brain, a recent study also demonstrated that insulin resistance in the brain induces dopaminergic dysfunction, leading to anxiety and behavioral disorders (Kleinridders *et al.*, 2015). To gain access to its receptor in the CNS, the insulin produced by the pancreatic beta cells is transported across the BBB through a saturable transport system (Banks, Owen and Erickson, 2012). Insulin crosses the BBB in the hypothalamus, medullary bridge, hippocampus, striatum, parietal cortex and frontal cortex, but not in the cerebral cortex, thalamus and occipital cortex (Rhea, Salameh and Banks, 2019). Several animal studies in animal models have shown that impairment of the BBB's insulin transport system is found in insulin resistance associated with obesity, as well as in various physiological extremes, including hunger, hyperglycemia, immune system activation and hibernation, suggesting an important function of the BBB in maintaining normal metabolic homeostasis (Tietz and Engelhardt, 2015; Konishi *et al.*, 2017; Rhea, Salameh and Banks, 2019).

There are some evidence indicates that insulin is also synthesized in the brain. It was detected the C-peptide immunoreactivity in human CNS neurons (Dorn A et al., 1983; Dorn A et al., 1982) and pro-insulin or pre-pro-insulin mRNAs in animals and cell culture systems in mouse *foetal fetal* brain (Birch, Christie and Renwick, 1984; Ghasemi *et al.*, 2013). Indications that neurons are capable of producing and secreting insulin have been reported for the first time in a primary neuronal culture from the rat brain, but not in primary glial cultures (Clarke *et al.*, 1986). More recently, insulin secretion has also been reported in cultured astrocytes (Takano *et al.*, 2018). Recent studies have shown for the first time, in rodents, that insulin is produced by the choroid plexus epithelial cells (EChP), which is a highly vascularized tissue found in the cerebral ventricles. The production and release are modulated at least by serotonin, but not by glucose (Mazucanti *et al.*, 2019). However, the source of insulin from the CNS, peripheral, central or both, is still much debated and more evidence is needed to confirm this in humans (Spinelli, Fusco and Grassi, 2019).

An intracerebroventricular insulin injection increased pancreatic insulin secretion in dogs (Chen et al., 1975) and decreased food intake and body weight in baboons (Woods et al., 1979). Likewise, intranasal insulin also decreased circulating glucose concentrations in dogs (Harai et al., 1978) and rhesus monkeys (Kumar et al., 1980). Inactivating the brain-

specific insulin receptor did not affect the brain development or neuronal survival but caused diet-sensitive obesity with increased body fat (Bruning *et al.*, 2000). Recently, several studies have focused on elucidating the neuronal circuitry underlying the action of insulin on body weight and glucose homeostasis in the hypothalamus. The arcuate nucleus of the medio basal hypothalamus, adjacent to the third ventricle and median eminence, contains important populations of neurons that respond to afferent signals of hormones and nutrients. Insulin receptors are highly expressed in neurons that express proopiomelanocortin (POMC) or agouti-related peptide (AgRP) and neuropeptide Y (NPY) in the arcuate nucleus (Hill *et al.*, 2010; Varela and Horvath, 2012). These first-order neurons project to second-order neurons in other hypothalamic areas (for example, paraventricular hypothalamus, lateral hypothalamus and ventromedial hypothalamus) or extra-hypothalamic areas (for example, the solitary tract nucleus) to alter eating behavior or energy metabolism. Insulin decreases the expression of orexigenic AgRP and NPY, leading to decreased food intake (Sipols, Baskin and Schwartz, 1995). In addition, insulin increases POMC expression, resulting in increased levels of α-melanocyte stimulating hormone (α-MSH), which promotes anorexia and increases energy expenditure (Benoit *et al.*, 2002), presumably through of neurons that express melanocortin-4 (MC4R) receptors (Balthasar *et al.*, 2004). In AgRP neurons, once is Akt translocated to the nucleus in response to insulin, it inhibits the transcriptional activity of the fork box O1 protein (FOXO1) by phosphorylating FOXO1 and leads to the exclusion of FOXO1 from the nucleus, resulting in reduced AgRP expression (Kim *et al.*, 2006; PENG *et al.*, 2019). In unstimulated POMC neurons, FOXO1 improves the recruitment of histone acetylases and co-suppressors in the promoter region of the *POMC* gene to suppress their expression. After stimulation with insulin, the phosphorylated FOXO1 is translocated from the nucleus, resulting in the disinhibition of the POMC promoter, thereby increasing POMC expression (Kitamura *et al.*, 2006; Belgardt, Okamura and Brüning, 2009; Ruud, Steculorum and Bruning, 2017).

A recent study investigated the effect of intra-hippocampal infusion of insulin on spatial cognition and associated neuroinflammation in a context of diet-induced obesity in rodents. In this study, it is demonstrated that HFD impairs the performance of spatial memory accompanied by inflammation of the hippocampus. These harmful effects of HFD are attenuated in rodents receiving an intra-hippocampal infusion of insulin, which also reduces inflammation of the hippocampus. These findings may be of great interest to human subjects suffering from obesity, who are now considered with an increased risk

factor for dementia (Gladding *et al.*, 2018). In fact, it has been reported that insulin signaling is impaired in the brain of AD, which (Alzheimer's disease) rand represents a major breakthrough and advance in the current understanding of the pathophysiology of AD disease. In addition, evidence demonstrates that the molecular mechanisms that lead to cerebral insulin resistance in AD share a substantial similarity with those involved in peripheral insulin resistance in diabetes and obesity. This includes chronic low-grade inflammation, TNF-α-mediated IRS-1 inhibition and stress in the endoplasmic reticulum (Diehl, Mullins and Kapogiannis, 2017). Central insulin resistance potentially predisposes for the development of neurodegeneration, creating a clinical link between type 2 diabetes and nonvascular dementia (Hölscher, 2020). It signals (Thambisetty *et al.*, 2013; Vieira *et al.*, 2017; Gabbouj *et al.*, 2019; Miriam Longo *et al.*, 2019)downstream in the brain is an important research topic, as is its suggested involvement in brain function in aging and in various diseases. There are substantial epidemiological data that support the role of central insulin resistance related to the onset of metabolic disorders, type 2 diabetes, neurodegenerative diseases such as Alzheimer's disease and reduced life span in humans (Thambisetty *et al.*, 2013; Vieira *et al.*, 2017; Gabbouj *et al.*, 2019; Miriam Longo *et al.*, 2019).

1.10 Leptin and Obesity

As mentioned, obesity is characterized by the expansion of white adipose tissue, with subsequent imbalance in the production and signaling of adipokines, accompanied by the development of chronic low-grade inflammation (Unamuno *et al.*, 2018). Moreover, obesity is a well-characterized factor for increasing the risk of type 2 diabetes and an increase in leptin levels is a direct indicator of obesity. Therefore, numerous studies show a positive association between leptin and type 2 diabetes in adults (Fischer *et al.*, 2002; Chen, Qin and Ye, 2014), however, the molecular mechanisms that link leptin to the development of type 2 diabetes are not yet fully understood (Chobot *et al.*, 2018).

Recently, a study investigated leptin's ability to modulate adipogenesis in a murine strain of NIH3T3-L1 pre-adipocytes and primary adipose-derived stromal cells (ASCs) from subcutaneous and visceral deposits of WAT. The study demonstrated that leptin is able to accelerate the differentiation of pre-adipocytes and induces adipogenesis, even in the absence of insulin that is considered an essential hormone for the induction of

adipogenesis (Green and Kehinde, 1975; Palhinha *et al.*, 2019). WAT is recognized as an essential immunoendocrine organ that controls energy balance and metabolism (Vegiopoulos, Rohm and Herzig, 2017). The concentration of leptin in the deposits of WAT may be much higher than its circulating serum levels. Although adipocyte hypertrophy and hyperplasia occur in this leptin-rich environment, the paracrine and autocrine effects of leptin on adipocyte differentiation are still unclear (Palhinha *et al.*, 2019).

Several studies have shown that the responsiveness to leptin decreases with obesity, aging and neurodegenerative diseases, a phenomenon called leptin resistance. Leptin resistance affects several processes, such as food intake, insulin sensitivity, inflammation and cognition. In obesity, leptin resistance leads to an increase in the production of leptin by adipocytes and hyperleptinemia, in an attempt to compensate for the low responsiveness to leptin. The decrease in leptin signaling in the CNS may be related to defective leptin transport through the BBB, negative regulation of LepR and / or deficiency of leptin signal downstream LepRs (Myers *et al.*, 2012; de Git and Adan, 2015; Hoffmann *et al.*, 2016; Banks *et al.*, 2018). In rodents with free access to a HFD, leptin resistance manifests itself after just a few weeks (Frederich *et al.*, 1995) and leptin transport in the blood is reduced (Banks, 2001). However, more recent data from rodents suggest that impaired transport of BBB leptin is acquired during the development of obesity and is therefore a secondary defect (Banks *et al.*, 2004; Izquierdo *et al.*, 2019). It is suggested that the excess triglycerides present in the HFD diet can impair leptin transport through the BBB, causing central leptin deficiency (Banks et al., 2004). In addition, it has recently been shown that triglycerides can cross the BBB to directly induce resistance to hypothalamic leptin and the insulin receptor, leading to decreased satiety and cognitive impairment in mice (Banks *et al.*, 2018). In fact, deficient leptin transport through the BBB, leading to reduced leptin entry into the brain, has also been described in elderly mice and in AD mouse models (Carro *et al.*, 2005; Dietrich *et al.*, 2008).

The increasing prevalence of obesity in Western society is paralleled by a significant increase in autoimmune diseases. Currently, many studies are being performed regarding the effects of leptin on innate and adaptive immunity. In innate immunity, leptin increases the cytotoxicity of natural killer cells (NK) and promotes the activation of granulocytes, macrophages and DCs. Leptin also regulates the polarization of the M1 or M2 phenotype and modulates DCs, allowing them to initiate type 1 helper T cells (Th1). In adaptive

immunity, leptin increases the proliferation of naive T cells and B cells while reducing that of regulatory T cells (Treg). Leptin promotes the switch to a pro-inflammatory Th1 phenotype (which secretes IFNγ) instead of the anti-inflammatory Th2 (IL-4 secretory). Thus, it is suggestive that leptin at dysfunctional levels, specifically in obesity, somewhat reduces the body's immune defense and could predispose to autoimmune and immune-metabolic diseases such as Lupos and Type 2 Diabetes, respectively (Francisco *et al.*, 2018). In fact, the plasma leptin concentration can be a biological marker of the inflammatory state in obesity and the appearance onset and evolution development of pathologies associated with dysregulation of the immune system, and further evaluations will be essential to establish leptin as a clinical biomarker. In addition, the control of bioactive leptin levels by high-affinity leptin-binding molecules, leptin-directed miRNAs, LEPR antagonists or humanized monoclonal antibodies against LEPR are likely to be viable therapeutic approaches (Otvos *et al.*, 2011). Recombinant leptin is now available for use in patients with congenital leptin deficiency, while synthetic analog leptin metreleptin has been approved for the treatment of lipodystrophy (Tchang, Shukla and Aronne, 2015).

1. 11 Insulin and Obesity

Obesity is directly or indirectly associated with several metabolic dysfunctions, including chronic low-grade inflammation and insulin resistance, which are causally related to the development and progression of type 2 diabetes (Thaler and Schwartz, 2010b; Gregor and Hotamisligil, 2011; Boden *et al.*, 2015). Insulin-sensitive tissues, including adipose tissue, skeletal muscle and liver, are deeply affected by obesity at molecular and functional levels (Barazzoni *et al.*, 2018). Therefore, numerous studies have been dedicated to the understanding of the relationship between obesity, inflammation and insulin resistance (Afshin *et al.*, 2017; Yazıcı and Sezer, 2017; Barazzoni *et al.*, 2018; Kolb *et al.*, 2018). For example, it was shown that an increased production of tumor necrosis factor α (TNFα) and non-esterified fatty acids (AGNE) by hypertrophied adipocytes leads to reduced levels and dysregulation of insulin and dysregulation of signaling molecules, such as in the insulin receptor substrate (IRS), resulting in resistance to insulin in the liver and skeletal muscle (de Luca and Olefsky, 2008; Barazzoni *et al.*, 2018). A high-fat diet predisposes to obesity, insulin resistance and low-grade

inflammation (Gregor and Hotamisligil, 2011). HFD consumption altered the hypothalamic response to leptin and insulin, leading to deregulation of energy homeostasis control. In fact, these two hormones are anorectic and considered to be the main regulators of energy homeostasis (Könner, Klöckener and Brüning, 2009; Belgardt and Brüning, 2010).Increased oxidative stress and inflammation associated with obesity are also involved in the onset and exacerbation of insulin resistance (Yazıcı and Sezer, 2017). The inflammation associated with obesity begins in the adipose tissue and in the liver, with the infiltration of macrophages and expression of pro-inflammatory cytokines. Inflammation inhibits insulin signaling activity in adipocytes and hepatocytes through several mechanisms. For example, the inhibition of IRS-1 (insulin receptor substrate 1) and insulin receptor in the insulin signaling pathway (Boucher, Kleinridders and Ronald Kahn, 2014). Another mechanism involves the inhibition of the PPARγ function (Ye, 2008; Varga, Czimmerer and Nagy, 2011). PPARγ is a nuclear receptor that boosts lipid synthesis and fat storage in cells. Its activity is dependent on ligands, which include long-chain fatty acids and thiazolidinedione (TZD). It induces the expression of enzymes or proteins in lipogenesis or storage through transcriptional activation (Giorgino *et al.*, 2009). The reduction of PPARγ activity contributes to insulin resistance. The third other mechanism involves the is to increase of free plasma fatty acid (FFA) by stimulating lipolysis and blocking TG synthesis(Ormazabal *et al.*, 2018). These three pathways mechanisms mediate these effects of inflammation, which are mainly. These effects are seen observed mainly in adipose tissue and the liver. The action of muscle insulin does not appear to be sensitive to inflammation (Wu and Ballantyne, 2017).

In obesity, inflammation of the liver is associated with hepatic steatosis (fatty liver), a result of the accumulation of lipids in hepatocytes. Pro-inflammatory cytokines and accumulation of Kupffer cells (hepatic macrophages) are elevated in the inflammatory response (Serviddio, Bellanti and Vendemiale, 2013). Although these cytokines are known to block the insulin signaling pathway in hepatocytes, the biological significance of inflammation remains to be investigated. Non-alcoholic fatty liver disease (NAFLD) represents the result of impaired lipid metabolism, redox imbalance and insulin resistance in the liver (Engin, 2017). The inflammation of the liver appears to be a feedback response in an effort to mitigate the accumulation of lipids in hepatocytes. One possibility is to increase the conversion of fatty acid into glucose in hepatic gluconeogenesis, in which acetyl-CoA derived from fatty acid is used as a building material in the production of glucose. Gluconeogenesis is normally inhibited by insulin and is increased under insulin

resistance. Inflammation induces hepatic insulin resistance to promote gluconeogenesis. Overexpression of IKKβ in the liver leads to liver inflammation and hepatic insulin resistance (Cai *et al.*, 2005), whereas the knockout of IKKβ in the liver reduced inflammation and protected rodents from insulin resistance (Arkan *et al.*, 2005). Although inflammation is well documented in the pathogenesis of insulin resistance, it seems not a good target in the treatment of insulin resistance (Ye, 2013). This conclusion was supported by the following observations, i) anti-inflammatory therapies failed to generate a satisfactory result in improving insulin sensitivity in most clinical trials, although hyperglycemia parameters have been improved in some studies (Gao and Ye, 2012); ii) Inflammation did not impair insulin sensitivity in several models of transgenic mice with chronic inflammation (Jiao *et al.*, 2012; Tang *et al.*, 2012) and iii) elevated levels of IL-6 in the plasma is associated with improved insulin sensitivity during exercise (Pedersen, Febbraio and Mooney, 2007).

Historically, it is described that the prevalence of insulin resistance is increased in individuals with obesity (Olefsky, Farquhar and Reaven, 1974). In a recent study, there is evidence that obesity, by itself, was an independent predictor of fasting hyperinsulinemia (Kim *et al.*, 2015). Moreover, previous studies have shown that hyperinsulinemia in individuals with obesity is related to an increase in the secretion of insulin and a decrease in insulin release (Ferrannini *et al.*, 1997; Lorenzo *et al.*, 2013). Hyperinsulinemia means a constant high level of plasma insulin in fasting conditions. Although it is generally accepted that hyperinsulinemia is a result of insulin resistance, several results also suggest that a high level of insulin can lead to insulin resistance, especially in the presence of fatty acids (Ye *et al.*, 2007). On the other hand, hyperinsulinemia is due to overproduction or decreased insulin clearance in obesity. The balance between the rate of insulin production and the rate of elimination of insulin determines the level of insulin in the plasma. β cells in pancreatic islets are a single source of insulin. In obesity, there is a greater stimulation of the function of β cells, generating a substantial increase in the number of cells in the pancreatic islets; during weight gain, there is considerable stimulation of β cells by fatty acids and glucose (Czech, 2017). Resistance to leptin in β cells can contribute to the excessive production of insulin in these cells (Gray *et al.*, 2010). Furthermore, leptin inhibits insulin production in β cells (Zhao AZ et al., 1998). In knockout mice for β-specific leptin receptors, this inhibition was abolished, and hyperinsulinemia was observed. It was shown that rodents first exhibited hyperinsulinemia and then developed insulin resistance. This study provides an optimal

response to hyperinsulinemia in models of leptin-resistant mice, such as ob/ob mice (leptin deficient) and db/db mice (deficient in leptin receptors) (Gray *et al.*, 2010). Other evidence for this is that mice with extra copies of the insulin gene had two to four times an increase of insulin in their blood, although they presented normal weight, but with insulin resistance, hyperglycemia and hypertriglyceridemia. In fact, both in rodents and humans, increasing doses of insulin induce hyperinsulinemia and insulin resistance (Shanik *et al.*, 2008).

On the other hand, the reduction of insulin production in β cells by decreasing the expression of the insulin gene prevented insulin resistance in mice with access to HFD (Mehran *et al.*, 2012). Several studies consistently suggest that excess insulin production or supply leads to hyperinsulinemia and causes insulin resistance in animal models (King, 2012; Wong *et al.*, 2016). The insulin signaling pathway has a negative feedback loop to control the activity of the pathway precisely in response to insulin stimulation. This loop is activated by the insulin signal to prevent the activation of responses to insulin-induced stress (Ye and Kraegen, 2008). This mechanism has received consistent support from studies in humans and animals (Shanik *et al.*, 2008; Sah *et al.*, 2016).

1.12 Hypothalamus

The hypothalamus is a crucial region of the CNS present in the anterior brain. It is a superordinate master regulator and integrator of fundamental aspects of the physiological behavior and energy homeostasis of the entire body (Myers & Olson 2012). Moreover, it controls the firing of the autonomic nervous system, as well as the functioning of the endocrine system and internal homeostasis. Therefore, it plays a central role in controlling most of the essential life processes (Rodríguez *et al.*, 2005; Mayer *et al.*, 2009; Buckman and Ellacott, 2014).

Anatomically, the hypothalamus is located in the most ventral position of the anterior brain, with functional connections with the pituitary gland, through which controls the release of the endocrine hormone (Burbridge, Stewart and Placzek, 2016). It is centrally located in the brain and it also connects to the brain stem through the longitudinal dorsal fascicle, the cerebral cortex through the anterior bundle of the medial brain, the hippocampus through the fornix, the amygdala through the terminal stripe, the thalamus through the mammillothalamic tract, the pituitary via the median eminence, and the retina

through the retinohypothalamic tract (Coenen *et al.*, 2018). The median eminence (ME) is located in the mid-basal hypothalamus and on the dorsal side of the third ventricle and has blood vessels without a barrier, which act as a window to release hypothalamic metabolic signals (Yin and Gore, 2010).

Structurally, the hypothalamus is composed of a cluster of neurons arranged in different nuclei, which send and receive fibers to other parts of the brain. A bilateral collection of these nuclei is divided into three zones around the third ventricle and the nipple bodies (Xie and Dorsky, 2017; Bear and Bollu, 2020): (i) the periventricular zone formed by the preoptic area (POA), paraventricular nucleus (PVN), arcuate nucleus (AN) and posterior nucleus; (ii) the medial zone formed by medial PON, anterior hypothalamic nucleus (ANH), ventromedial nucleus (VMN), dorsomedial nucleus (DMN) and pre-mammary nucleus and (iii) the lateral hypothalamic area (LHA) formed by the lateral pre-optic nucleus, tuberomammillary nucleus and supraoptic nucleus (**Figure 1.7**) (Elizondo-Vega *et al.*, 2015; Gabriela Pop, Crivii and Opincariu, 2018).

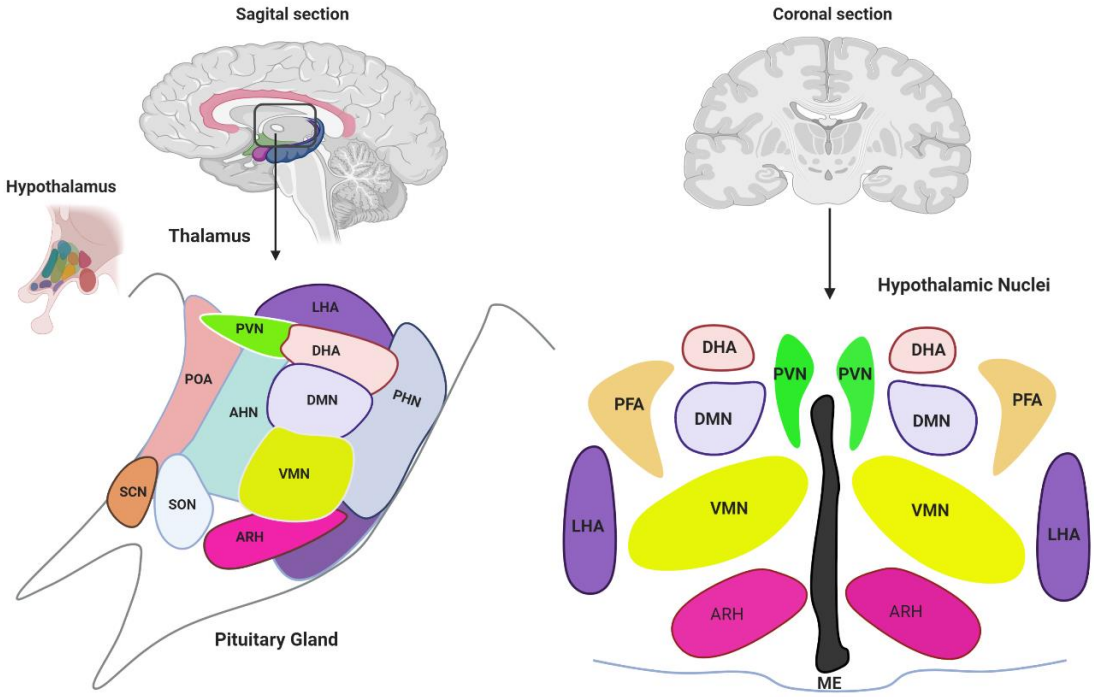


Figure 1.7. The different nuclei of each area are metabolically important and play crucial roles in several physiological functions **(Table 1.0),** including nutrient detection, appetite control and energy metabolism (Coll & Yeo 2013). The hypothalamus receives metabolic signals from the periphery through hormonal and cellular communication, allowing it to control the metabolism of the entire body (Urso & Bollu 2019; Alquier & Kahn, 2004)

Nucleus	Zones(s)	Region(s)	Functions
Paraventricular	Periventricular, Medial	Anterior,Tuberal	Fluid balance, milk let-down, parturition, autonomic & anterior pituitary control
Preoptic	Medial, Lateral	Anterior	Lateral anterior thermoregulation, sexual behavior
Anterior	Medial	Anterior	Lateral anterior thermoregulation, sexual behavior
Suprachiasmatic	Medial	Anterior	Biological rhythms
Supraoptic	Medial, Lateral	Anterior	Fluid balance, milk let-down, parturition
Dorsomedial	Medial	Tuberal	Emotion (rage)
Ventromedial	Medial	Tuberal	Appetite, body weight, insulin regulation
Arcuate	Periventricular, Medial	Tuberal	Control of anterior pituitary, feeding
Posterior	Medial	Posterior	Thermoregulation
Mammillary	Medial	Posterior	Emotion and short-term memory
Lateral Complex	Lateral	Tuberal	Appetite and body weight control

Table 1.0 – Specific functions of the hypothalamic nuclei (adapted from Urso MH & PC Bollu 2019; Miana Pop & Carmen Crivii 2018).

The hypothalamus integrates internal and external information about the state of the organism and governs patterns of action to maintain body homeostasis. The sensory areas of the cerebral cortex provide abstract sensory perceptions. The limbic system provides powerful emotional stimuli. The spinohypothalamic tract provides information on pain and temperature. The brain stem provides serotonin and norepinephrine. The hypothalamus integrates all these stimuli and activates action patterns and postures in the cerebral cortex and brain stem. These signals travel down the spine to the muscles and produce behaviors (Parent and Perkins, 2018).

The hypothalamic regions regulate functions that integrate metabolic detection, together with neuroendocrine and neural controls of systemic physiology (Cai and Liu, 2011). In this sense, the neuronal and non-neuronal cells of the hypothalamus are essential to perform the hormonal and homeostatic balance of the organism. However, conditions of cerebral and hypothalamic inflammation result in intracellular stresses induced by super nutrition or systemic inflammatory factors associated with disease. Thus, the extracellular and intracellular environments of hypothalamic cells will present disrupted hypothalamic circuits, leading to central metabolic dysregulations that could underlie a disease state (Morselli *et al.*, 2016; Valdearcos *et al.*, 2017). Recent studies started to elucidate the impact of hypothalamic inflammation as an etiological factor of obesity induced by HFD, or to contribute to other components of the metabolic syndrome. These new evidence expand the knowledge about the brain inflammatory response as an etiological factor for several diseases, such as neurodegenerative diseases, infections and cancer (Posey *et al.*, 2009; Cai and Liu, 2011; Cesar and Pisani, 2017).

1.13 Hypothalamus and ARC

The hypothalamus is the main area of the brain that controls eating behavior and energy homeostasis, implying complex neuronal circuits that project towards several brain regions and of the brainstem (Woods *et al.*, 2000; Andermann and Lowell, 2017). The ARC, which is close to the ME and the third ventricle in the MBH, constitutes the main hypothalamic area that integrates peripheral hormonal and nutritional metabolic signals (Belgardt, Okamura and Brüning, 2009; Campbell *et al.*, 2017). In the arcuate nucleus there is a special modification in the blood-brain barrier, to facilitate the entry of nutrients,

hormones and other blood molecules. Its 'privileged' location allows it to be the first sensor of peripheral signals (Cone *et al.*, 2001). The ARC contains two distinct functionally antagonistic neuronal populations, the orexigenic neurons that express the agouti-related peptide (AgRP) and neuropeptide Y (NPY) and the anorexic neurons that include cocaine and amphetamine (CART) and transcription regulated neurons opiomelanocortin (POMC) neurons. NYP positive neurons are present in several hypothalamic nuclei and other regions of brain. However, POMC/CART neurons are exclusively localized in the ARC (Cowley *et al.*, 2003; Lee and Blackshaw, 2012; Timper and Brüning, 2017). More recently it was identified that there was a third neuronal population in the ARC, which expressed tyrosine hydroxylase (TH) and had orexigenic characteristics (Zhang and Van Den Pol, 2016). The population of POMC and CART neurons provide a strong anorexigenic effect, as the secretion of the POMC and CART neuropeptides from these neurons decreases food intake and body weight. On the other hand, the second population of AgRP and NPY neurons has a potent orexigenic effect, with the release of AgRP and NPY that increases food intake. Thus, in a situation of negative energy balance, for example, fasting, the expression of AgRP is increased and the expression of POMC is decreased (Cordeira *et al.*, 2014; Gautron, Elmquist and Williams, 2015). However, during an energy surplus state, AgRP levels are reduced and POMC levels are high. Importantly, both hormones are also important regulators of peripheral metabolism; in fact, recent studies show the importance of intact AgRP signaling in adult mice to maintain normal lipid and glucose homeostasis in peripheral tissues, such as the liver, muscles and pancreas (Dietrich and Horvath, 2013). The NPY and AgRP, are co-expressed in the medial part of the arcuate nucleus (ARC) of the rodent, while POMC-derived peptides originate from more laterally arched neurons, constituting a cell group clearly distinct from the neurons that express AgRP (Hahn *et al.*, 1998). The messenger ribonucleic acid of AgRP and NPY (mRNA) is markedly increased in the ARC during fasting, simultaneously with the reduction in the pro-TRH mRNA in the PVN. On the other hand, POMC mRNA is reduced by fasting neurons in the ARC and is increased by leptin administration (Mizuno *et al.*, 1998).

The POMC anorexigenic neurons are activated by adiposity signals, such as leptin, insulin, and some nutrients. The binding of leptin to its receptor induces phosphorylation of the transcription factor STAT3, which upregulates POMC expression (Håkansson *et al.*, 1998; Lee *et al.*, 2018). Neuronal insulin signaling induces phosphorylation of FOXO1, which is also a transcription factor that increases the of POMC (Belgardt *et al.*,

2008). POMC is a neuropeptide that is post-translationally processed in several active peptides, including alfa and beta melanocyte-stimulating hormone (MSH) and beta-endorphins (Wardlaw, 2011). After, activation, neuronal projections of POMC neurons activate second-order neurons in the PVN, acting on the melanocortin receptors (MC3R/MC4R) leading to a decrease in food intake and to an activation of energy expenditure. The activation of POMC neurons has been shown to decrease body weight (Mountjoy, 2015).

The population of AgRP/NPY neurons is inhibited by leptin and activated by fasting and ghrelin and has been shown to have a potent orexigenic effect increasing food intake and decreasing energy expenditure (Wu et al., 2014). In a fed state, AgRP expression is suppressed by leptin. Under fasting conditions (no leptin release) there is an increase in AgRP and consequent increase in food behavior and adiposity. Activation of this neuronal populations leads to a release of AgRP, which is an antagonist of the MC4 receptor and inhibits PVN neurons. This process blocks the satiety feeling and stimulates feeding behavior through activation of lateral hypothalamic neurons (Luquet *et al.*, 2005; Mountjoy, 2015).

The hypothalamic ARC is well established as the main target of leptin's central actions to regulate energy homeostasis in response to fasting or feeding states (Bjørbæk, Elmquist, Michl, *et al.*, 1998; Baskin, Hahn and Schwartz, 1999). This region of the brain contains a high concentration of leptin receptors expressed in several populations of neurons, including NPY, AgRP and neurons involved in appetite regulation, reproductive function, autonomic regulation and thermogenesis (Hillebrand, De Wied and Adan, 2002). In fact, ARC neurons are first order neurons, which have also high levels of other hormone receptors, including receptors for insulin, ghrelin, GLP-1 and nutrients, which act mainly integrating peripheral signals in relation to metabolic status of the organism and allowing the control of the body homeostasis (Belgardt and Brüning, 2010; Fu *et al.*, 2019).

Several studies have shown that HFD in rodents initiates hypothalamic inflammation, mainly in the ARC (Zhang *et al.*, 2008; Milanski *et al.*, 2009). Only one to three days of HFD are sufficient to induce the activation of microglia and astrocytes, leading to an increase in inflammatory markers in the ARC (Thaler *et al.*, 2012; Waise *et al.*, 2015). The inflammatory process in ARC predisposes obesity and metabolic dysfunction (Pimentel, Ganeshan and Carvalheira, 2014; Timper and Brüning, 2017).

1.14 Hypothalamus and PVN

The PVN in the hypothalamus is one of the most important autonomic control centers in the brain, with roles in several homeostatic responses (Pyner, 2009; Barrett-Jolley *et al.*, 2011). Anatomically, the PVN is located dorsally on both sides of the third ventricle; it participates not only in the regulation of the energy balance, but also in the formation of the hypothalamic-pituitary (HP) axis (Scott and Dinan, 1998; Hill, 2012). In rodents and primates, neurons in PVN connect extensively with a variety of neurons in the hypothalamus, brainstem, limbic regions and prefrontal cortex (Sawchenko and Swanson, 1983). The PVN comprises several anatomical subdivisions, being typically divided into parvocellular and magnocellular subnuclei. The rodent parvocellular area includes approximately 1000 small neurons that project to regions of the central nervous system involved in autonomic control (Lovick, Malpas and Mahony, 1993; Shafton, Ryan and Badoer, 1998; Pyner and Coote, 2000). The magnocellular area contains larger neurons that project to the posterior pituitary. The cells within this area generally have a neuroendocrine function, for example, secretion of vasopressin and oxytocin. In fact, thirty or more neurotransmitters have been identified in this region (Pyner, 2009). The PVN integrates signals from neuropeptides from different regions of the brain, including ARC. Thyrotropin releasing hormone (TRH) neurons, corticotropin releasing hormone (CRH) neurons and oxytocin (OXY) neurons are located in the PVN and their active state is modulated by the ARC neurons (Wynne *et al.*, 2005). Importantly, the activation of POMC / CART neurons inhibits PVN activity, while NYP / AgRP activation increases PVN activity (Hill, 2012; Roh, Song and Kim, 2016). PVN neurons control the sympathetic flow to the peripheral metabolic organs, resulting in increased fatty acid oxidation and lipolysis (Foster, Song and Bartness, 2010). The destruction of PVN and haploinsufficiency of Sim1, a critical transcriptional factor in the development of PVN, causes hyperphagia and obesity, highlighting the inhibitory role of PVN in food intake and weight gain (Ahima and Antwi, 2008; Roh, Song and Kim, 2016). The functions of the PVN nucleus in the modulation of energy homeostasis and in the hypothalamic-pituitary system have been extensively investigated (Shi *et al.*, 2013; O'Hare and Zsombok, 2016). In fact, PVN is a multifunctional nucleus, with autonomic roles, including (but not limited to) coordination of cardiovascular, thermoregulatory,

metabolic, circadian and stress responses. However, the cellular mechanisms underlying these multifunctional roles remain poorly understood (Feetham, O'Brien and Barrett-Jolley, 2018). The dysfunction of PVN can lead to human diseases such as obesity, short stature, hypertension, cardiovascular dysfunction, diabetes insipidus and many others (Ferguson, Latchford and Sanon, 2008; Kawabe *et al.*, 2009).

Magnocellular neurons project mainly into the posterior lobe of the pituitary, where they secrete vasopressin arginine (AVP) and OXT in the hypothalamic-neurohypophysial (HNS) system, which regulates fluid balance, the release of breast milk and uterine contraction and ejaculation (Sawchenko and Swanson, 1983). In addition, AVP neurons participate in the regulation of eating behaviors (Pei *et al.*, 2014). Long-projected neurons mainly express the 4 melanocortin receptor (MC4R) and OXT, which are projected mainly in the posterior brain to regulate energy balance (Pei *et al.*, 2014; Shah and Vella, 2014). Most likely, OXT neurons are the main mediator of hyperphagic obesity in the heterozygous mutant mouse 1 (Sim1) and are also postsynaptic targets for agoutis-related protein (AgRP) positive neurons in ARC (Kublaoui *et al.*, 2008; Atasoy *et al.*, 2012).

Parvocellular neurons send axons to the ME and mainly secrete HRT and CRH into the portal vasculature to initiate the hypothalamic-pituitary-thyroid (HPT) axis and the hypothalamic-pituitary-adrenal (HPA) axis, respectively (Sawchenko and Swanson, 1983). Interestingly, CRH neurons in PVH are associated with the food preference for carbohydrates over fat, through the activation of AMP-activated protein kinase (Okamoto *et al.*, 2018). PVN also contains some neurons positive for somatostatin (SST) that project to the ME and inhibit the secretion of GH and TSH in the anterior pituitary (Morales-Delgado *et al.*, 2014).

PVN is a multifunctional nucleus, with autonomic roles, including (but not limited to) coordination of cardiovascular, thermoregulatory, metabolic, circadian and stress responses. However, the cellular mechanisms underlying these multifunctional roles remain poorly understood (Feetham, O'Brien and Barrett-Jolley, 2018).

1.15 Hypothalamic inflammation with HFD

As mentioned, the hypothalamus is the main area of the brain that controls eating behavior and energy homeostasis, implying complex neuronal circuits that project towards various brain regions and the brainstem (Kleinridders *et al.*, 2009; Williams and Elmquist, 2012). The ARC, VMH, DMH and PVN are critical for the control of energy homeostasis (Jais and Brüning, 2017). In particular, two hypothalamic nuclei are involved in maintaining the energy balance: the ARC and the PVN. ARC integrates peripheral signals in relation to the body's metabolic status, such as insulin, leptin or other hormones and thus monitors the energy status of the entire body. ARC stimulates melanocortinergic pathways in PVN, which activate or repress food intake and energy expenditure (Woods *et al.*, 2000). Several studies demonstrate that HFD in rodents' initiates inflammation in the hypothalamus, mainly in the ARC (De Souza *et al.*, 2005; Milanski *et al.*, 2009; McLean *et al.*, 2019), irreversibly affecting the neurons in this region (Waise *et al.*, 2015). In fact, the inflammation in ARC is suggested as an essential mechanism of hypothalamic dysfunction associated with obesity and metabolic disease (Lee *et al.*, 2012; Jais and Brüning, 2017). Experimental studies have shown that, after prolonged feeding of HFD, hypothalamic neurons suffer severe damage that can result in apoptosis (Moraes *et al.*, 2009; Thaler *et al.*, 2012). Several different studies have identified the POMC neurons as the main targets of apoptosis associated with inflammation. Moreover, it was shown that the early inhibition of POMC is enough to transform obesity-resistant rodents into rodents prone to obesity (Souza *et al.*, 2016). HFD activates hypothalamic inflammatory signaling pathways, resulting in increased food intake and nutrient storage (Thaler and Schwartz, 2010). With HFD, metabolites such as diacylglycerols and ceramides accumulate in the hypothalamus and induce resistance to leptin and insulin in the CNS (Holland *et al.*, 2011). Part of this effect is mediated by saturated FAs, which activate the JNK and NF-kB neuronal signaling pathways, with direct effects on leptin and insulin signaling (Zhang *et al.*, 2008; Thaler *et al.*, 2012; Koch and Horvath, 2014). Increased FAs induce the activation of immune cells and elicit an inflammatory response that affects the

hypothalamus. The innate immune response is mediated by toll-like receptors that activate two different transcription factors, NF-kB and activator protein-1, which in turn increase the expression of pro-inflammatory mediators, such as cytokines, such as IL1B, IL6, TNF-α and chemokines (Manousopoulou *et al.*, 2016; Guillemot-Legris and Muccioli, 2017). Chemokines can influence, at the hypothalamic level, the dysregulation of energy balance and body weight. In fact, in addition of being essential mediators of the inflammatory response, chemokines play important roles at the central level, activating and attracting immune cells, regulating neuronal survival and death and also modulating the activity of certain neurons (Rostène *et al.*, 2011). Therefore, the chemokinergic system may be responsible for the dysregulation of eating behavior associated with inflammation, both in appetite and in weight loss, and in the development of obesity (Le Thuc *et al.*, 2017; Luquet, Vaudry and Granata, 2019).

Hypothalamic inflammatory signaling occurs before the onset of weight gain and peripheral inflammation, suggesting a neuroprotective response induced by the detection of hypothalamic nutrients, rather than adipokines signaling to the hypothalamus. In addition, both reactive glucose and markers suggestive of neuronal injury were evident in the hypothalamic arcuate nucleus of rodents in the first week of HFD feeding (Thaler *et al.*, 2012). Current research raises the hypothesis that microglia partially leads to "neuroinflammation" associated with obesity (Valdearcos *et al.*, 2017). This hypothesis is supported by the fact that there is a higher activation and proliferation of glial cells when in contact with hypothalamic inflammatory cytokines IL6 and TNF-α (**Figure 1.8**). This process was recorded even before substantial weight gain induced by the administration of HFD food in rodents (Stein *et al.*, 2020).

The effects of brain inflammation on the metabolic function of peripheral tissues are extensive. Regardless of obesity, hypothalamic inflammation can impair the release of insulin by β cells, impair the peripheral action of insulin, and enhance hypertension (Kang *et al.*, 2009; Purkayastha *et al.*, 2011). Many of these effects are generated by signals from the sympathetic nervous system, which are also capable of inducing inflammatory changes in adipose tissue in response to neuronal injury (Purkayastha *et al.*, 2011).

Over the last years, hypothalamic inflammation has been linked to the development and progression of obesity, emerging not only as an important driver of impaired energy balance, but also as a contributor to obesity-associated insulin resistance (Jais and Brüning, 2017).

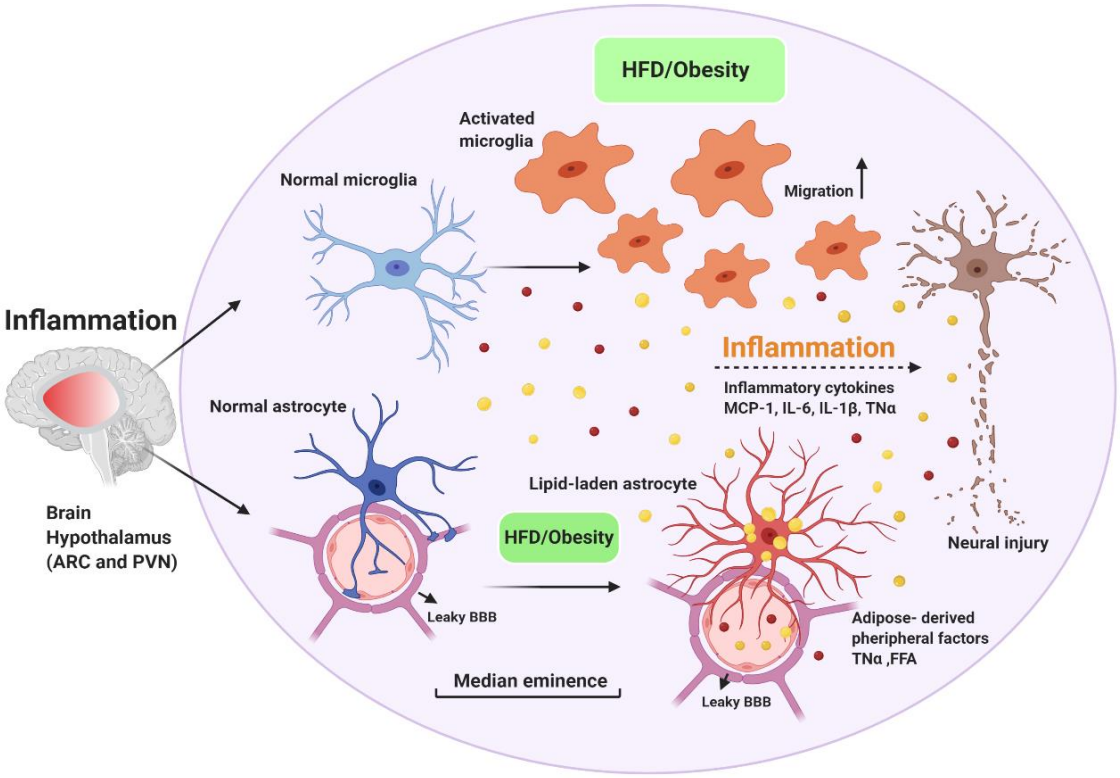


Figure 1.8. Hypothalamic Inflammation. The activation of the microglia is mediated by lipid-laden astrocytes under conditions of HFD-induced obesity. Astrocytes accumulate droplets of lipids in obese environments rich in fatty acids. Lipid-laden astrocytes release inflammatory cytokines, leading to microglia migration and activation. Lipid-laden astrocytes can play a crucial role in obesity-induced hypothalamic inflammation, increasing astrocyte / microglia-mediated inflammatory responses, causing neuronal injury in the hypothalamic neurons of the arcuate or paraventricular nucleus.

1. 16 Hypothalamus and Leptin

Leptin is a hormone secreted mainly by white adipose tissue and the main messenger that carries information about peripheral energy stores to the hypothalamus (Bjørbaek and Kahn, 2004). The primary site of the CNS involved in regulating appetite for leptin is the hypothalamus ARC. In fact, the injection of leptin directly into the ARC reduces food intake and body weight (Satoh *et al.*, 1997). Within the ARC, leptin acts on LepRb to inhibit neurons that express the AgRP, while simultaneously stimulating neighboring neurons that express the anorexigenic neuropeptide POMC. Both actions work together to reduce food intake (Cone *et al.*, 2001). In addition to direct homeostatic actions in the hypothalamus, leptin has gained recognition as a modulator of neural circuits that govern motivation and reward (Murray *et al.*, 2014). Leptin acts through the mesolimbic dopaminergic "reward system" to suppress the motivational impulse to seek and consume food (Hommel *et al.*, 2006; Evans, Rizwan and Anderson, 2014). Leptin also stimulates locomotion through signaling in POMC neurons (Huo *et al.*, 2009).

The first studies showing that a circulating hormone regulates food intake in a centralized manner benefited from spontaneous mutant mice with severe hereditary hyperphagia and obesity (Fisher *et al.*, 2010). One of these obese mice, the ob/ob was later used to identify the leptin gene (Zhang *et al.*, 1994). Leptin-activated receptors are highly expressed in several regions of the brain, especially the hypothalamus (Elias *et al.*, 1999; Cowley *et al.*, 2001). Genetic deficiency in leptin or in the LepRb is associated with hyperphagia, hypoactivity and obesity (Coppari *et al.*, 2005; Fernandes *et al.*, 2015). Leptin acts through the mesolimbic dopaminergic "reward system" to suppress the motivational impulse to seek and consume food (Hommel *et al.*, 2006; Evans, Rizwan and Anderson, 2014). The primary site of the CNS involved in regulating appetite for leptin is the arched nucleus of the hypothalamus (ARC). Within the ARC, leptin acts on LepRb to inhibit neurons that express the agouti-related peptide (AgRP), the orexigenic neuropeptide (appetite stimulant), while simultaneously stimulating neighboring neurons that express the anorexigenic neuropeptide proopiomelanocortin (inhibitor) of appetite). Both actions work together to reduce food intake (Elias *et al.*, 1999; Cone *et al.*, 2001). Leptin also stimulates locomotion through signaling in POMC neurons (Huo *et al.*, 2009). In the hypothalamic neurons, leptin causes induces several signaling cascades, such as the Janus kinase-STAT pathway, IRS-PI3K signaling, the mammalian target of rapamycin-S6-

kinase signaling, AMP-activated protein kinase (AMPK) signaling and ERK signaling (Kola, 2008). Of these, STAT3 signaling represents hypothalamic leptin signaling and is often used as a marker of leptin signaling activity. Leptin directs the expression of AgRP and POMC in opposite directions (Ibars *et al.*, 2017). The hormone acts on these two types of ARC neurons mainly through the same signaling cascades. The binding of leptin to LepRb, which has no intrinsic kinase activity, causes the receptor to recruit intracellular Janus kinase 2 (JAK2). The recruitment of JAK2, in turn, activates several cascades of signal transduction through autophosphorylation and phosphorylation of LepRb and STAT3 (Flak and Myers, 2016; Pan and Myers, 2018). The phosphorylation of STAT3 silences the *AgRP* gene and directs the expression of the *POMC* gene. The leptin-LepRb bond also activates intracellular PI3K (Niswender *et al.*, 2001), whose phosphorylation then causes the nuclear exclusion of the FOXO1 transcription factor (Kitamura *et al.*, 2006). The non-phosphorylated FOXO1 protein and phosphorylated STAT3 appear to act on the promoters of *AgRP* and *POMC* through a competition process, in this way, they will compete for binding sites within the two promoters (Kitamura *et al.*, 2006). JAK2-STAT3 and PI3K have been implicated in the anorexigenic effect of leptin (J. Sun *et al.*, 2016; Fruhwürth *et al.*, 2018). A recent study shows that the protein tyrosine phosphatase 1B (PTP1B) regulates leptin signaling in hypothalamic neurons, via the JAK2-STAT3 pathway. PTP1B has also been implicated in the regulation of inflammation in the peripheric tissues. This study also revealed an important role of the PTP1B in the signaling of JAK2-STAT3 in the microglia, which attenuates hypothalamic inflammation, regulating leptin levels (Tsunekawa *et al.*, 2017). Another recent study identifies a new essential pathway for canonical leptin signaling in hypothalamic neurons: the NSAPP pathway (oxide transport chain). This pathway fails in states of HFD overnutrition in rodents; helping to explain in some way the central resistance of leptin observed in the diet-induced obese phenotype (Fruhwürth *et al.*, 2018).

Hyperleptinemia is associated with leptin resistance, which is specifically resistance to the anorectic and weight-reducing effects of leptin. Hyperleptinemia and leptin resistance are components of common obesity (Yang *et al.*, 2015). Models of rodents and obese individuals usually exhibit high circulating levels of leptin because the total fat mass increases, but the hypothalamus no longer responds normally to leptin to suppress appetite (Münzberg, Flier and Bjørbæk, 2004; Enriori *et al.*, 2007). However, the exact molecular mechanisms responsible for the poor response to leptin in the brain remain unknown (Fruhwürth *et al.*, 2018). The resistance mechanism appears to be related with

defects in leptin transport across the BBB or to intracellular signaling mechanisms downstream of the LR. Other diseases associated with hyperleptinemia include NAFLD, Rabson-Mendenhall syndrome, neurodegenerative disorders, depression and others (Ramos-Lobo and Donato, 2017; Zou *et al.*, 2019)

1.16.1 Hypothalamic Inflammation and Leptin Resistance

Leptin signals to the different areas of the brain involved in energy homeostasis, especially the hypothalamus (Zhou and Rui, 2013). The brain responds to high plasma leptin levels, reducing food intake and increasing energy expenditure, as well as improving glucose metabolism, through the reduction of glucose production in the liver (Dardeno *et al.*, 2010; Berglund *et al.*, 2012). However, most obese individuals, despite having high circulating levels of leptin have an increased food intake (Afshin *et al.*, 2017). In this sense, leptin itself plays an important role in the development of its resistance, with a phenomenon called "leptin resistance induced by leptin". The development of leptin resistance increases patients' predisposition to diet-induced obesity, which in turn contributes to a further increase in leptin levels and to the worsening of existing leptin resistance in a vicious cycle (Gruzdeva *et al.*, 2019). Recent studies consider that over-nutrition, particularly a high-fat diet, can induce leptin resistance in the absence of obesity, acting on the hypothalamus ARC (Zhou and Rui, 2013; Sáinz *et al.*, 2015). Studies in rodent models with diet-induced obesity fed with HFD, presented not only peripheral inflammation, but also increased inflammatory signaling in the hypothalamus, which was associated with the development of central resistance to leptin and with the development of obesity **(Figure 1.9)** (Thaler *et al.*, 2012; Wang *et al.*, 2013). Several molecular mechanisms have been proposed to explain the inflammatory process that occurs especially in the hypothalamus, which triggers leptin resistance in diet-induced obesity, including impaired leptin transport in the BBB, LEPRb trafficking and leptin signaling (Morris and Rui, 2009; Zhou and Rui, 2013). A recent study highlighted the presence of several characteristic inflammatory pathways involved in leptin resistance in rodent hypothalamus, including: i) increased IKKβ/NF-κB signaling; ii) increased JNK signaling, including an increase in TLR4 in the microglia; iii) stress of the endoplasmic reticulum; and iv) disturbances in the autophagy. All of these events were observed in rodents exposed to HFD feeding in a chronic way and for a pre-determined time (de Git

and Adan, 2015). Mutations in the OB and DBU genes in humans are extremely rare and cause hyperphagia and obesity shortly after birth (Kilpeläinen *et al.*, 2016). All of these molecular changes result in structural changes in the leptin molecule and leptin signaling, accompanied by hypothalamic inflammation and endoplasmic reticulum stress (Morris and Rui, 2009; Gregor and Hotamisligil, 2011; Zhou and Rui, 2013; Gruzdeva *et al.*, 2019). Although the understanding of the mechanisms by which hypothalamic inflammation promotes central leptin resistance has been improved in recent years, the exact cellular mechanisms have yet to be elucidated (Stein *et al.*, 2020).

1.17 Hypothalamus and Insulin

Among the peripheral signs, the anorexigenic effect of insulin on the hypothalamus is widely documented, in which insulin modulates food intake and glucose homeostasis (Könner, Klöckener and Brüning, 2009). Insulin crosses the BBB in a receptor-dependent manner to reach the hypothalamus (Niswender, Baskin and Schwartz, 2004). In the hypothalamus, insulin receptors (IR) are highly expressed in the neurons POMC/CART and NPY/AgRP, and central insulin administration increases the hypothalamic expression of CART and αMSH (α-melanocyte stimulating hormone) and inhibits NPY and AgRP expression, reducing food intake and body weight (Benoit *et al.*, 2002; Niswender *et al.*, 2003). In addition, insulin regulates the electrical activity of the POMC/CART and NPY/AgRP neurons by stimulating the ATP-sensitive potassium channel (KATP), leading to membrane hyperpolarization and decreased firing of these neurons in a PI3K-dependent manner (Choudhury *et al.*, 2005). Insulin, through its action on ARC hypothalamic neurons, has also been reported to regulate hepatic glucose production (Könner *et al.*, 2007), glycogen synthesis and fat metabolism. The action of insulin on AgRP/NPY neurons suppresses hepatic glucose production, while its action on POMC neurons inhibits lipolysis of adipose tissue (Shin *et al.*, 2017). In addition to insulin, the hormone derived from the adipose tissue, leptin also plays a critical role in the hypothalamic control of energy and glucose homeostasis. The action of leptin on the hypothalamus improves glucose use and insulin sensitivity in adipose tissue, muscle and liver (Berthou *et al.*, 2011). It is important to note that leptin and insulin share several

signaling pathways in the hypothalamus and act synergistically to modulate the central regulation of food and energy homeostasis of the entire body (Benomar *et al.*, 2005).

The hypothalamus is an important mediator of energy balance, food intake and glucose homeostasis in the brain (Gray, Meijer and Barrett, 2014). Rodents with selective hypothalamic insulin resistance, achieved by an intra-hypothalamic injection of antisense oligodeoxynucleotides specific to the insulin receptor, failed to suppress hepatic glucose production and food intake, suggesting that hypothalamic insulin resistance plays a role critical in the effects of insulin on energy and glucose metabolism (Obici *et al.*, 2002). The selective knockdown of the insulin receptor in the rat hypothalamus also increases body weight and adiposity (Grillo *et al.*, 2007). More recently, studies have shown that chronic reduction of the insulin receptor in the VMH of rats leads to glucose intolerance due to islet dysfunction, although body adiposity has not been affected, suggesting that VMH insulin signaling may regulate glucose, but not energy homeostasis (Paranjape *et al.*, 2011).

The molecular mechanism by which insulin signaling in the hypothalamus suppresses food intake and mediates systemic glucose metabolism has been extensively studied (Dodd and Tiganis, 2017). The specific tyrosine residues of IR are phosphorylated by the IR itself when it binds to insulin, thus inducing tyrosine phosphorylation of insulin receptor substrate proteins (IRS). This process results in the activation of PI3-kinase, which in turn produces phosphatidylinositol (3,4,5) triphosphate (PIP3) (Sánchez-Alegría *et al.*, 2018). When PI3-kinase inhibitors are injected into the ventricle, insulin does not suppress food intake or improve glucose metabolism, showing that activation of PI-3-kinase is necessary for both effects of central insulin (Niswender *et al.*, 2003). A recent study modulated PTEN bidirectionally, the negative regulator of PI-3-kinase signaling, in the hypothalamus of rats and showed that hypothalamic PIP3 is in fact responsible for regulating food intake and glucose metabolism (Sumita *et al.*, 2014)

1.17.1 Hypothalamic Inflammation and Insulin Resistance

The consumption of HFD alters the hypothalamic responsiveness to insulin, deregulating the control of energy homeostasis (Gregor and Hotamisligil, 2011). HFD triggers local immune responses in the MBH, resulting in the production of pro-inflammatory cytokines and neuronal damage, affecting the control of energy homeostasis and hypothalamic insulin sensitivity (Kälin *et al.*, 2015). Several mechanisms have been proposed to explain the loss of hypothalamic insulin action induced by HFD. Indeed, it has been shown that this impairment of insulin action could result from impaired transport through the BBB, thus reducing hypothalamic insulin uptake (Urayama and Banks, 2008). Other study reported that HFD activates hypothalamic inflammatory pathways, notably NF-kB and JNK, and increases pro-inflammatory cytokines, leading to insulin resistance (Rehman and Akash, 2016). This brings further arguments for the role of hypothalamic inflammation as an important contributor to insulin resistance associated with HFD (Benomar and Taouis, 2019). Currently, some studies report that several cell types in the hypothalamus, including POMC and AgRP neurons, astrocytes, microglia, endothelial cells and others are related to hypothalamic insulin resistance through the hypothalamic inflammation process **(Figure 1.9)** (Ono, 2019).

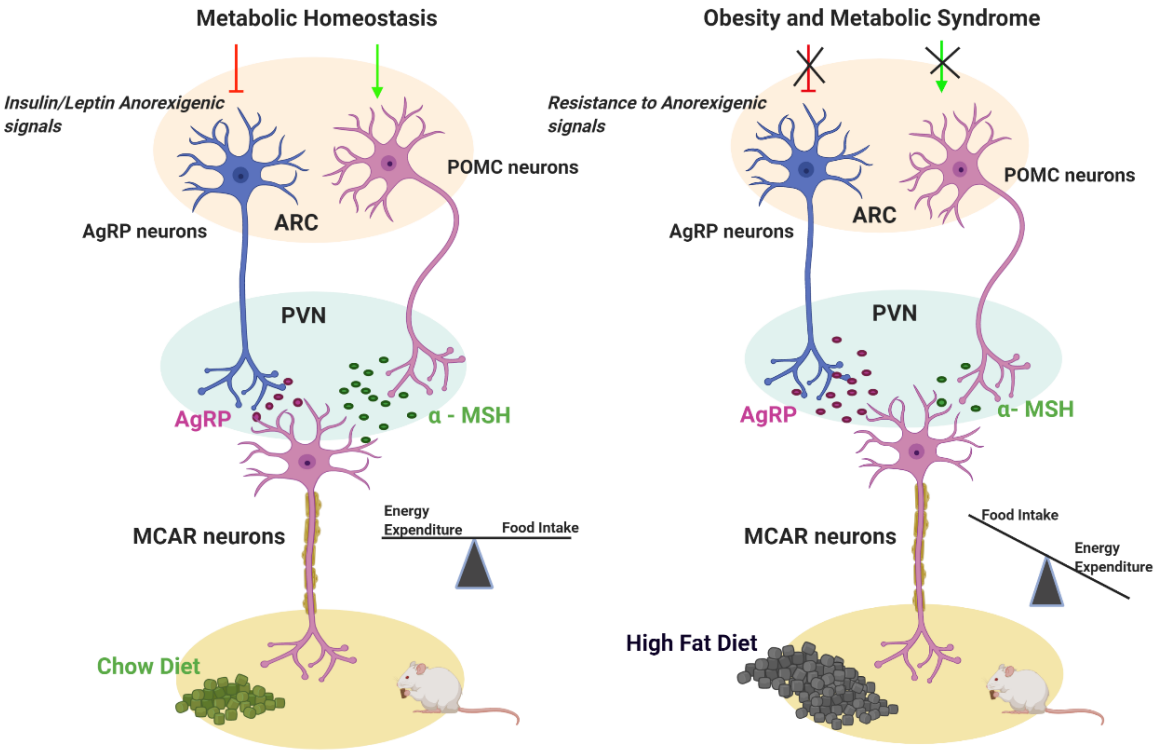


Figure 1.9. HFD induces hypothalamic Inflammation, Leptin and Insulin resistance. The hypothalamus senses and integrates feedback from adipostatic hormones, which circulate in levels proportionate to nutritional status and adipose tissue stores. Insulin and leptin act directly on neuronal subsets in the ARC of the hypothalamus to control energy homeostasis. Through activation of POMC neurons and inhibition of AgRP neurons, adipostatic signals activate MC4R-expressing neurons in the PVN. During fasting conditions, the expression of AgRP increases, whereas POMC expression is reduced, resulting in decreased MC4R signaling. In the fed state, AgRP levels are diminished and POMC levels increase, which triggers MC4R signaling and culminates in satiety and stimulation of energy expenditure. Neuronal inflammation and the subsequent insulin and leptin resistance of ARC neurons disrupts this metabolic feedback loop, further promoting increased food intake and body weight

1. 18 Brain and Lipids

The brain is a vital organ of the human body that orchestrates its main functions, being the key center of initiation, coordination, interpretation and integration of most nervous messages (Hussain *et al.*, 2013). To this end, it depends on a complex network of nerves that are wrapped in myelin made with lipids; thus, constituting an abundant source of lipids in the body (Hussain *et al.*, 2019). Lipids represent 50-60% of the dry weight of the brain (Luchtman and Song, 2013) and a substantial amount of lipids in the nervous system is present in the myelin sheaths (Cermenati *et al.*, 2015). The myelin sheath is an extended and highly specialized plasma membrane synthesized by myelinating glial cells: oligodendrocytes in the CNS and Schwann cells in the PNS. Myelin wrapping around an axonal segment increases axonal resistance and allows grouping of axonal ion channels in Ranvier nodes, allowing and refining the efficient propagation of neuronal impulses (Harayama and Riezman, 2018). Cholesterol synthesis is crucial for correct myelination (Saher and Stumpf, 2015). Brain lipids consist of glycerophospholipids, sphingolipids and cholesterol in approximately equal proportions (Korade and Kenworthy, 2008; Zhang and Liu, 2015). Lipids act as signaling molecules, as a source of energy, they contribute to synaptogenesis, neurogenesis, impulse conduction and many others (Cermenati *et al.*, 2015). All the vital events responsible for the development and maintenance of the functional activities of the nervous system depend on the unique lipid content found in the different membrane regions (lipid rafts) of neuronal cells (Tsui-Pierchala *et al.*, 2002; Korade and Kenworthy, 2008; Cermenati *et al.*, 2015). In addition, it has long been known that myelin structure and brain homeostasis depend on specific lipid-protein interactions and cell-to-cell signaling (Adibhatla and Hatcher, 2008; Hussain, Rasul, *et al.*, 2020).

Lipid-driven membrane domains function as dynamic platforms for signal transduction, protein processing, and membrane turnover. Essential events involved in the development and in the maintenance of the functional integrity of the brain depend on the organization of lipid-driven membrane domains, and alterations in lipid homeostasis, leading to deranged lipid-driven membrane organization (Aureli *et al.*, 2015). Any change in lipid metabolism results in altered lipid composition of the intracellular membrane compartments. Recently, lipids are being looked as biomarker in several major brain diseases (Adibhatla and Hatcher, 2008; Aureli *et al.*, 2015; Hussain, Anwar, *et al.*, 2020).

In fact, several CNS disorders and injuries are related to impaired lipid metabolism, including AD, Parkinson's disease (PD), Machado-Joseph disease (MJD), Huntington's disease (HD), schizophrenia, epilepsy and bipolar disorders in which progressive neuron degeneration occurs (Adibhatla and Hatcher, 2008; Schmitt *et al.*, 2014; Grassi *et al.*, 2020; Nóbrega *et al.*, 2020).

It is established that adequate supply and active lipid metabolism are of crucial importance in maintaining the highly specialized brain functions, including learning, memory, attention and several other physiological processes. However, there is still much to be studied about the lipidome of the brain (Yu *et al.*, 2018).

1. 19 Role of Cholesterol in the Brain

As mentioned, cholesterol is one of the main constituents of the human brain, being the richest organ in cholesterol, containing about 25% of the total cholesterol in the body (Björkhem, Meaney and Fogelman, 2004; Hussain *et al.*, 2019; Yutuc *et al.*, 2020). Cholesterol is the main lipid component of neuronal and glial membranes and plays essential roles in the compartmentalization of the plasma membrane, signaling, myelination, formation and maintenance of synapses and dendrites, and for axonal orientation (Fester *et al.*, 2009; Petrov, Kasimov and Zefirov, 2017; Hussain *et al.*, 2019).

Cholesterol is one the most important components and fundamental functional units of the mammalian cell membrane (Zou *et al.*, 2019). The cholesterol content of the plasma membrane plays a fundamental role in the formation of the complex that regulates the recycling of the synaptic vesicles to release neurotransmitters (Südhof, 2004). Most body cholesterol resides in the brain in the form of myelin, which contains almost 80% of the cholesterol found in the adult brain (Segatto *et al.*, 2013). Sufficient cholesterol availability is necessary for normal neuronal function and morphology. Cholesterol depletion in neurons impairs vital functions, including synaptic vesicle exocytosis, neuronal activity and neurotransmission and results in synaptic loss and neurodegeneration (Linetti *et al.*, 2010; Liu *et al.*, 2010). Nevertheless, the functions of neuronal cells are impaired not only due to the lack, but also due to an excess of cholesterol (Ko *et al.*, 2005; Pooler, Xi and Wurtman, 2006; Zhang and Liu, 2015). Cholesterol levels are strongly regulated among the brain cells, being an essential

constituent of steroid hormones and for the function of the hedgehog protein (Dietschy and Turley, 2001; Herz and Bock, 2002). It was also clearly demonstrated that cholesterol plays a key role in neuronal differentiation and plasticity (Goritz, Mauch and Pfrieger, 2005). In rodents, humans and others, cholesterol is actively synthesized in the CNS during the first weeks after birth, and at this neonatal stage, any interruption in its synthesis and supply can lead to the development of neurodegenerative disorders (Cunningham *et al.*, 2015). Moreover, cholesterol is an essential component of neuronal physiology in adulthood; being independently regulated in the brain, largely due to the existence of the BBB (Zhang and Liu, 2015). Cholesterol can be synthesized endogenously, or it can be supplied exogenously by the endocytosis of plasma lipoproteins; for example, low-density lipoproteins (LDLs) mediated by specific receptors (Vance, 2012). The rate of cholesterol synthesis depends on the ongoing myelination process and the excess cholesterol is exported mainly in the form of 24-hydroxycholesterol to maintain cholesterol homeostasis (Dietschy *et al.*, 1983; Lund *et al.*, 2003). There is a constant flow of cholesterol in the brain, however in the form of oxysterols (Björkhem, Meaney and Fogelman, 2004). Since cholesterol cannot cross the blood brain barrier (BBB) and be imported from the periphery, essentially all cholesterol in the brain is biosynthesized in the brain (Bjoandrkhem *et al.*, 2006; Kanungo *et al.*, 2013; Yutuc *et al.*, 2020).

Oxysterols are oxidized forms of cholesterol, or its precursors, and represent the initial metabolites in cholesterol catabolism (Schroepfer and Wilson, 2000). In fact, it is now known that cholesterol is oxidized, mainly due to an oxidative process by specific enzymes called CYPs to act on brain homeostasis (Testa *et al.*, 2016). One of the most studied oxysterols in the brain is 24S-hydroxycholesterol (24-HC). In mammals, 24-HC is synthesized mainly in neurons by the cytochrome P450 (CYP) 46A1 enzyme (cholesterol 24S-hydroxylase, CYP46A1) and acts as a form of cholesterol transport (Lund, Guileyardo and Russell, 1999; Russell *et al.*, 2009). Unlike cholesterol, 24-HC can cross the BBB (Björkhem, Meaney and Fogelman, 2004). Oxysterol 24-HC is a ligand for liver X receptors (LXRs)(Lehmann *et al.*, 1997).

In the CNS, cholesterol is transported by special lipoproteins, such as apolipoprotein-E (Apo-E), secreted by astrocytes (Pfrieger and Ungerer, 2011; Mahley, 2016). Astrocytes are critical for the regulation of cholesterol homeostasis in neurons (Czuba *et al.*, 2017), as neurons can synthesize only a small amount of cholesterol and depend mainly on

lipoproteins containing cholesterol secreted by astrocytes (Pfrieger, 2003). The cholesterol-Apo-E complex accelerates axonal extension when applied to the distal end, but not to the cell body of neurons (Vance, Hayashi and Karten, 2005). ApoE not only plays an important role in glial lipid-neuronal metabolism, but also acts as a ligand for multiple receptors in neurons, which interact with several downstream physiological processes (Beffert *et al.,* 2006). In particular, several recent studies have shown that brain ApoE is an important regulator of peripheral energy homeostasis. In rats, the intracerebroventricular injections of ApoE significantly decreased food intake, while the infusion of ApoE antiserum stimulated feeding, suggesting that ApoE may be an important satiety factor in the hypothalamus (Huang and Mahley, 2014). In this context, the regulation of lipids, particularly cholesterol, can provide relief against neurodegenerative diseases (Petrov, Kasimov and Zefirov, 2016). Recent studies demonstrate that the need for cholesterol is increased in the case of nerve regeneration, as it is an important modulator of axon regeneration after nerve injury (Petrov, Kasimov and Zefirov, 2016; Panagopoulos, Megaloikonomos and Mavrogenis, 2017). This availability is met by an increase in the supply of cholesterol in the form of lipoproteins from macrophages that recycle cholesterol from degenerating neurons (Mar *et al.*, 2016). Cholesterol plays a crucial role in nerve regeneration after CNS injuries. The local availability of cholesterol in nerve damage is necessary for nerve regeneration (Goodrum *et al.*, 2000). The carrier lipoprotein rich in Apo-E cholesterol has been reported to accumulate at the site of injury after crushing the nerve (Martín, Pfrieger and Dotti, 2014). The delay in axon regeneration is a major obstacle to functional recovery after these injuries (Park *et al.*, 2013), which can result in a long-term disability (Schmitt *et al.*, 2014). Cholesterol regulates a large number of pathways that play important roles in brain health (Hussain *et al.*, 2019). Since cholesterol cannot cross the blood brain barrier (BBB) and be imported from the periphery, essentially all cholesterol in the brain is biosynthesized in the brain (Kanungo *et al.*, 2013; Yutuc *et al.*, 2020). Inborn errors of cholesterol biosynthesis generally result in neurodevelopmental disorders and delayed syndromes (Kanungo *et al.*, 2013). Oxysterols are oxidized forms of cholesterol, or its precursors, and represent the initial metabolites in cholesterol catabolism (Schroepfer and Wilson, 2000). This catabolism is activated by the neuron-specific cytochrome P450 oxidase Cyp46a1 (Russell *et al.*, 2009; Petrov, Kasimov and Zefirov, 2017). In fact, from a body of evidence, it is now known that cholesterol is oxidized as an example 24-

hydroxycholesterol and 27-hydroxycholesterol, mainly due to an oxidative process by specific enzymes called CYPs to act on brain homeostasis (Testa *et al.*, 2016).

Defects in cholesterol metabolism lead to structural and functional diseases of the CNS, such as HD, AD, and MJD, among others (Strittmatter *et al.*, 1993; Di Paolo and Kim, 2011; Vance, 2012; Martín, Pfrieger and Dotti, 2014; Nóbrega *et al.*, 2019, 2020). These metabolic defects affect different pathways, such as cholesterol biosynthesis, lipid transport and lipoprotein assembly, receptors that measure cell uptake of lipids and signaling molecules (Orth and Bellosta, 2012). Significant changes in brain cholesterol metabolism have also been observed in other different pathological conditions, such as depression, amyotrophic lateral sclerosis, stroke, head trauma, insulin resistance in the brain and normal aging (Gamba *et al.*, 2019). An age-dependent loss of cholesterol was seen in the human brain (Söderberg *et al.*, 1990; Svennerholm *et al.*, 1991, 1994) and in the hippocampus of rodents, *in vivo* and *in vitro* (Martin *et al.*, 2008, 2011; Sodero, Trovò, *et al.*, 2011; Sodero, Weissmann, *et al.*, 2011).

1. 20 BBB and Cholesterol

The brain is isolated from the entire body by BBB, which substantially restricts the exchange of molecules between the brain and the bloodstream (Saint-Pol and Gosselet, 2019). The exchange of cholesterol in the BBB is very limited and occurs through the transport of LDLs (Dehouck *et al.*, 1997; Candela *et al.*, 2008). Oxysterols on the other hand are able to cross the BBB and regulate cholesterol exchanges through the LXRs (Saint-Pol and Gosselet, 2019).

The BBB, formed by narrow junctions between capillary endothelial cells, separates the circulating blood from the extracellular fluid in the CNS. BBB cells have the potential to absorb low-density lipoprotein (LDL) cholesterol through luminal endothelial receptors, followed by translocation through the endothelial cell. This uptake, however, is not relevant under physiological conditions and the lipoprotein receptor-mediated cholesterol uptake in plasma does not regulate cholesterol in the brain (Dehouck *et al.*, 1994). The BBB is of particular importance for cholesterol metabolism in the brain, as it effectively prevents the uptake of cholesterol linked to lipoprotein from the bloodstream (Björkhem, Meaney and Fogelman, 2004). Consequently, the cholesterol level in the brain seems to be independent of that in peripheral tissues; therefore, the cholesterol requirement in the

CNS is met with locally synthesized cholesterol (Peet *et al.*, 1998). The brain does not seem to respond to many of the regulatory mechanisms that operate in maintaining cholesterol extracerebral homeostasis. Interestingly, the half-life of most cholesterol in the brain has been estimated to be about 5 years and 4 months in humans and mice, respectively (Andersson *et al.*, 1990; Dietschy and Turley, 2001; Björkhem, Meaney and Fogelman, 2004; Bjoandrkhem *et al.*, 2006), which contrast to the plasma half-life, measured in hours (Dietschy and Turley, 2001; Pfrieger, 2003; Fünfschilling *et al.*, 2007; Liu *et al.*, 2010). A recent study using a pericyte-deficient mouse model, *Pdgfb* ret/ret, demonstrated increased BBB permeability. Increased BBB permeability can lead to a significant flow of cholesterol in the brain and / or loss of cholesterol from the brain to the circulation. The animals showed that cholesterol synthesis was increased by about 60% and that there was an increase in leakage of 24-OHC from the brain to the circulation. This study was an important demonstration that a defective BBB can lead to an increase in the flow of a lipophilic compound from the brain (Saeed *et al.*, 2014)

1.21 HFD-induced hypercholesterolemia affects cerebral homeostasis in rodents

As one of the most cholesterol-rich organs, brain cholesterol homeostasis is heavily regulated; however, there is growing evidence that the lipid profile of the brain can be modified by HFD-induced hypercholesterolemia in rodents (Czuba *et al.*, 2017). Long-term feeding of rodents with HFD results in increased plasma cholesterol and, more importantly, disrupts cholesterol homeostasis in the brain, leading to Aβ accumulation, tau hyperphosphorylation and neuronal death (Vance, 2006). In this sense, HFD triggers astrocytes activation in the rodent's hippocampus and increases the expression of proteins involved in cholesterol transport through brain cell membranes, such as Apo-E (Zhong *et al.*, 2016).

A recent study showed that brain levels of 27-OHC transported from the systemic circulation increased, in rabbits fed with high cholesterol diet, leading to neurodegeneration in the hippocampus (Brooks, Dykes and Schreurs, 2017). The influence of HFD on the development of cerebral insulin resistance (IR) was also demonstrated by the presence, in the hippocampus of rodents fed with HFD, of high levels of phospho-IRS1 (Ser616) (Arnold *et al.*, 2014), phospho-Akt (Ser473) and phospho-GSK3β (Ser9) (Spinelli *et al.*, 2017). Therefore, both the short-term high-fat diet and the long-term, moderate-fat diet substantially interfere with insulin signaling pathways and induce IR in the brain (Arnold *et al.*, 2014). In addition to the increased risk of AD induced by HFD due to cerebral cholesterol metabolism dysfunction (Stapleton *et al.*, 2008), hypercholesterolemia has also been shown to induce hepatic insulin resistance and impaired synaptic plasticity (Liu *et al.*, 2015). Previous studies have indicated a coexistence of hypercholesterolemia with pathological signs of AD, but the mechanisms involved are still unclear (Ehehalt *et al.*, 2003).

Hypercholesterolemia and altered cholesterol homeostasis affect IR, but IR can also inversely affect cholesterol metabolism. In fact, insulin can activate the SREBPs transcription factors involved in cholesterol biosynthesis (Suzuki *et al.*, 2010). It has been shown that in diabetic mice with insulin deficiency, there is a reduction in the expression of the main transcriptional regulator of cholesterol metabolism, SREBP-2, in the hypothalamus and other areas of the brain, leading to a reduction in the brain cholesterol synthesis. In addition to the reduction of SREBP-2 in the hypothalamus of mice using shRNA results in increased feeding and weight gain (Suzuki *et al.*, 2010; Gamba *et al.*, 2019). A recent study shows that astrocyte activation is initiated in the early stages of

hypercholesterolemia and that the long-term cholesterol-rich diet triggered astrocyte activation in the rodent hippocampus. This led to increased expression of proteins involved in cholesterol transport across cell membranes, such as ApoE and aquaporin 4 (AQP4), as well as increased expression of the pro-inflammatory cytokine IL-1β. This high expression of ApoE during the early stages of hypercholesterolemia also impacts cholesterol homeostasis in the brain (Y. L. Chen *et al.*, 2016).

The homeostasis of cerebral cholesterol and its dysregulation has acquired a considerable impact; mainly caused by the connection of metabolic dysfunctions of cholesterol with numerous pathological processes: such as memory and cognitive impairment, neurodegenerative diseases, stroke and other cerebrovascular diseases and insulin-dependent metabolic brain disorders. A growing body of evidence suggests that there are even more connections between hypercholesterolemia and some pathological processes in the brain (Czuba *et al.*, 2017). New questions still arise, and the pathological mechanisms related to cholesterol in the brain require further investigation, suggesting that the use of cholesterol as a potential therapeutic target requires extensive investigation.

1. 22 Cholesterol, Hypothalamus and HFD

Cholesterol homeostasis is essential for brain function (Yutuc *et al.*, 2020), being highly regulated to maintain the structure and function of neurons (Bruce, Zsombok and Eckel, 2017). The circuits in the CNS, especially in the hypothalamus, control most of the peripheral metabolic responses that are triggered during energy imbalance (Morton *et al.*, 2006; Myers and Olson, 2012). In this sense, the consumption of HFD by rodents, promotes an inflammatory process and substantial changes in the hypothalamic neuronal circuits thus changing the homeostasis of brain (Thaler *et al.*, 2012; McLean *et al.*, 2019). It is becoming increasingly evident that the CNS is a major contributor to the regulation of systemic metabolism and lipid homeostasis (Bruce, Zsombok and Eckel, 2017).

When FAs levels are increased, as in the case of a HFD, there is evidence to suggest that the excess FAs detected in hypothalamic neurons via its accumulation or of its metabolites, can signal nutritional sufficiency and decrease the production of hepatic glucose, lipogenesis and secretion of VLDL-TG (Bruce, Zsombok and Eckel, 2017). In addition, recent studies have highlighted the existence of liver-related hypothalamic

neurons that have the potential to direct these signals through the activity of the parasympathetic and sympathetic nervous system (Yue *et al.*, 2015; O'Hare and Zsombok, 2016). The hypothalamus neurons express many molecular components of the lipid catabolism pathways, suggesting that lipid utilization is a critical process for neuronal function (Bruce, Zsombok and Eckel, 2017). For example, VMH neurons express enzymes involved in the intracellular metabolism of FAs, including long-chain acyl-CoA synthase (SCA), palmitoyltransferase-1a and 1c (CPT-1a and 1c) and uncouple protein-2 **(**UCP2) and enzymes involved in de novo lipogenesis, such as fatty acid synthase (FAS) (Le Foll *et al.*, 2009). AMPK-activated protein kinase can also act as a cell energy sensor within neurons to link neuronal lipid metabolism to systemic lipid metabolism and energy balance. AMPK is widely expressed in the hypothalamus ARC, PVN and VMH and is capable of detecting intracellular energy status by the AMP/ATP ratio and the level of adipokines (e.g., leptin and ghrelin) (López *et al.*, 2016).

It has been study has shown that the hypothalamus participates in cholesterol control, as it has been observed that electrical stimulation of the hypothalamus causes changes in the level of cholesterol in the blood, however the study has shown no molecular mechanisms to explain this observation (Bogach and Kosenko, 1964). Currently, it is known that circulating cholesterol in blood plasma is the balance between the absorption of cholesterol in the diet, the synthesis and hepatic secretion and the metabolism of lipoproteins by various tissues (Perez-Tilve *et al.*, 2010). A recent study investigated the role of hypothalamic PSA (polysialic acid) in regulating plasma cholesterol levels and distribution (Brenachot *et al.*, 2017). Hypothalamic PSA coordinates many aspects of metabolism and energy balance, including peripheral lipid metabolism and food intake (Benani *et al.*, 2012), osmoregulation (Theodosis *et al.*, 1999), circadian rhythm (Shen *et al.*, 1997; Glass *et al.*, 2000; Prosser *et al.*, 2003) and sleep (Black *et al.*, 2009). The study suggested that hypothalamic PSA, a modulator of neuronal function, is essential to maintain the LDL/HDL ratio through the short-term consumption of HFD. Given the role of PSA in the control of synaptic plasticity (Rutishauser, 2008), these findings postulate that blood cholesterol homeostasis can be regulated via neuronal reconnection in hypothalamic circuits. Moreover, the results demonstrated that the acute overfeeding induced by the consumption of HFD, rapidly increased plasma cholesterol levels in mice (Brenachot *et al.*, 2017). Cholesterol is mainly composed of low-density lipoprotein (LDL), very low-density lipoprotein (VLDL) and high-density lipoprotein (HDL) (Mao, Li and Zhang, 2018). Substantial changes were demonstrated in the characterization of

the main circulating cholesterol lipoproteins, revealing that VLDL, LDL and HDL cholesterol was elevated in rodents after 1 day of feeding with HFD. However, the mechanisms by which hypothalamic PSA affects plasma LDL and HDL cholesterol levels have yet to be elucidated (Brenachot *et al.*, 2017).

1. 23 Mechanism conversion of cholesterol in oxysterol

The brain is the richest organ in cholesterol, as it contains a quarter of the pool of unesterified cholesterol in the entire body (Dietschy, 2009). However, cholesterol metabolism in the brain is distinct from that of other tissues, due to the fact that cholesterol itself is unable to cross the blood-brain barrier (Milagre *et al.*, 2012). In order to maintain cholesterol homeostasis in the brain, excess cholesterol is converted into oxysterols, which are metabolites resulting from the enzymatic oxidation or auto-oxidation of cholesterol (Liao, Yoon and Kim, 2017).

Cholesterol in the brain is catabolized to 24-OHC by the specific neuron enzyme CYP46A1 (Björkhem *et al.*, 1997b, 1997a; Lund, Guileyardo and Russell, 1999; Boussicault *et al.*, 2016; Kacher *et al.*, 2019). 24S-OHC flows from the brain to the circulation through the BBB (∼99%), driven by the concentration gradient and therefore is subsequently excreted by the liver in the form of bile acids (Björkhem, Leoni and Svenningsson, 2019; Dosch, Imagawa and Jutric, 2019; Kacher *et al.*, 2019). The remaining 1% of 24-OHC flows to the CSF **(Figure 1.10)** (Lütjohann *et al.*, 1996; Dosch, Imagawa and Jutric, 2019).

Cholesterol in the brain is also oxidized to 27-OHC by the enzyme CYP27A1, expressed by neurons and glial cells. Unlike 24-OHC, most of the cerebral 27-OHC is derived from the peripheral circulation, since CYP27A1 is expressed in most organs and tissues (Marwarha and Ghribi, 2015). 24-OHC and 27-OHC can regulate the synthesis and transport of glial cholesterol to neurons, acting on the nuclear LXR, which regulates the expression and synthesis of ApoE and ABCA1/ABCG1 (Milagre *et al.*, 2012; Czuba *et al.*, 2017). In addition to enzymatic oxidation, cholesterol self-oxidation can be induced by different compounds, such as lipid peroxides, free radical species and metallic cations,

resulting in the formation of various oxysterols. As an example: 7α-OHC, 7β-OHC, 7-KC, 25-OHC, α-EPOX and β-EPOX are the most representative. Both 7α-OHC and 25-OHC can also be derived from enzymatic oxidation of cholesterol, respectively by CYP7A1 and CH25H **(Figure 1.10)** (Leoni and Caccia, 2013).

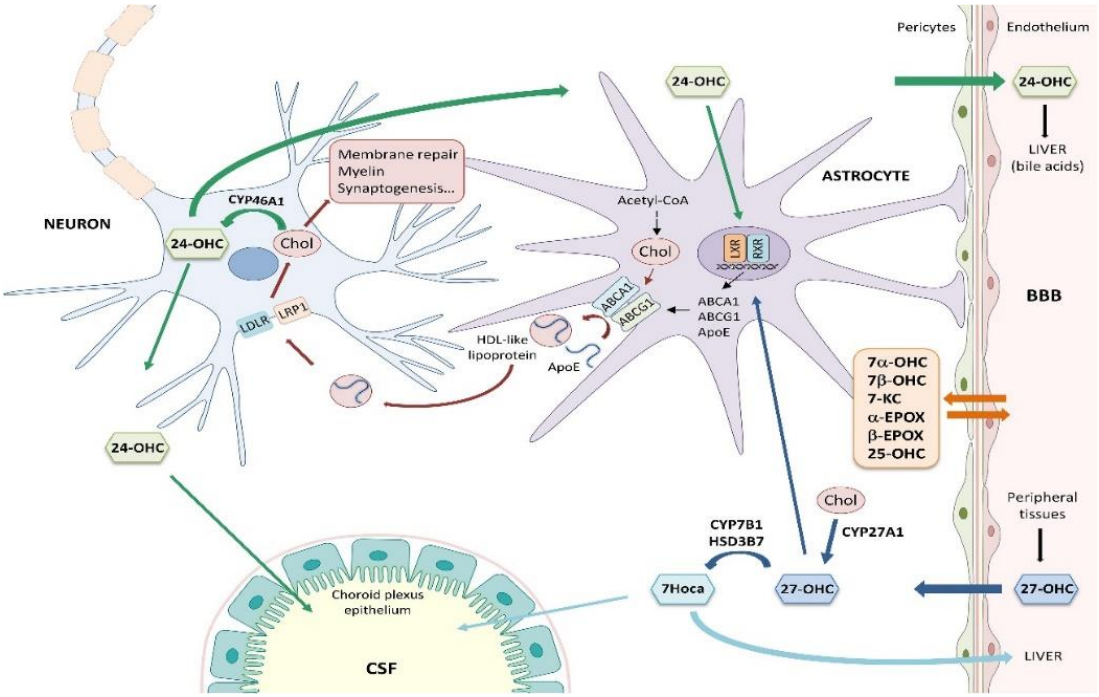


Figure 1.10. Mechanism conversion of cholesterol. The main mechanisms involved in cholesterol homeostasis in the brain. The pathways of cholesterol metabolites (oxysterols) are indicated by arrows and the important enzymes involved in cholesterol metabolism are demonstrated. (Source: Gamba *et al.*, 2019)

1. 24 Oxysterols

Oxysterols are oxidized forms of cholesterol, or its precursors, and represent the initial metabolites in cholesterol catabolism (Schroepfer and Wilson, 2000), being one of the most potent negative regulators of cholesterol biosynthesis (Björkhem and Diczfalusy, 2002). In addition, in contrast to cholesterol itself, oxysterols are considered forms of cholesterol transport, since additional hydroxyl groups facilitate the flow of cholesterol

through lipophilic membranes and are able to overcome the BBB (Meaney *et al.*, 2002; Björkhem, 2006; Režen, 2011; Poli, Biasi and Leonarduzzi, 2013; M. Y. Sun *et al.*, 2016). The suppression of cholesterol synthesis is dependent on the formation of 25-OHC, which plays an important role in viral infection and inhibition of replication. In addition, oxysterols act as LXR ligands (Janowski *et al.*, 1996) that induce the expression of genes involved in cholesterol efflux, including the ATCA (ABC) binding cassette ABCA1 and ABCG1 and ApoE transporters (Calkin and Tontonoz, 2012). ApoE disks potently accelerate the cholesterol efflux from primary human neurons and cell lines. Thus, the majority of cholesterol (approximately 75%) appeared to have an outflow of neurons in a native state via a transport route (Kim *et al.*, 2007; Koldamova, Fitz and Lefterov, 2014).

In general, oxysterols fall into two main categories; the oxygenated ones in the sterol ring, mainly in the 7 position (for example, 7α / β-hydroperoxicolesterol (7OOHC), 7-ketocholesterol (7KC) and 7α / β-hydroyxcholesterol (7HC) and the oxygenated ones in the side chain (for example, 24S- hydroxycholesterol (24-OHC), 25-hydroxycholesterol (25-OHC) and 27-hydroxycholesterol (27-OHC). Generally, ring oxygenated sterols tend to be formed non-enzymatically, while side chain oxygenated sterols generally have an enzymatic origin. Exceptions to this rule are for example, 25HC and 7αHC that can be produced by enzymatic and non-enzymatic routes (Olkkonen, Béaslas and Nissilä, 2012; Luu *et al.*, 2016).

The 25-hydroxycholesterol (25-OHC) regulates the expression of genes related to lipid metabolism. addition, 25-OHC activates a cascade of caspase mediated by the release of cytochrome c and acts in the generation of inflammasomes cell death (Xu *et al.*, 2003; Jang *et al.*, 2016).

The ATP-binding cassette that induce the expression of genes involved in cholesterol efflux, including the ATCA (ABC) binding cassette ABCA1 and ABCG1 and apolipoprotein E (apoE) transporters (Calkin and Tontonoz, 2012). ApoE disks potently accelerate the cholesterol efflux from primary human neurons and cell lines. Thus, the majority of cholesterol (approximately 75%) appeared to have an outflow of neurons in a native state via a transport route (Kim, Weickert and Garner, 2008; Koldamova, Fitz and Lefterov, 2014). 27-OHC is an endogenous oxysterol with multiple biological functions, including activity as a selective estrogen receptor modulator (SERM) (a specific estrogen receptor (ER) tissue-specific agonist-antagonist) and as an agonist of LXRs (Nicod *et al.*, 2015). It was shown to be elevated by pharmacological treatments,

diet-induced hypercholesterolemia or genetic manipulations (Umetani *et al.*, 2007; DuSell *et al.*, 2010). In breast tumors, 27-OHC acted as a partial ER agonist and stimulated tumor growth in several models of breast cancer in mice. Through action on LXRs, 27-OHC also increased the metastasis of breast tumors (Nelson, Chang and McDonnell, 2014). In bone, 27-OHC attenuated the action of estrogen and had negative effects on bone mineralization (DuSell *et al.*, 2010). 27-OHC has a potent effect on several important systems in connection with neurodegeneration (Björkhem *et al.*, 2009).

The main metabolite of cholesterol in the brain, 24-hydroxycholesterol (24-OHC), serves as a vehicle for cholesterol removal. It outperforms all others in the mature brain: the oxidation product of the 24-OHC side chain (Russell *et al.*, 2009; Meljon *et al.*, 2012). 24-OHC is so prevalent in the brain that it is known as "cerebrosterol" (M. Y. Sun *et al.*, 2016). Both 24-OHC and 27-OHC are effective inhibitors of cholesterol synthesis *in vitro* (Schroepfer and Wilson, 2000; Wang *et al.*, 2008). Recent studies with transgenic mouse models are consistent with the possibility that both the 27-OHC flow in the brain and the size of the 24-OHC pool in the brain are of regulatory importance for cholesterol synthesis (Maioli *et al.*, 2013; Saeed *et al.*, 2014). In mammals, 24-OHC is synthesized mainly in neurons by the cytochrome P450 (CYP) enzyme 46A1 (cholesterol 24-hydroxylase, CYP46A1) and acts as a form of cholesterol transport (Lund, Guileyardo and Russell, 1999; Björkhem, 2006). Side-chain oxysterols such as 24-OHC also alter the accessibility of membrane cholesterol, altering the membrane structure and indirectly influencing neuronal excitability (Bielska *et al.*, 2012, 2014).

The levels of the 24-OHC brain are variable throughout development. In rodents, brain 24-OHC nad other oxysterols levels are quite low in the early stages (Meljon *et al.*, 2012). With maturity in young adulthood, 24-OHC levels far exceed other metabolites, and during aging reduces its levels in relation to cholesterol (Lütjohann *et al.*, 1996). Recently, in several neurodegenerative diseases of aging, such as AD and PD, it was reported altered levels of 24-OHC compared to age-matched controls (Björkhem *et al.*, 2013; Leoni and Caccia, 2013). These observations suggest that 24-OHC may be a clinically useful biomarker for neurodegenerative diseases. These altered levels may play an important role in the pathological etiology of neurodegenerative diseases, in addition to functioning as a current biomarker; 24-OHC may become a nexus for therapeutic development.

It has been shown that about two thirds of cholesterol synthesis in the brain, is balanced by the neuronal formation of 24-OHC, which is able to cross the BBB into the circulation (Björkhem *et al.*, 1997b; Xie *et al.*, 2003). Several studies have reported the importance of the main biological oxysterols and the routes for their formation (Brown and Jessup, 1999; Schroepfer and Wilson, 2000; Gill, Chow and Brown, 2008; Korade and Kenworthy, 2008). However, the excess of oxysterols is toxic to cells; therefore, oxysterols are transported to the liver, where they are metabolized to bile acid products and excreted from the body (Chiang, 2013). FXR is a nuclear bile acid receptor and is also the main regulator of bile acid synthesis. Thus, FXR directly or indirectly regulates the expression and activity of oxysterol-producing enzymes (Režen, 2011). The figure shows the structures of several major biological oxysterols and their relationship to the structure of cholesterol **(Figure 1.11)**.

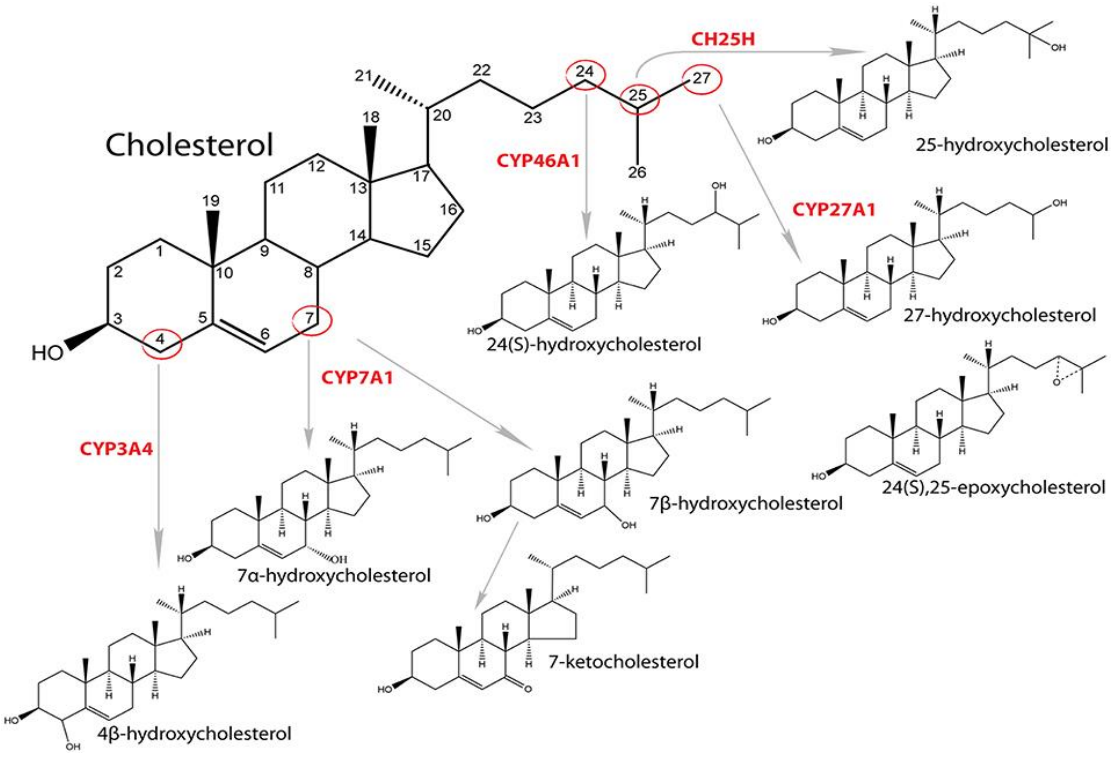


Figure 1.11. Cholesterol and Oxysterols. The synthesis of oxysterols by catalyst enzymes. Demonstration of the structure and origin of each type of oxysterol. Source: (Kovač *et al.*, 2019)

Currently, it is known that immune cells have the ability to recognize different oxysterols using intra and extracellular receptors. Immune cells express "generic" oxysterol receptors, such as the LXRs, as well as receptors expressed specifically by immune cells, such as the receptor coupled to the G 183 protein (GPR183) (Spann and Glass, 2013). Consequently, oxysterols seem to control several aspects of immune function including cell migration, production of cytokines, differentiation of T-cells, proliferation of lymphocytes, phagocytosis of macrophages, activation of inflammasomes and antiviral activity (Fessler, 2016).

1. 25 Oxysterols and Obesity

The current knowledge of oxysterol metabolism in the context of obesity is very limited (Wooten *et al.*, 2014). Obesity is associated with several pathologies known collectively as the metabolic syndrome (Guh *et al.*, 2009). Currently, it is known that among several etiologies, hyper lipidic diet and cholesterol metabolism are substantially important factors in the genesis of these disorders (Lupattelli *et al.*, 2012). In this sense, changes in cholesterol synthesis during obesity have been studied, including as a means of treatment (Wang and Peng, 2011; Guillemot-Legris and Muccioli, 2017).

A recent study aimed to determine the effect of diet-induced obesity, on the tissue distribution of several oxysterols and on the expression of the mRNA of key enzymes involved in the metabolism of oxysterol. This study demonstrated that obesity induced by HFD diet, resulted in markedly increased concentrations of enzymatically and non-enzymatically derived oxysterols in the circulation, as well as liver and adipose tissues. The increase in circulating oxysterols was associated with an increase in cholesterol, triglycerides and phospholipids in all fractions of lipoproteins and the presence of LDL/HDL, a phenotype typically observed in obese mice (Wooten *et al.*, 2014). Another important study also sought to investigate the levels of oxysterols, in models of obesity, induced by diet or of genetic origin, characterizing the most abundant oxysterols in the liver, hypothalamus, adipose tissue, and plasma. The results of this study showed marked changes in hepatic oxysterols levels in obesity models, in addition to altered levels of oxysterols in plasma, hypothalamus and adipose tissue. However, it was not possible to

establish a clear relationship between oxysterols levels and CYP enzyme mRNA levels (Guillemot-Legris and Muccioli, 2017). Cholesterol 25-hydroxylase (CH25H) converts cholesterol to 25-HC, an oxysterol that modulates immune responses. A recent study suggests a critical role for CH25H/25-HC in the progression of meta-inflammation and insulin resistance in obese humans and models of obesity in mice. Obesity induced by a high-fat diet in mice activates the expression of CH25H and the production of 25-HC in visceral adipose tissue. In addition, 25-HC increases inflammatory gene expression in macrophages and pre-adipocytes (Russo *et al.*, 2020). However, it is important to clarify to what extent the metabolism of oxysterols is altered during diet-induced obesity and how oxysterols can alter cholesterol homeostasis in obesity or predispose to the worsening of additional pro-inflammatory mechanisms involved in obesity (Wooten *et al.*, 2014; Guillemot-Legris and Muccioli, 2017; Russo *et al.*, 2020).

1. 26 Oxysterols and Human diseases

Oxysterols were first identified as intermediates in bile acid metabolism, but it is now becoming clear that oxysterols have pleiotropic roles in immunity and inflammation (Yu *et al.*, 2018). These metabolites have been suspected for some time to play key roles in various pathologies, mainly in cardiovascular diseases (Colles *et al.*, 2001). In fact, 7-ketocholesterol and 7β-hydroxycholesterol, which are the main components of oxidized LDL (oxLDL), are recognized for contributing to the genesis of atherosclerosis (Brown and Jessup, 1999) and are found at increased levels in atherosclerotic lesions and in the plasma of patients with cardiovascular diseases and individuals after a high-fat meal (Colles *et al.*, 2001; Virginio *et al.*, 2015; Brzeska, Szymczyk and Szterk, 2016; Wielkoszyński *et al.*, 2018). It was also shown that oxysterols can have cytotoxic and pro-inflammatory activities (Lemaire-Ewing *et al.*, 2005), and that these molecules can stimulate the differentiation of mesenchymal cells, monocytes, keratinocytes, lens epithelial cells and osteoblasts (Hayden *et al.*, 2002). Therefore, in addition to atherosclerosis, it is suggested that oxysterols may contribute to the development of numerous other degenerative diseases, such as age-related macular degeneration (Malvitte *et al.*, 2006), AD (Cao *et al.*, 2007) and osteoporosis (Liu *et al.*, 2005). It has

also been suggested that oxysterols may play a role in malignant diseases, such as breast, prostate, colon and bile duct cancer (Poli, Biasi and Leonarduzzi, 2013; Kloudova, Guengerich and Soucek, 2017).

1.27 Cytochrome P450 enzymes

Cytochrome P450 enzymes (P450s) are widely distributed among living organisms and play crucial roles in the biosynthesis of natural products, degradation of xenobiotics, steroid biosynthesis and drug metabolism. P450s are considered the most versatile biocatalysts in nature; due to the wide variety of substrate structures and the types of reactions they catalyze (Li *et al.*, 2020). The several P450s are very diverse, despite sharing common structural characteristics, with some such as human P450 3A4 having thousands of reported substrates (Rendic and Guengerich, 2015; Saravanakumar *et al.*, 2019). Prokaryotic P450s synthesize important secondary metabolites, such as antibiotics, and have also been used as model enzymes for the study of all aspects of the general P450 catalytic cycle (Khmelevtsova *et al.*, 2017). The use of prokaryotic P450s to catalyze several chemical reactions that are difficult to perform synthetically has also shown to be promising (Girvan and Munro, 2016). This includes the use of H2O2 and high-valence oxygen compounds as oxygen substitutes, for example, peroxides and iodosylbenzene, for chemical reactions (Albertolle *et al.*, 2017).

1. 28 Cytochrome P450 enzymes and Cholesterol

Cholesterol plays an important role in many biological processes that are considered central to the well-being of most living organisms. In addition of being a precursor to steroid hormones and bile acids, cholesterol plays a structural role and is an important component of cell membranes. The excess of cholesterol in many tissues is prevented in part by the action of different enzymes of cytochrome P450 that hydroxylate cholesterol in specific positions to facilitate its elimination (Pikuleva, 2006). These enzymes include 7-, 24 and 27-hydroxylases (CYP7A1, CYP46A1 and CYP27A1, respectively), as well

as the cholesterol side chain cleavage enzyme CYP11A1 (Pikuleva, 2006; Tempel *et al.*, 2014). CYP7A1 and CYP46A1 are microsomal enzymes, while CYP27A1 and CYP11A1 are mitochondrial monooxygenases. CYP7A1 is expressed only in the liver, CYP46A1 is found mainly in neurons, CYP27A1 is ubiquitous and CYP11A1 is the main cholesterol hydroxylase in steroidogenic tissues. In order for cholesterol to be metabolized by these P450s, they need NADPH as a source of reducing equivalents and proteins that transfer electrons from NADPH to P450 (Luthra, Denisov and Sligar, 2011).

The main intermediate in P450-mediated hydroxylation was identified as Compound I (Cpd I), the highly reactive hydroperoxy-Fe (IV) = O + • species (Rittle and Green, 2010). In addition to P450 hydroxylases, several tissues express cholesterol CH25H, a protein linked to the endoplasmic reticulum and a member of lipid hydroxylases that do not use oxygen as a cofactor (Russell et al., 2003). In recent years, there have been important advances in understanding the function of 25-hydroxycholesterol (Rittle and Green, 2010) as a key modulator of immune cell function and inhibitor of viral entry (Cyster *et al.*, 2014). This oxysterol can also be produced in cholesterol-rich tissues via auto-oxidation (Vejux, Malvitte and Lizard, 2008), a possibility that has received much less attention.

Many diseases are associated with specific P450 variants, and other diseases result from the lack of genes or the replacement of functionally inactive mutants endogenous and exogenous; as in the pathogenesis of cancer, cardiovascular diseases, atherosclerosis, hypertension, ischemia/reperfusion injury, diabetes mellitus, neurodegenerative diseases, rheumatoid arthritis and even normal aging (Zordoky and El-Kadi, 2010; Rendic and Guengerich, 2015; Petrov, Kasimov and Zefirov, 2016; Albertolle *et al.*, 2017; Petrov and Pikuleva, 2019).

1.29 Cytochrome P450- Drug-Metabolizing enzymes

CYP450 enzymes are a superfamily of enzymes that contain heme, classified based on the similarity of the amino acid sequence (McDonnell, PharmD, BCOP and Dang, PharmD, BCPS, 2013). The CYP enzymes superfamily are encoded by approximately 57 genes that have been found in the human genome (Fujikura, Ingelman-Sundberg and Lauschke, 2015; Ramírez *et al.*, 2019). Therefore, it constitutes the most relevant set of

isoenzymes responsible for the metabolism of endogenous and exogenous compounds and is located on the cytoplasmic side of the endoplasmic reticulum (Allegaert and van den Anker, 2019).

Human CYPs enzymes participate in the biotransformation of most xenobiotics, including more than two-thirds of all drugs in clinical use (Brill *et al.*, 2012). CYP genes are highly polymorphic, and variability in CYP content and activities has a considerable impact on drug pharmacokinetics and clinical response (Nebert and Russell, 2002). The genetic polymorphisms of CYPs are of particular relevance for *CYP2A6, CYP2C9, CYP2C19* and *CYP2D6* genes and lead to different pharmacokinetic phenotypes, for example, weak, intermediate, extensive and ultra-fast metabolizers (Zhou, Liu and Chowbay, 2009; Zanger and Schwab, 2013).

The most common form expressed in the human liver that metabolizes almost half of the drugs currently marketed is CYP3A4 (Zanger and Schwab, 2013; Rodríguez-Morató *et al.*, 2019). CYP3A, CYP2D6 and CYP2C9 together represent about 85% of all human oxidation activities of medicines (Zanger and Schwab, 2013; Tomankova, Anzenbacher and Anzenbacherova, 2017; Allegaert and van den Anker, 2019; Rodríguez-Morató *et al.*, 2019). In fact, most CYPs enzymes in the human body are mainly expressed in the liver (Zanger and Schwab, 2013), but they are also present in the small intestine, heart, lungs, placenta, kidneys and other organs (Lynch and Price, 2007). Drug metabolism occurs in many parts of the body, including the liver, intestinal wall, lungs, kidneys and plasma (Manikandan and Nagini, 2017). As the main site of drug metabolism, the liver works to detoxify and facilitate the excretion of xenobiotics (drugs or foreign chemicals), enzymatically converting fat-soluble compounds into more water-soluble compounds (Guengerich, Waterman and Egli, 2016). The most important CYPs enzymes that participate in the metabolism of drugs include CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A4 (Guengerich, 2003; Zanger and Schwab, 2013; Tomankova, Anzenbacher and Anzenbacherova, 2017). Drug metabolism is achieved through phase I reactions, phase II reactions, or both. The most common reaction of phase I is oxidation, which is catalyzed by the CYP system (Anzenbacher and Anzenbacherová, 2001; Ghose, 2016). Phase I of the biotransformation of foreign compounds includes oxidation, reduction or hydrolysis, converting the parent substance into a more polar metabolite (Fujikura, Ingelman-Sundberg and Lauschke, 2015; Ramírez *et al.*, 2019;Ogilvie *et al.*, 2020). CYPs enzymes

are essential for the detoxification of foreign chemical substances and for the metabolism of many drugs (Lynch and Price, 2007; Zanger and Schwab, 2013; Tomankova, Anzenbacher and Anzenbacherova, 2017).

1.30 Obesity and CYPs

Obesity is a serious metabolic disorder that has become a global health problem (Finkelstein *et al.*, 2012; Blüher, 2019). Obesity affects the properties of CYPs enzymes, in addition to being associated with the development of different diseases (Tomankova, Anzenbacher and Anzenbacherova, 2017; Rodríguez-Morató *et al.*, 2019). As mentioned, obesity it is often considered a low-grade inflammatory condition, resulting in an increased propensity for several chronic human diseases (Morrish, Pai and Green, 2011; Tahergorabi and Khazaei, 2013; Carbone *et al.*, 2019), including hypertension, diabetes (Khaodhiar, McCowen and Blackburn, 1999; Verma and Hussain, 2017), neurodegenerative diseases (Uranga et al., 2019; Mazon et al., 2017) and several types of cancers (Kompella et al., 2019; Stone et al., 2018; De Pergola & Silvestris, 2013).

Obese patients generally suffer from obesity-related illnesses and, therefore, depend on the concomitant use of diverse medication (Zanger & Schwab, 2013), which can lead to an increased risk of adverse effects and deleterious drug interactions (Ghose, 2013). Physiological and metabolic changes associated with obesity can also affect the pharmacokinetics of drugs, including absorption, distribution, metabolism and excretion (Smitet al., 2018; Ghose 2013; Morrish et al., 2011; Hanley et al., 2010; Lynch et al., 2007; Blouin et al., 1999).

In general, the study of human samples revealed that obesity is associated with a decrease in CYP activities, except enzymes CYP2C and CYP2E1. Similar results were obtained for most CYPs results in ob/ob mice (Tomankova et al., 2017). Other studies have documented that animal models of obesity exhibited altered expression of CYPs (Tomankova V et al., 2015; Patoine D et al., 2014; Cheng Q et al., 2008; Martignoni M et al., 2006; Watson AM et al., 1999). However, there are differences within an animal species, in gender specific differences or those depending in on the type of obesity induction (Veronika Tomankova et al., 2017). In a recent study, morbidly obese individuals were reported to have less CYP3A4 activity than healthy controls with normal

weight. However, the CYP3A4 activity found in morbidly obese patients is normalized after bariatric surgery. Among the carriers of two functional alleles for CYP2D6; morbidly obese individuals have a higher CYP2D6 activity than those with normal weight. Based on these findings, dose adjustments may be necessary to treat patients with morbid obesity with drugs that are substrates for CYP3A4 (Rodríguez-Morató et al., 2019). In fact, new studies should evaluate the changes observed in the activity of CYPs related to obesity, as there is currently no substantial data on how obesity can affect enzyme activity and the expression of CYP enzymes in an individual. Therefore, it is important to use animal obesity models as models for human obesity, as well as future clinical trials in humans to better understand the change in CYPs in obesity (Rodríguez-Morató et al., 2019; Tomankova et al., 2017).

1.31 CYPs and human diseases

As mentioned, in humans, CYPs participate in several innate metabolic pathways, for example such as, cholesterol biosynthesis, bile acids, eicosanoids and steroid hormone synthesis and catabolism; they are also involved in the biotransformation of xenobiotics, such as drugs and environmental pollutants (Bernhardt R, 2006; Nelson, DR et al., 2004; Anzenbacher, P. & Anzenbacherová, E., 2001). This considerable involvement in a wide range of biological processes requires fine tuning of CYPs to function properly (Košir et al., 2013). Consequently, any imbalance in the availability of enzymes or their malfunction, for example, due to a genetic mutation, can lead to a disease state in humans (Nebert et al., 2013; Pikuleva et al., 2013) or can alter an individual's susceptibility to environmental insults (Thier et al., 2003). The human genome encodes 57 P450 genes grouped into 18 families (Christ et al., 2019; Fujikura et al., 2015). Despite the significant sequence diversity among CYP families, all proteins exhibit common 3D structural elements shared with CYPs from other biological kingdoms (Masters, 2005; Graham & Peterson, 1999). The specificity of the substrate is important, as well as its physical-chemical characteristics and the composition of amino acids in the substrate recognition sites (Johnson & Stout, 2005). Missense mutations that alter the enzymatic activity can occur at the heme binding site, in the protein core and at the protein interface involved in the transfer of electrons from the redox partners, ferredoxin and oxidoreductase P450

(Robins et al., 2006; Crespi & Miller, 1997). However, not all mutations in CYPs have functional implications and result in a disease phenotype (Fechter et al., 2014). Genetic polymorphisms of CYPs can affect the enzyme's catalytic activity and have been reported in different populations to be associated with various diseases and adverse drug reactions (Elfaki et al., 2018).

Several studies have demonstrated a large number of pathways in which CYPs are involved, including drug and xenobiotic metabolism, show significant circadian variation in their activity (Chen et al., 2020; Zhanget al., 2018; Green et al., 2012; Froy, 2009; Gachon et al., 2006). Studies and meta-analysis data indicate that several CYPs exhibit a circadian pattern of expression not only in the liver, but also in other peripheral tissues (Froy, 2009; Yan et al., 2008). In fact, changes in CYP enzymes, in relation to the intricate connections between the mammalian circadian system and many metabolic pathways in the liver and other organs; demonstrated that the interrupted circadian rhythm can contribute to the most varied types of diseases, such as: cardiovascular diseases, ulcer, hypertension, night asthma, hyperlipidemia, cancer, rheumatoid arthritis, among others (Košir et al., 2013). In this way, the activities mediated by CYPs enzymes in xenobiotic metabolism (governed by the circadian clock), as well as in metabolic signals, can return to the circadian system and modulate its rhythm, predisposing or not to the condition of health or disease in humans (Lin, 2019; Košir et al., 2013; Green et al., 2008).

1.31.1 CYPs and Cancer

P450 cytochromes (CYPs) are key enzymes in the formation and treatment of cancer. They mediate the metabolic activation of various procarcinogens and participate in the inactivation and activation of anticancer drugs. Some forms of P450 are also selectively expressed in tumors, and this could provide a mechanism for drug resistance, but future therapies using these enzymes as drug targets can also be envisaged. The metabolism of various anticancer drugs is catalyzed by specific polymorphic forms of CYP, such as CYP2B6, CYP2C19 and CYP2D6 (Rodriguez-Antona et al., 2006).

Cytochrome P450 (CYP3A) forms have been shown to be consistently expressed in kidney cancer cells using immunohistochemistry, western blot analysis and reverse transcriptase PCR (Murray et al., 1999). This study suggested that expressed CYP3A may

be involved in the development of kidney cancer (Murray et al., 1999), and that these forms of CYP3A are the cause of the multiple drug resistance seen in this cancer. Cytochrome CYP1B1 was also found in renal cell carcinoma (McFadyen et al., 2004). Recently, it has been suggested that CYP1B1 is significantly unregulated in renal cell carcinoma and that it promotes this cancer progression (Mitsui et al., 2015).

CYP2A13 belongs to the member of the subfamily CYP2A and has been shown to be expressed predominantly in the respiratory tract, brain, mammary gland, prostate, testicles and uterus (Zhu et al., 2006). The highest expression quantified by PCR was in the nasal mucosa, trachea and lung tissues (Sun & Fan, 2013; Su et al., 2000). It has been shown that there is a negative regulation of CYP2A13 expression in adenocarcinoma and suggested that CYP2A13 is implicated in the development and progression of lung adenocarcinoma (Sun & Fan, 2013).

It has been suggested that there is an association between estrogen metabolism and smoking and there is growing evidence to suggest that estrogen levels are elevated in lung cancer (Slowikowski et al., 2017; Chakraborty et al., 2010). CYP1B1 is induced by tobacco (Port et al., 2004) and can convert tobacco procarcinogens into carcinogenic intermediates (Port et al., 2004). It has been proposed that CYP1B1 is implicated in prostate carcinogenesis due to its ability to activate metabolic carcinogens, as there is an association between exposure to PAHs and an increased risk for prostate cancer (Tokizane et al., 2005). CYP1A1 is involved in estrogen metabolism and can activate procarcinogens (He and Feng, 2015). CYP1A1 polymorphisms has been reported to be associated with the development of prostate cancer (Ding et al., 2013).

CYP27B1 has antitumor activity and, when deregulated by the epidermal growth factor, prostate cancer develops (Chen, 2008; Chen et al., 2012). Another CYP isoform of interest in prostate cancer is CYP17A1. CYP17A1 is involved in androgen biosynthesis and has been implicated in the proliferation of prostate cancer (Gomez et al., 2015). The use of CYP17A1 inhibitors (such as abiraterone acetate) is a recent strategy for the treatment of castration-resistant prostate cancer (Gomez et al., 2015).

Polymorphisms in *CYP1A1* gene has been associated with increased activity of aryl hydrocarbon hydroxylase, which in turn can alter the individual's susceptibility to breast cancer (Li et al., 2004; Ishibe et al., 1998). A recent study investigated the correlation between smoking and genetic polymorphisms of CYP1A1m1 T6345C and CYP1A1m2 A4889G with breast cancer risk in Iraqi women. The results demonstrated that the genetic

polymorphisms of the mutant genotypes CYP1A1m1 CC and CYP1A1m2 GG and the frequencies of the C, G allele were significantly associated with a higher risk of breast cancer when compared to healthy controls (Naif et al., 2018).

1.31.2 CYPs and Diabetes

CYP2E1 has been shown to be overexpressed in alcohol-induced liver damage as well as in non-alcoholic steatosis (Niemela et al., 2000). Diabetes is commonly associated with fat mobilization, as it will be the first source of energy that will lead to the development of non-alcoholic fatty liver disease (Hazlehurst et al., 2016). It has been shown that there is high activity of CYP2E1 in the liver of obese type 2 diabetic patients (Wang et al., 2003).

In an experiment carried out on non-obese T2D Goto-Kakizaki rats (Oh et al., 2012), it was shown that the hepatic expression of CYP3A2 was increased, while the expression of CYP1A2 and CYP3A1 was reduced. In another experiment carried out on streptozotocin-induced diabetic male Sprague-Dawley rats (Sindhu et al., 2006), it was reported that CYP1B1, CYP2B1, CYP1A2 and CYP2E1 is increased in diabetic rats, in contrast to CYP2C11, which has been decreased further 90% in diabetic rats. Both studies above indicated that, in diabetes, CYP expression is isoform specific and that this altered expression can be partially treated with insulin (Oh et al., 2012; Sindhu et al., 2006). In addition, it has been reported that *CYP2C8* * 3 (rs10509681), *CYP2C9* * 2 (rs1799853), *CYP3A4* (Ile118Val), *CYP2C19* * 2 and *CYP2C19* * 2 and *CYP1B1* * 2 (rs1056827) susceptibility were associated with increased susceptibility to diabetes type e 2 in Indians, Japanese, Mexicans and Saudi populations, respectively (Elfaki et al., 2018; Mahdi et al., 2016; Hoyo-Vadillo et al., 2010; Yamada et al., 2007).

1.31.3 CYPs and Cardiovascular disease

Many CYPs enzymes have been identified in the heart, kidneys, endothelium and smooth muscle of blood vessels. In addition, growing evidence points to the role of CYP-dependent endogenous metabolites, such as epoxyieicosatrienoic acids (TSEs), 20-hydroxyeicosatetraenoic acid (20-HETE), thromboxane A2 (TxA2) and prostacyclin (PGI2), in maintaining vascular physiology and cardiovascular homeostasis. The link between the genetic polymorphism of *CYPs* and its pathological impact on cardiovascular disorders, such as hypertension and myocardial infarction, has been established in recent years. Therefore, there are numerous studies indicating the involvement of CYP in atherosclerotic and cardiovascular diseases (Ong et al., 2017).

CYP7A1 plays a protective role against atherosclerosis (Li et al., 2011); it catalyzes the compromised stage in the synthesis of cholesterol and bile acids in hepatocytes and is critical for its homeostasis (Li et al., 2011). CYP7A1 maintains this homeostasis by improving the formation of bile acids from cholesterol and increases free cholesterol secreted in bile, without increasing the reabsorption of cholesterol by intestinal cells (Li et al., 2011). In humans, a frame-shift mutation in the *CYP7A1* gene (L413fsX414) that leads to loss of enzyme function would result in hypercholesterolemia and premature coronary and peripheral vascular disease (Pullinger et al., 2002). Recently, it has been shown in mice that overexpression of CYP7A1 attenuates atherosclerosis by increasing the secretion of bile acid (Li *et al.*, 2011). CYP4A11 also has an anti-atherosclerosis effect and metabolizes arachidonic acid to 20-hydroxyeicosatetraenoic acid vasoactive (20-HETE) (Fu et al., 2013; White et al., 2013). It has been reported that the SNP in the *CYP4A11* promoter (rs9332978 T> C) is associated with coronary artery disease in women, in a Russian cohort (Sirotina et al., 2018). I Elfaki et al., 2018). It has been reported that CYP polymorphisms confer disease susceptibility (Meng, Ma et al. 2015; Mittal et al., 2015; Pikuleva and Waterman, 2013), however, paradoxically, they can also act as protection against disease or reduced risk (Ur Rasheed et al. 2017; Yamada et al., 2007; Silvestri et al., 2003).

1.31.4 CYPs and Neurodegenerative disease

In adults, brain cholesterol is produced in situ and is unable to cross the blood-brain barrier, being mainly exported as 24-OHC, a reaction mediated by CYP46A1 enzyme (Bjorkhem et al., 2006), which expressed only in neurons (Guileyardo & Russell, 2006). A recent study confirms that CYP46A1 is the main enzyme that allows cholesterol efflux and activates cholesterol exchange in the brain (Nóbrega et al., 2019). This study demonstrated for the first time both *in vitro*, in a cell model of MJD, and *in vivo*, in the mouse brain, that CYP46A1 activates autophagy, which is impaired in MJD, leading to decreased deposition of mutant ataxin-3, and more broadly, also demonstrated the beneficial effect of CYP46A1 with mutant ataxin-2 aggregates. Thus, this study first identified CYP46A1 as a relevant therapeutic target, not only for MJD, but also for other SCAs (Spinocerebellar ataxias) (Nóbrega et al., 2019).

Other studies report that hippocampal protein CYP46A1 and 24-OHC levels were reduced in a mouse model of AD type Tau neuropathology (Burlot et al., 2015; Burnouf et al., 2013). The compromised turnover of cerebral cholesterol and the altered regulation of cerebral cholesterol metabolism have been combined with some neurodegenerative diseases, including HD (Nóbrega et al., 2020; Vance, 2012; Korade & Kenworthy, 2008). It has recently been demonstrated that CYP46A1 reduces the amount and size of mutant huntingtin aggregates in cells, in addition to mutant huntingtin protein levels. The results of this study suggest that the observed beneficial effects of CYP46A1 in HD cells are linked to the activation of autophagy, making CYP46A1 a promising target to stop or delay HD progression (Nóbrega et al., 2020).

Currently, the importance of CYPs enzymes in several physiological processes is becoming increasingly clear; as well as there is substantial evidence of its correlation to the etiology of various types of diseases, such as: various types of cancer, hypertension, atherosclerosis, cardiovascular and vascular problems, type 2 diabetes, and several neurodegenerative diseases (Nóbrega et al., 2020; Nóbrega et al., 2019; Lin et al.,2019; Naif et al., 2018; Sirotina et al., 2018; Elfaki et al., 2018; Ong et al., 2017; Navarro-Mabarak et al., 2018; Boussicault et al., 2016; Burlot et al., 2015; Oh et al., 2012). Therefore, there is the need for a continuos research effort in this field, trying to further understand the CYP role in disease and their suitability as therapeutic targets.

1.32 CYP silencing in the hypothalamus

As already mentioned, the conversion of cholesterol to 24-hydroxycholesterol (24-OHC) is the main mechanism for the removal of cholesterol from the brain and the reaction catalyzed by cytochrome P450 46A1 (CYP46A1), a specific CNS enzyme, produced by neurons (Y Chen et al., 2020; Lutjohann et al., 1996). *CYP46A1* is a crucial gene for effective cholesterol homeostasis in the brain (Y Chen et al., 2020; Nóbrega et al., 2019; Dosch et al., 2019; Björkhem et al., 2018; Sun et al., 2016). In fact, it has been shown that the mechanism of cerebral cholesterol homeostasis has a profound impact on the maintenance of brain health and substantially influences the maintenance of the whole-body metabolism (Gamba et al., 2019; Guillemont-Legris et al., 2016).

Recently, it has been shown that the accumulation of cholesterol in neurons in the hippocampus, induced by the silencing of *Cyp46a1* gene expression, leads to neurodegeneration with a progressive neuronal loss associated with the AD type phenotype in wild-type mice. This study also demonstrated that the inhibition of cholesterol 24-hydroxylase, a key enzyme in cholesterol metabolism, leads to a complex deregulation of lipid homeostasis (Ayciriex et al., 2017).

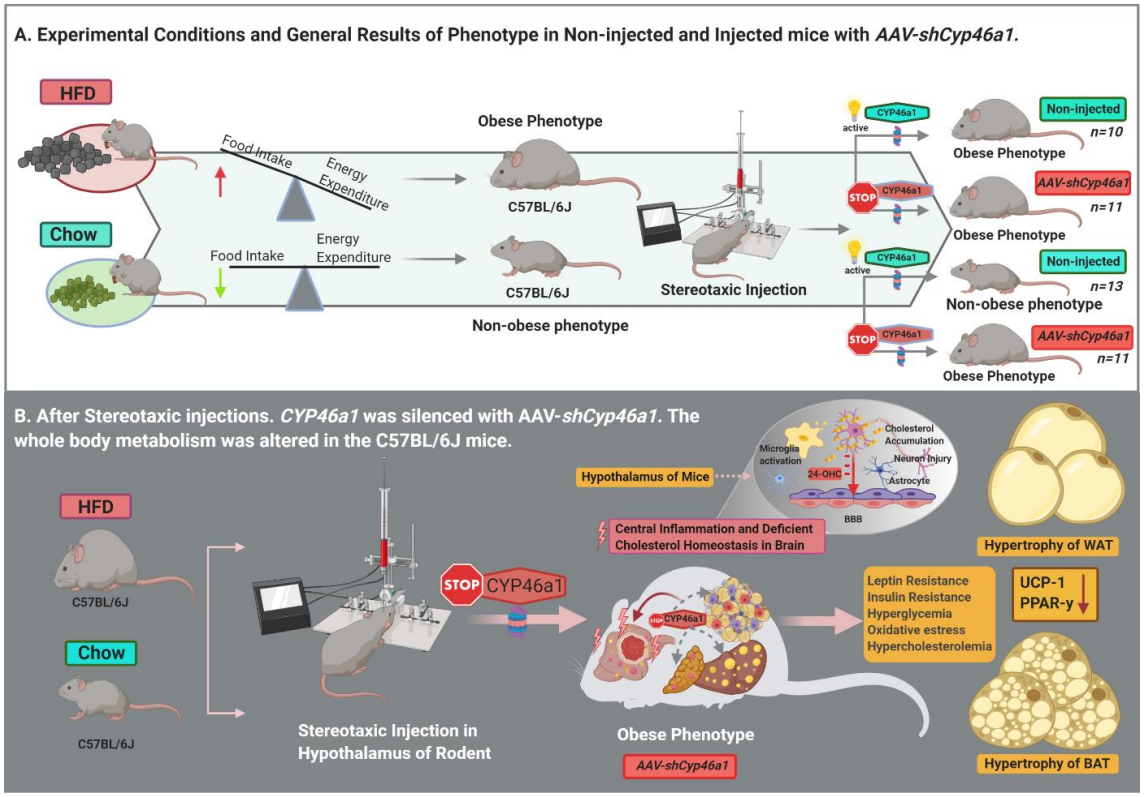
The hypothalamus, especially the arcuate nucleus, is the region of the brain responsible for regulating body energy homeostasis, playing an important crucial role in the balance between energy consumption and expenditure (Andermann ML & Lowell BB 2017). Diet-induced hypothalamic inflammation, more specifically in the arcuate nucleus, substantially alters cholesterol homeostasis in the brain (Guillemot-Legris et al., 2017). In this line of research, recent studies by our group sought to investigate the effects of silencing *Cyp46a1* on the hypothalamus of mice C57BL/6J fed on a Chow or HFD diet to understand its impact on whole-body metabolism. Therefore, we hypothesized that silencing of *Cyp46a1* gene in the hypothalamus, specifically in the ARC, leads to a reduction in the *Cyp46a1* enzymatic activity, as consequence to a reduction of 24-OHC levels and cholesterol accumulation in the ARC neurons. As the ARC is implicated in the control of whole-body energy metabolism, the silencing of *Cyp46a1* gene could lead to obesity type 2 diabetes development. The study (Pereira, 2019) was conducted during a 12-week period, in which, on the fourth week, the two groups were divided into four

subgroups. Two groups were submitted to bilateral stereotaxic injection in the ARC, constituting the treated groups, (Chow *AAV5-shCyp46a1* and HFD *AAV5-shCyp46a1*) and the other groups were not submitted to stereotaxic injection, constituting the control group **(Figure 1.12 A)**.

The results of the study could be resumed as follows:

- Silencing *Cyp46a1* gene in the hypothalamus (arcuate nucleus) of wild-type mice fed with Chow diet (Chow *AAV5-shCyp46a1)* seems to mimic the effects of an HFD diet, as it resulted in increased body weight, food and water intake; reduced glucose tolerance, reduced insulin sensitivity and substantial changes in several metabolic organs. These changes observed are mainly an increase on liver weight and hepatic steatosis, fatty pancreas and inflammatory changes, in addition to substantially modifying the adipocyte morphology of white and brown adipose tissue. Moreover, the study found a significant hypertrophy of white and brown adipose tissue, as well as a marked decrease in protein levels of PPAR-y and UCP-1. Indicating, pathological remodeling of the adipose tissue, with general increase in volume and loss of the thermogenic capacity of the brown adipose tissue **(Figure 1.12 B)**.

- Silencing *Cyp46a1* gene in the hypothalamus (arcuate nucleus) of mice fed with HFD diet (HFD *AAV5-shCyp46a1)* resulted in increased body weight, decreased food intake, decreased insulin sensitivity and decreased glucose tolerance; in addition to significant changes in metabolic organs, showing changes in pancreas, liver and white (WAT) and brown adipose tissue (BAT), and also a significant alteration in PPAR-y and UCP-1 protein levels in white and brown adipose tissue. The impact of the effect of silencing *Cyp46a1* on mice fed with HFD appears to exacerbate the obese phenotype, as they were already metabolically dysregulated.



1.12. Study of silencing *Cyp46a1* in the hypothalamus. (A) Experimental conditions and General results of phenotype in non-injected animals and injected animals. **(B)** The silencing of Cypx in hypothalamus led to: an obesity phenotype, even in control diet (Chow), the development of diabetes mellitus, an alteration in the morphology and histology of several metabolic organs (e.g. liver, pancreas, WAT and BAT) and decrease the levels of UCP-1 and PPARy in WAT and BAT.

CHAPTER 2 - Objective

Objective

Previous studies by our group have shown that silencing Cyp46a1 in the hypothalamus (arcuate nucleus) of obese mice induced by diet (C57BL/6J) has strong effect on the metabolism of the entire body. The Cyp46a1 silencing, results in a dysfunction of whole-body metabolism, including increased body weight, hypertrophy of adipocytes in white adipose tissue (WAT) and hypertrophy of adipocytes of brown adipose tissue (BAT).
Therefore, in this study, our main goal was to control the effect of the Cyp46a1silencing in the hypothalamus, by injecting a control gene (GFP) mediated by AAV, in both control and HFD fed mice. From this general goal, several other were outlined:

- To investigate the impact of the injection in inflammation and its impact in whole-body metabolism.
- To demonstrate that GFP protein does not interfere with whole body metabolism, in both control and HFD fed mice.
- To provide further evidence for the role of Cyp46a1 in the hypothalamus in the control of whole body-metabolism.

CHAPTER 3 - Methodology

Materials and Methods

3.1 *In vivo* studies

All experimental procedures were performed in accordance with European Union Directive 86/609/EEC for the care and use of laboratory animals. In addition, animals were housed in a licensed animal facility (International Animal Welfare Assurance number 520.000.000.2006). Researchers received adequate training (Federation of Laboratory Animal Science Associations (FELASA) certified course and certification to perform the experiments from the Portuguese authorities (Direção Geral de Alimentação e Veterinária – DGAV). The experimental procedures were approved by DGAV, project ALBUM reference 421/2019.

3.1.1 Animals and Diets

As the complications of obesity, such as diabetes and cardiovascular diseases, usually take decades, animal models are important to study the molecular aspects of obesity and its pathophysiological effects (Wang CY & Liao JK, 2013). One of these models with higher face validity is the diet-induced obesity model in the mouse (Speakman J et al., 2007). High-fat food have been shown to increase body weight and diabetes in several strains of mice and rats (Sclafani A, 1984). When fed a high-fat diet, the C57BL / 6J mouse is a particularly good model that mimics human metabolic disorders that are seen in obesity because, when fed *ad libitum* on a high-fat diet, these mice develop obesity, hyperinsulinemia, hyperglycemia and hypertension (Collins S et al., 2006; Rossmeisl M et al., 2003; Surwit et al., 1988).

For the experiments, adult C57BL/6J wild-type mice were obtained from in-house breeding (founders were bought from Charles River, Barcelona, Spain), and maintained in the animal pathogen-free facility of Centre for Biomedical Research (CBMR) of the

University of Algarve. Twelve week-old mice, with an average body weight of 20-25g, were housed 2-3 or 4 per cage under a 12 hours light/ 12 hours dark cycle in a temperature/humidity controlled (22+-2C) room with *ad libitum* access to water and food.

Initially, the entire cohort of animals were 14 mice (n = 14), all animals at twelve weeks of age, were randomly divided into two groups, corresponding to two different diets. One group (n = 7) (3 females and 4 males) had access to a low-fat control diet (Chow, D12450J, 10% fat, Research Diets, USA) and the other group (n = 7) (3 females and 4 males) had access to a high fat diet (HFD, D122492, 60% fat, Research Diets, USA). Both diets, Chow and HFD, and autoclaved water were provided *ad libitum*. This study was performed for 12 weeks. The stereotaxic surgery was performed in the 4th week of study, aiming to deliver the AAV-GFP into hypothalamus of mice. Mice for the two experimental groups were subjected to a GTT, an ITT and to a behavior test, at 12th weeks. The animals were sacrificed at 12 weeks. The percentage of Kcal derived from proteins, fats and carbohydrates from both diets is detailed in **Table 1.2.** During the 12 weeks of the study, the animals had access to the described foods and were carefully maintained in the described conditions. The mice were accommodated to the diets for four weeks, until stereotaxic surgery. **(Figure 1.13).**

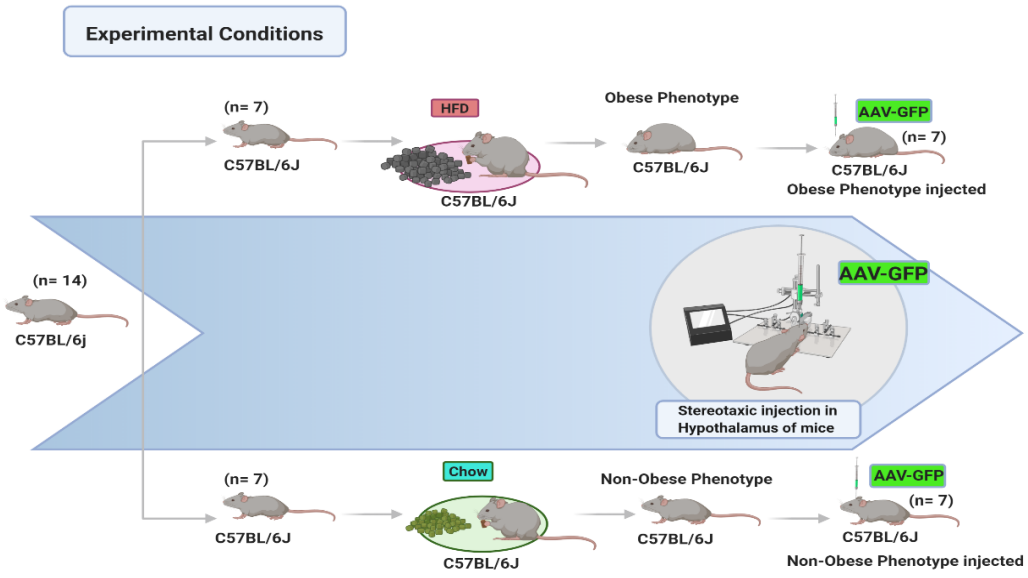


Figure 1.13. Experimental Conditions. At the beginning of the study, 12 weeks-old C57BL/6J *(n=14)* were divided into two groups of animals corresponding to the two different diets, a low-fat control diet (Chow, 10%fat) (n = *7*) and high-fat diet (HFD, 60% fat) *(n = 7).* After this period, both groups (n = 6 Chow) and (n = 7 HFD) received a bilateral stereotaxic injection in the hypothalamus (arcuate nucleus) with AAV-GFP, in the fourth week of the study.

	Low-fat control diet (Chow) -D12450J	High-fat diet (HFD) -D12492
Protein	20% Kcal	20% Kcal
Fat	10% Kcal	60% Kcal
Carbohydrate	70% Kcal	20% Kcal
Energy Density	3,82 Kcal/g	5,21 Kcal/g

Table 1.2 Caloric information of diets: Shows the caloric information (kcal) of each diet, Chow and HFD. **Abbreviations: Chow:** low fat control diet; **HFD:** high fat diet. All cohorts (n = 7 Chow) and (n = 7 HFD) underwent bilateral stereotaxic injection in the hypothalamus (arcuate nucleus) in the fourth week of the study.

However, after stereotaxic surgery (4 weeks of the study), one mouse (female) from the Chow diet group died due to post-anesthetic complications. Thus, after this, the entire cohort consisted of 13 animals (n = 13). After 10 weeks of the study, two females in the group fed the HFD diet showed, surprisingly, a remarkable resistance in gaining weight; presenting lower weight gain than the animals in the Chow group (n = 6). At this time, these two females were removed from the study, but continued to be fed and cared for until the end of the study to collect all tissue and organ data for future analysis. Finally, the total study cohort were 11 animals (n = 11). Animals fed by Chow n = 6 and animals fed by HFD ration n = 5.

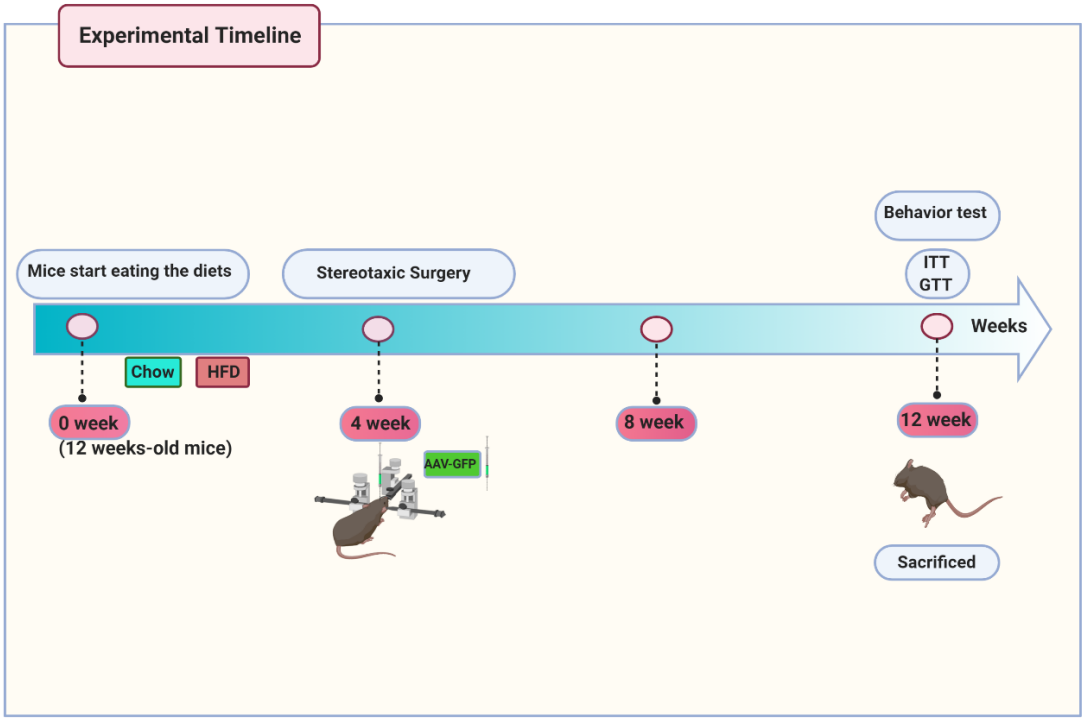


Figure 1.14. Representation of experimental timeline

The study was performed for 12 weeks. Initially, the C57BL/6J mice were accommodated to the diets (Chow and HFD). The stereotaxic surgery was performed in the 4th week of study, aiming to deliver the AAV-GFP into hypothalamus of mice. Mice for the two experimental groups were subjected to a GTT, an ITT and to a behavior test, at 12th weeks. The animals were sacrificed at 12 weeks. **Chow:** low fat control diet; **HFD:** high fat diet; **ITT:** insulin tolerance test; **GTT:** glucose tolerance test; **AAV:** adeno-associated viral vectors; **GFP:** green fluorescent protein.

3.1.2 Production of viral vectors

The AAV vectors were produced and purified by Atlantic Gene therapies (Inserm U1089, Nantes, France). All the viral vectors encoding for the different constructs were produced in human embryonic kidney (HEK) 293T cells using a four-plasmid system described previously (de Almeida et al., 2002). The cDNA encoding GFP (Nóbrega et al., 2013); the GFP reporter gene driven by the phosphoglycerate kinase 1(PGK1) promoter in the adeno-associated viral vector serotype 5 (AAV5) (Boussicault et al., 2016). Viral stocks were stored at -80 °C until use.

3.1.3 Stereotaxic Surgery

For the stereotaxic injections of the AAV, the concentrated viral stocks were thawed on ice and resuspended by vortexing. The stereotaxic injections were performed in the 4th week of study (Figure 8), aiming to deliver the AAV-GFP to the hypothalamus (arcuate nucleus) of the mice. First, the animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/ml, Dechra) and medetomidine (1 mg/ml, Esteve) (50-75 mg/kg + 1-10 mg/kg IP) and placed in an stereotaxic structure (Stoelting Company, USA) with the teeth and ears of the upper incisors on the support bars to immobilize the skull.
After the immobilization of the mice skull, the bregma point was identified and used as a reference point for the origin of the coordinates to access specifically to the arcuate nucleus of the hypothalamus. The hypothalamus coordinates were defined using The Paxinos and Franklin's Mouse Brain Atlas and the stereotaxic injection was performed bilaterally: 0,5 mm lateral to middle line (in each side of the brain), 1,65 mm posterior to bregma and -5,8 mm ventral to the brain surface: (AP= -1,65; DL= -0,5; MV= -5,8) (Franklin and Paxinos 2019).

The skull was drilled using a surgical drill and the two treated groups (Chow-AAV-GFP and HFD-AAV-GFP) received 1x10 a 9 v.g. (viral genomes) of AAV-GFP in each side of the arcuate nucleus of hypothalamus. The injection was performed at a rate of 0.5 µl/min with a 10 mL-Hamilton syringe attached to a Quintessential Stereotaxic Injector (QSI TM) (Stoelting Company). Finally, after the end of the administration, needle was kept in the plce for 5 min to minimize backflow (Aveleira et al., 2015). After, the animal was sutured, a reverser of anesthesia was administered; Atipamezole (5 mg/ml, Esteve) (1-2,5 mg/kg IM or SC) and post injection care performed. The health status of the animal was carefully monitored in the following days of the stereotaxic surgery. Mice were allowed to recover for two days before body weight and food intake analysis. At the end of this study (12 weeks after the food treatment and 8 weeks after stereotaxic injection), the mice were sacrificed. Mice were anesthetized using volatile anesthesia with 100% isoflurane (Zoetis) and sacrificed by decapitation.

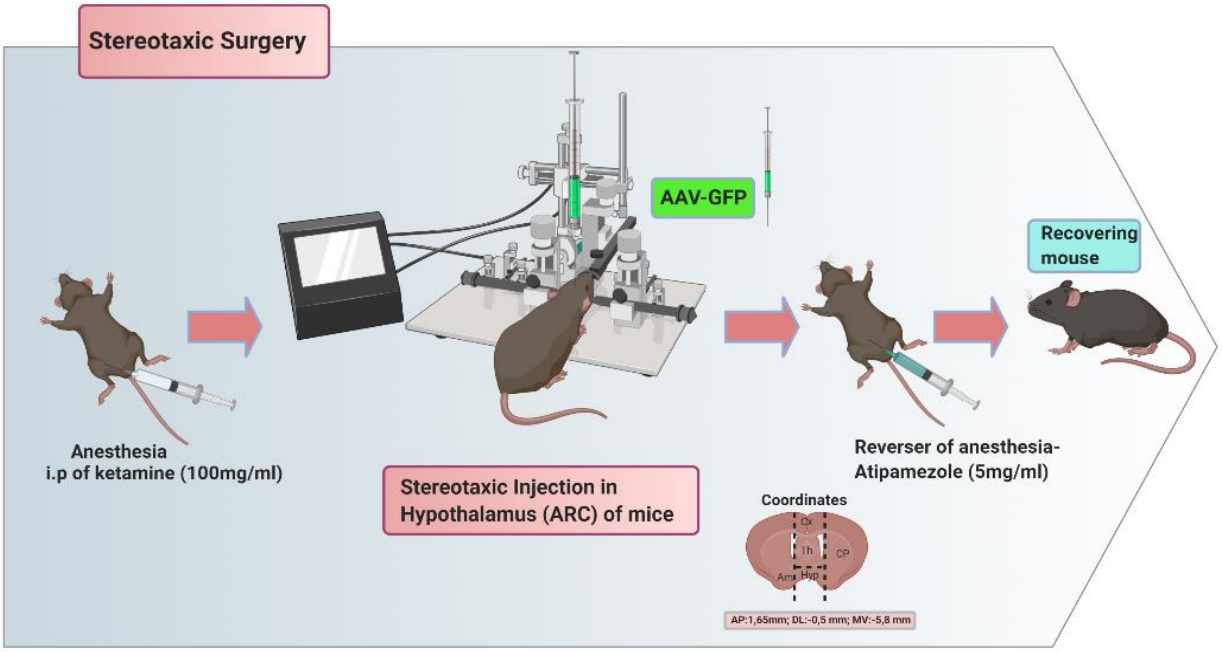


Figure 1.15. Stereotaxic Surgery. Representation of the entire process performed during stereotaxic surgery in the mice.

3.1.4 Animal behavior tests

The open field test (OFT) is a common measure of exploratory behavior and general activity in both mice and rats, where both the quality and quantity of the activity can be measured. Distance moved, time spent moving, rearing, and change in activity over time are among many measures that can be tabulated and reported (de TD Gould et al., 2009). Animals from all the experimental groups were subjected to an open field behavior test at the 12 weeks for the assessment of locomotor and anxiety-like behavior. The test consists of gently placing the mouse in the center of a transparent square box, without roof, with walls 40x40 cm and 40 cm high, in a controlled room with sound attenuation, lit with white light during the tests. Posteriorly, their movement activity was recorded by video for 10 min in day-time period (*n*=11) and 5 min in the nighttime period *(n*=11) using a GoPro Hero camera (GoPro Inc, USA). After, the following parameters were

measured using the ANY-maze behavioral tracking software (Stoelming Company, Europe): total distance, mean speed, time spend immobile, immobile episodes, number of lines crossings, number and time of rearing, number and time of grooming, number of entries and time in the middle. These parameters were scored from the video recordings. The number of rearing and grooming were counted as a vertical activity and rearing was only scored when mice raised both of front paws from the floor and leaned against a wall.

Figure 1.16. The square transparent box used in the open field tests.
The floor of transparent box was divided by lines, into nine equally sized squares, and was defined one square as the center zone and the eight squares along the walls as the periphery zones.

3.1.5. Glucose tolerance test

A glucose tolerance test is used to determine the mouse's ability to metabolize glucose. The test can demonstrate whether the animal can metabolize a standardized measured amount of glucose. Being used to diagnose type 1 diabetes mellitus and type 2 diabetes mellitus (de Eyth, 2019).

The animals were randomly divided into two groups for the GGT test: 4 animals from the HFD AAV-GFP and 4 animals from the Chow AAV-GFP were also selected. Briefly, animals were subject to starvation for about 12-16 hours (overnight period). In the morning, after this period, each animal was weighted and had a very small (2mm) portion of its tail cut off in order to start measuring blood glucose levels (time 0). The glycemia levels were measured using the FreeStyle Precision Neo glucometer (Abbot) (time 0). Then, mice were injected intraperitoneally with a 20% glucose solution (Fisher Chemical-G/0500/60), dissolved in saline solution, 0,9% NaCl. The injection volume of glucose solution to each animal was calculated according the formula: Body weight (BW) (g) X 10µl of 20% glucose solution (Andrikopoulos et al., 2008). Posteriorly, glycemia levels were measured at 5,15, 30, 60, 90 and 120 minutes after glucose administration.

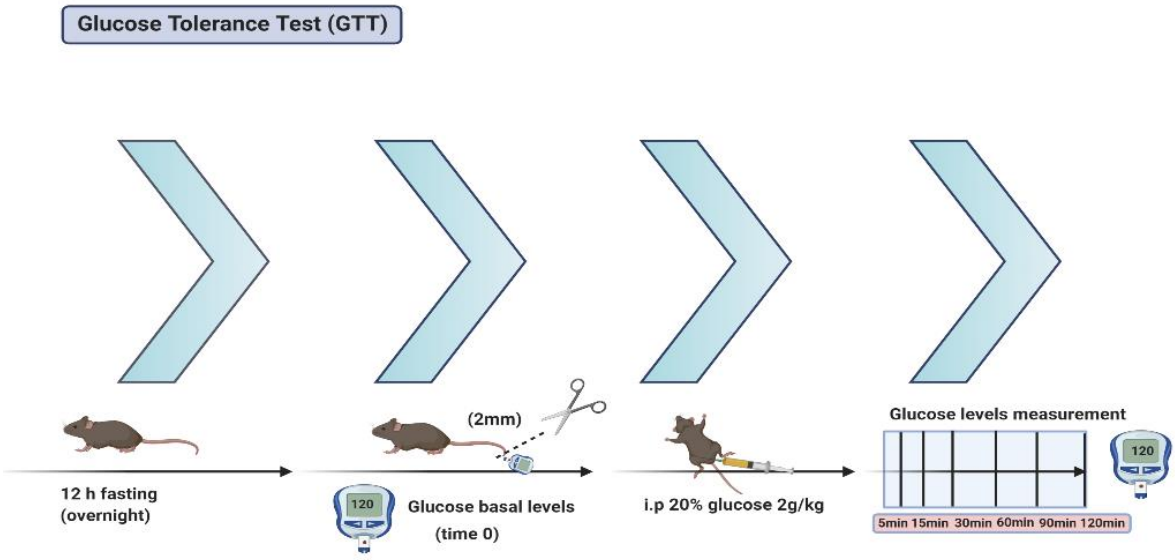


Figure 1.17. Schematic representation of GTT process. Mice were maintained during 12-16 hours in starvation, and glycemia basal were measured (time 0). Glucose was administered via I.p. and glycemia was measured 5,15, 30, 60, 90 and 120 minutes after glucose injection.

3.1.6 Insulin Tolerance test

Insulin resistance (IR) is predictive for type 2 diabetes and associated with various metabolic abnormalities in fasting conditions (de Q Wang et al., 2019). The ITT measures changes in blood glucose levels within 1 to 2 hours after the administration of intraperitoneal insulin to the mouse (de JE Ayala et al., 2010). The animals used in the GTT were also selected to perform the ITT test. Briefly, animals were subject to starvation for about 4-5 hours (in the morning). At the evening, after this period, each animal was weighted and had a very small (2mm) portion of its tail cut off in order to start measuring blood glucose levels (time 0). The glycemia levels were measured using the FreeStyle Precision Neo glucometer (Abbot) (time 0). Then, mice were injected intraperitoneally with 4 mg/mol human insulin solution (GibicoTM) in a phosphate-buffered saline solution (PBS X1). The injection volume of insulin solution was calculated using formula: BW (g) X 7,5µl of 4mg/ml insulin solution. Posteriorly, glycemia levels were measured at 5, 15, 30, 60, 90 and 120 minutes after insulin administration. At the end of ITT test, the animals were injected with 20% glucose solution to revert insulin effect and prevent animal death. The plasma glucose t1/2 was calculated from the slope of the least square's analysis of the plasma glucose concentration during the linear phase of decline (5-15 min) (Bonora et al., 1989).

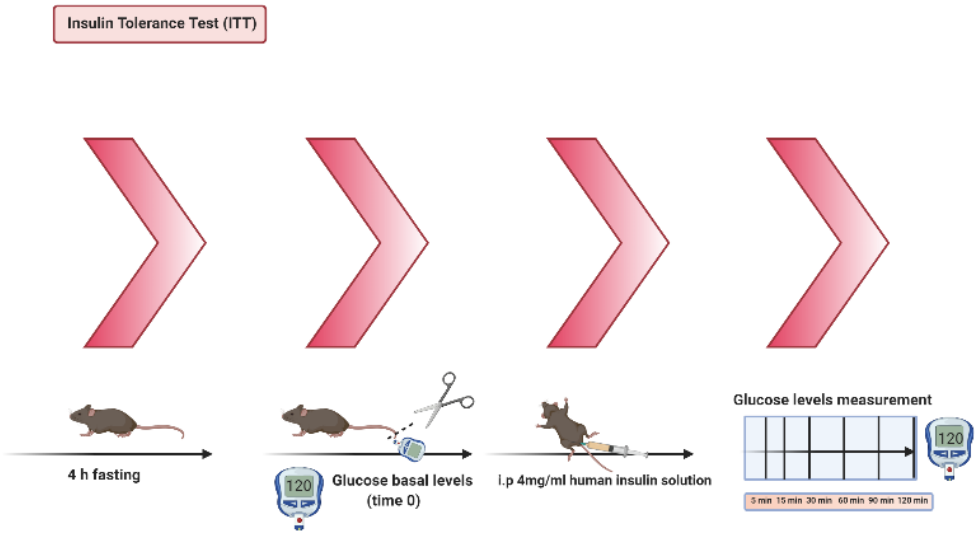


Figure 1.18. Schematic representation of ITT process. Mice were maintained during 4-5 hours in starvation, and glycemia basal were measured (time 0). After, mice were injected via i.p. with a 4mg/ml human insulin solution. Posterioly, glycemia was measured 5, 15, 30, 60, 90 and 120 minutes after insulin injection.

3.1.7. Tissues and blood collection

At week 12, mice were anesthetized with halothane (2-bromo-2-chloro-1, 1, 1-trifluoroethane) followed by decapitation. The blood was collected upon decapitation of the animals, and the serum was separated by centrifugation at 2000 rpm for 20 minutes at 4C. The serum samples were stored at – 80C for posterior analysis. The important metabolic organs and tissues were collected: brain, hypothalamus, liver, pancreas, spleen, muscle, white adipose tissue (WAT) and brown adipose tissue. The WAT, liver and spleen were weighed after organ collection with an analytical balance (AA-200 by Denver Instrument Company). The brain was individually collected, and the hypothalamus dissected for molecular analysis. The WAT, BAT, liver, pancreas and muscle of each animal were divided in three different portions for posterior analysis and histological processing. For the histological processing, the organs and tissues were fixed in 3,7%-4% formaldehyde (w/v) buffered (ph = 7) and stabilized with methanol fixative solution and stored at room temperature until the processing.

For the molecular analysis, small portions of the collected organs and tissues were stored at -80 C for posterior analysis.

3.1.8 Body weight, food and water intake analyses

During the 12 weeks of the experiment, mice were weighted twice a week for BW analysis, namely calculation of total BW gain (g) and cumulative BW gain (g). Additionally, the food was weighted, twice a week, and water measured, once a week, to calculate food and water intake per g/ml of BW. The ratio of food ingested (g) per total BW for cage (g) was calculated and then multiplied for the body weight of each mouse to obtain the food intake [(food ingested/total BW for cage X BW of each mouse)]. The ratio of water ingested (ml) per total body weight for cage (g) was calculated and then body weight of each mouse obtains the water intake.

Analysis

3.1.9 Histology

3.1.9.1 Tissue processing and paraffin inclusion

For tissue processing and paraffin inclusion, the organs and tissues previously fixed in a formaldehyde solution were divided into smaller pieces and placed in tissue processing/embedding cassettes. After, the dehydration of tissues was performed with 70% ethanol (v/v) (dilution from Fisher Chemical) for 1 hour; 95% ethanol (v/v) for 45 minutes; 95% ethanol (v/v) for 40 min and two series of 100% ethanol (v/v) for 1 hour each, follow by the clearing with two series of xylene (Fisher Chemical) for 1 hour each and by the infiltration with two series of paraffin (Luso Palex) in the incubator, at 56 C for 1 hour each. After, the tissue processing/embedding cassettes (Labor Spirit) were removed from the incubator. The organs and tissues samples were mounted in embedding molds (Tebu-bio) at the desired orientation and filled with liquid paraffin at 56 C. The cassettes, with the tissues, were placed on top and block was allowed to cool down and hardened. Afterwards, the block was removed from the mold and stored at room temperature in a dry place until use.

3.1.9.2 Sectioning

Paraffin blocks were sectioned in paraffin sections on HM 325 Rotary Microtome (Thermo Fisher Scientific) at room temperature.

The paraffin blocks containing white or brown adipose tissue were sectioned in paraffin sections with 4 or 5 µm of thickness. Other organs, such liver and pancreas, were sectioned in paraffin sections with 3 to 4wm to thickness. The paraffin sections were placed into microscopy slides and stored at room temperature until use.

3.1.9.3 Hematoxylin-eosin staining

Hematoxylin-eosin staining was performed according to the, manufacture´s guidelines (Merk Milipore). Briefly, the paraffin sections were placed in microscopy slide holders, deparaffinized with two series of xylene for 3 and 2 min and rehydrate with 100% ethanol (v/v), 95% ethanol (v/v) for 4 and 2 min, respectively, and with two series of distilled water for 30 seconds each. Posteriorly, the sections were stained with a hematoxylin solution modified, according to Gill III (Merk Milipore), for 30 seconds, washed two series of distilled water for 2 and 1 minute, counterstained with a 0,5% aqueous eosin Y solution for 1 minute and washed again in two series of distilled water for 1 minute each. For the dehydration and clearing, the sections were submitted to 95% ethanol (v/v), two series of 100% ethanol (v/v) for 1 minute each and two series of xylene for 2 min. The sections were allowed to dry and next mounted with Richard-Allan Scientific Mounting Medium (HM325, Thermo Fisher Scientific) and microscopy slide cover slips. Upon hematoxylin-eosin staining, the cells nuclei will stain dark purple and the cells cytoplasm will stain pink (acidophilic cytoplasm) or will stain light purple (basophilic cytoplasm).

3.1.9.4 Histological analysis of liver, pancreas, muscle, WAT and BAT.

Images of hematoxylin-eosin staining were acquired in the Axio Imager Z2 microscope (Carl Zeiss), using the Plan-Neo FLUAR 20X/0.5 Ph2 objective, the AXIOCAM-ICC3 camera and the AxioVision software (Carl Zeiss). The images obtained were analyzed using Zen 3.2 blue edition software (Carl Zeiss). Images of WAT were analyzed to calculate the adipocytes area, respective mean and the relative frequency in percentage.

3.10 Statistical analysis

Statistical analyses and graphing were performed using GraphPad Prism 7 (GraphPad Prism Software). All results are expressed as mean ± SEM. Values identified as outliers by Grubb´s test (alfa = 0.05) was excluded from analysis. The comparison of two independent groups the unpaired two-tailed Student´s t-test was used. For the comparison of more than two groups was performed by two-way analysis of variance (ANOVA), with Bonferroni´s multiple comparisons test. Statistical significance defined as $p<0,05$. [*P*-value < 0,05 (*), *P*-value < 0,01 (**), *P*-value < 0,001 (***), *P*-value < 0,0001 (****)].

CHAPTER 4 - Results

Results

1. High fat diet administration results in metabolic changes, increases food consumption and body weight gain of C57BL7/6J wild-type mice.

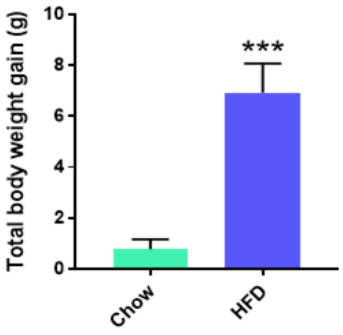
Overnutrition with HFD can dysregulate key hypothalamic processes, through an elicited inflammatory response. Currently, hypothalamic inflammatory state is regarded as the primary cause for obesity and affecting whole-body energy homeostasis (Milanski *et al.*, 2009; Guillemot-Legris and Muccioli, 2017). The C57BL/6J mouse is a particularly good model mimicking human metabolic derangements that are observed in obesity (Collins et al., 2004).

In this study, C57BL/6J wild type mice (*n* = 14) were divided into two groups corresponding to two different diets. One group (n = 7) was fed with a low-fat control diet (Chow) containing 10% fat, and the other group (n = 5) was fed a high-fat diet (HFD) containing 60% fat. The food was carefully stocked and weighed twice a week, as well as the water, and the animals for the analysis of body weight during the study period (12 weeks). In the 4th week of food access and before the stereotaxic injection with *AAV-GFP*, the total and cumulative BW gain was analyzed **(Figure 1.19 – A/B)** to observe the impact of HFD consumption in the body weight. The group fed with HFD showed a significant increase on total body weight gain comparatively to animals fed with a Chow diet. [Chow (0,7857 ± 0,3842); *n=7* versus HFD (6,93 ± 1,139); *n=5* – *P*-value <0,002] **(Figure 1.19 – A)**. In the cumulative gain of BW **(Figure 1.19 – B)**, it was observed that the HFD group gains weight compared to controls in each week of the study. It is important to note that at the beginning of the study, all C57BL / 6J wild type mice were around 20 to 25g, and in the 1st, 2nd, 3rd and 4th week, the HFD animals exhibited a significant increase on body weight gain [Chow; *n=7* versus HFD; *n=5* – 0: *P*-value >0,9999; 1: *P*-value =0,0002; 2: *P*-value <0,0001; 3: *P*-value <0,0001; 4: *P*-value <0,0001].

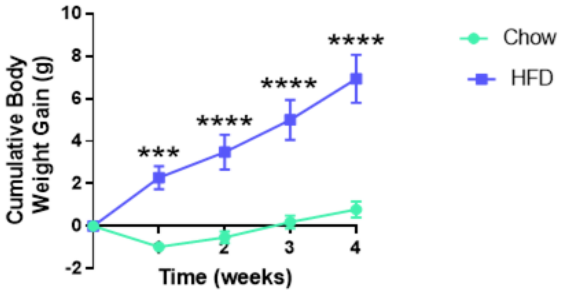
In the analysis of total food intake, the HFD group showed a significant increase on total food intake comparatively to the Chow group [Chow (91,02 ± 2,906); *n* =7 versus HFD (281,2 ± 19,82); *n*=5 – P-value <0,0001] in 4th week **(Figure 1.19 – C)**. In the cumulative food take analysis, the HFD group also presented a significant increase of food intake in the 2nd, 3rd and 4th week, comparatively to the Chow animals [Chow; *n* = 7 versus HFD; *n* = 5 – 0: *P*-value >0,9999; 1: *P*-value = 0,0125; 2: *P*-value <0,0001; 3: *P*-value <0,0001; 4: *P*-value <0,0001] **(Figure 1.19 – D)**.

In total water intake analysis, the HFD group presented a significant decrease relatively to the Chow animals [Chow (114 ± 10,27); *n* =7 versus HFD (82,01 ± 8,68); *n* = 5 – *P*-value =0,0487] (**Figure 1.19 – E)**. In the cumulative water intake analysis, the HFD group showed a decrease in water intake, in 3rd and 4th week, relatively to the Chow group **(Figure 1.19 – F).**

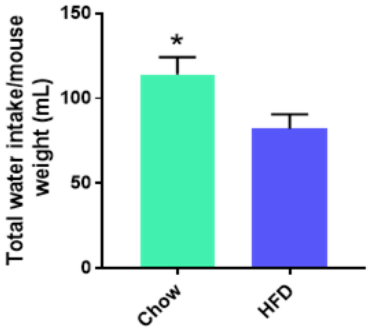
A)


Total body weight gain (g)
10
8
6
4
2
0
***
Chow
HFD

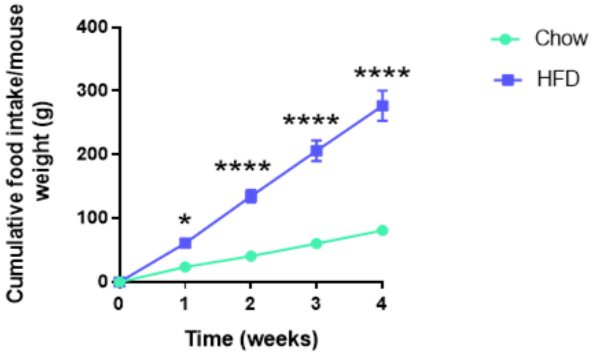
B)


Cumulative Body Weight Gain (g)
10
8
6
4
2
0
-2
***
****
****
****
2
3
4
Time (weeks)
Chow
HFD

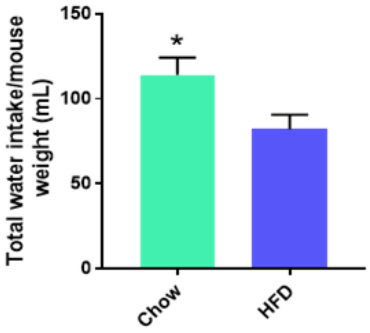
C)


Total water intake/mouse weight (mL)
150
100
50
0
*
Chow
HFD

D)


Cumulative food intake/mouse weight (g)
400
300
200
100
0
*
****
****
****
0
1
2
3
4
Time (weeks)
Chow
HFD

E)


Total water intake/mouse weight (mL)
150
100
50
0
*
Chow
HFD

F)

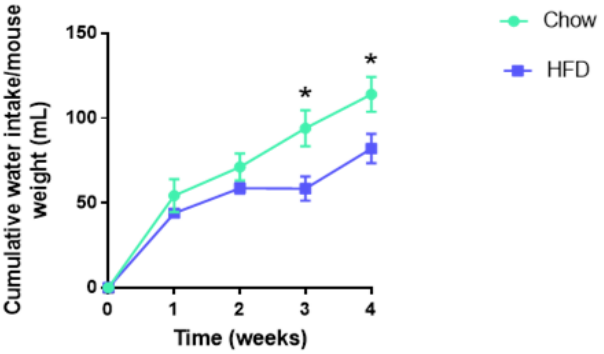

Cumulative water intake/mouse weight (mL)
150
100
50
0
*
*
0
1
2
3
4
Time (weeks)
Chow
HFD

Figure 1.19. HFD administration increases body weight, food intake and induce obesity phenotype in C57BL/6J wild-type mice. (A) The C57BL/6J mice fed with HFD diet, presented a statistically significant increase on total BW gain comparatively to the mice fed with Chow diet. **(B)** The HFD animals presented a statistically significant increase on cumulative BW gain comparatively to the Chow animals. **(C)** The HFD animals presented a statistically significant increase in total food intake comparatively to the Chow animals. **(D)** The HFD animals presented a statistically significant increase of the cumulative food intake, in 1st, 2^{n}, 3rd and 4th week, comparatively to the Chow animals. **(E)** The HFD animals presented statistically significant reduction of the total water intake comparatively to the Chow animals in the 4th week. **(F)** The Chow animals presented statistically significant increase of cumulative water intake, in 3rd and 4th week, comparatively to HFD animals. Data were expressed as the mean ± SEM. [*P*-value < 0,05 (*), P-value < 0,01 (**), *P*-value < 0,001 (***), P-value < 0,0001 (****) - Unpaired Student's t-test: A-C-E and Two-way ANOVA with Bonferroni's multiple comparisons test: B-D-F.

2. Stereotaxic injections with AAV-GFP did not alter the respective phenotypes achieved with the HFD diet and the Chow diet in C57BL7/6J wild type mice.

This study was conducted during a 12-week period; at the beginning of the study the mice (C57BL/ 6J) were randomly divided into two groups, Chow (control) and HFD. After this moment, each group was fed a specific diet for 12 weeks (HFD and Chow). In the fourth week of the study, the two groups underwent bilateral stereotaxic injection in the ARC. The two groups were composed by both females and males (Chow AAV-GFP: *n* =7; female: *n* = 3 and male: *n* = 4; HFD AAV-GFP: *n* = 5; female: *n* = 1 and male: *n* = 4). Importantly, before stereotaxic surgery there were 7 mice in the Chow group, however after stereotaxic surgery, 1 mouse died due to post-surgical complications regarding the effects of anesthesia. Then, after the stereotaxic injection, the chow group had 6 mice (Chow AAV-GFP: *n* = 6; female: *n* = 2 and male: *n* = 4). The analysis of total and cumulative body weight gain was carried out from the 4th to the 12th week of the study, in order to demonstrate that the stereotaxic injections of a control gene (GFP) mediated by lentiviral vectors AAV-GFP in the ARC did not interfere in the physiology of the hypothalamus **(Figure 1.21 – A/B).**

The HFD AAV-GFP animals presented a significant increase in total BW gain comparatively to the group of Chow AAV-GFP, after the stereotaxic injection [Chow AAV-GFP (0,4983 ± 0,9125); *n*=6 versus HFD AAV-GFP (15,38 ± 4,082); *n*=5- *P*-value=0,0036] **(Figure 1.21 – A) (Figure 1.20).** In the cumulative BW gain analysis **(Figure 1.21 – B)**, the HFD AAV-GFP animals exhibited a significant increase of body weight gain relatively to Chow AAV-GFP animals in the 3th, 6th and 8th week, after the stereotaxic injection [Chow AAV-GFP: *n* = 6 versus HFD AAV-GFP: *n* = 5 – 0: *P*-value

> 0,9999; 3: P-value=0,0188; 6: *P*-value=0,0002; 8: *P*-value <0,0001]. In the total food intake analysis **(Figure 1.21 – C**), the HFD AAV-GFP animals continue to present a significant increase of food intake after stereotaxic injection comparatively with Chow AAV-GFP animals [Chow AAV-GFP (142,5 ± 4,032); *n* = 6 versus HFD AAV-GFP (455,3 ± 89,49); *n* = 5 – *P*-value=0,0038]. In the cumulative food intake analysis **(Figure 1.21 - D)**, the HFD AAV-GFP animals continue to present a significant increase comparatively to Chow AAV-GFP in the 3rd, 6th and 8th week after stereotaxic injection [Chow AAV-GFP: *n* = 6 versus HFD AAV-GFP: *n* = 5 – 0: *P*-value >0,9999; 3: *P*-value=0.0056; 6: *P*-value <0.0001; 8: *P*-value <0,0001].

The Chow AAV-GFP animals **(Figure 1.21 – E)**, after stereotaxic injection, presented a decrease in total water intake relatively to the HFD AAV-GFP animals [Chow AAV-GFP (126,1 ± 5,433); *n* = 6 versus HFD AAV-GFP (146,9 ± 20,65); *n* = 5 *P*- value=0,3169]. In the cumulative water intake analysis, after stereotaxic injection, the HFD AAV-GFP continue to present a higher water intake relatively to the Chow AAV-GFP animals, although it is not statistically significant [Chow AAV-GFP; *n* = 6 versus HFD AAV-GFP; *n* =5 – 0: *P*-value >0,9999; 3: *P*-value=0,6611 >0,9999; 6: *P*-value=0,5337] **(Figure 1.21 – F).**

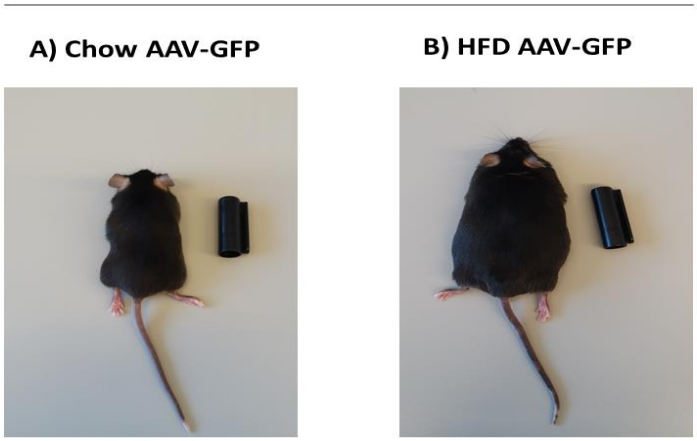


Figure 1.20. The difference between Chow AAV-GFP and HFD AAV-GFP animals in body weight gain with different diets (HFD diet and Chow diet). The notable difference at 12 weeks (end of study), after stereotaxic injection (*AAVGFP*), between the Chow AAV-GFP and HFD AAV-GFP animals in body weight gain due to the administration of different diets.

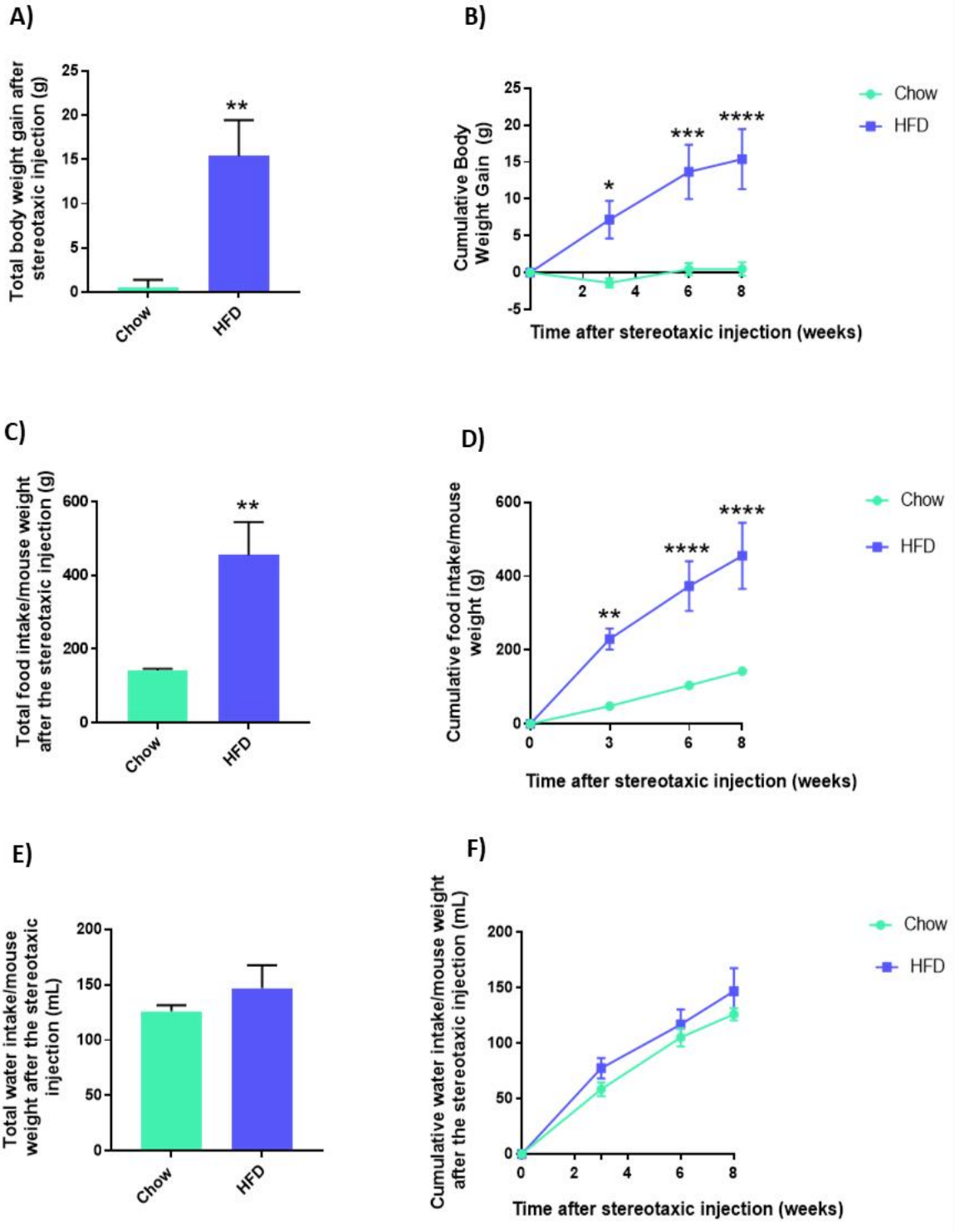

A)
Total body weight gain after stereotaxic injection (g)
25
20
15
10
5
0
**
Chow
HFD
B)
Cumulative Body Weight Gain (g)
25
20
15
10
5
0
-5
*
***
****
2
4
6
8
Time after stereotaxic injection (weeks)
Chow
HFD
C)
Total food intake/mouse weight after the stereotaxic injection (g)
600
400
200
0
**
Chow
HFD
D)
Cumulative food intake/mouse weight (g)
600
400
200
0
**
****
****
0
3
6
8
Time after stereotaxic injection (weeks)
Chow
HFD
E)
Total water intake/mouse weight after the stereotaxic injection (mL)
200
150
100
50
0
Chow
HFD
F)
Cumulative water intake/mouse weight after the stereotaxic injection (mL)
200
150
100
50
0
0
2
4
6
8
Time after stereotaxic injection (weeks)
Chow
HFD

Figure 1.21. Stereotaxic injections with AAV-GFP did not alter body weight, food intake and water intake. (A) After stereotaxic injection, HFD AAV-GFP animals presented a significant increase in total BW gain comparatively to the group of Chow AAV-GFP. **(B)** The HFD AAV-GFP animals exhibited a significant increase of body weight gain relatively to Chow AAV-GFP animals in the 3rd, 6th and 8th week, after the stereotaxic injection. **(C)** The HFD AAV-GFP animals presented a statistically significant increase of food intake after stereotaxic injection comparatively with Chow AAV-GFP animals. **(D)** The HFD AAV-GFP animals presented a statistically significant increase of the cumulative food intake after stereotaxic injection comparatively with Chow AAV-GFP animals. **(E)** The Chow AAV-GFP animals presented lower water intake analysis after stereotaxic injection; however, it was not statistically significant comparing to the HFD AAV-GFP animals. **(F)** The HFD AAV-GFP presented a slight increase in cumulative water intake analysis relatively to the Chow AAV-GFP animals, but it was not statistically significant. Data were expressed as the mean ± SEM. [*P*-value < 0,05 (*), P-value < 0,01 (**), *P*-value < 0,001 (***), P-value < 0,0001 (****) - Unpaired Student's t-test: A-C-E and Two-way ANOVA with Bonferroni's multiple comparisons test: B-D-F.

3. HFD administration induces glucose response alterations, hyperglycemia and a reduction of insulin sensitivity in C57BL/6J wild-type mice.

Obesity is closely related to hyperinsulinemia and insulin resistance (Kahn et al., 2006; Kahn & Flier, 2000). Insulin resistance is an integral part of the metabolic syndrome and the cause of type 2 diabetes. To further characterize the metabolic status of the mice and study the impact of feeding the HFD diet on glucose levels, a tolerance test to glucose (GTT) and an insulin tolerance test (ITT) were performed in 12th week of the study. These tests are indicators of the body's response to glucose release and an indication of the diabetic state, as well as allows assessing the sensitivity of the body to insulin and a possible resistance to insulin. Briefly, for the GTT the animals were subjected to starvation period for about 12-16 hours (overnight), and then the blood glucose was measured (t=0 min). After, the mice were injected intraperitoneally with a glucose solution (20%) and blood glucose levels were measured at 5, 15, 30, 60,90, and 120 min after the injection. The HFD AAV-GFP animals showed higher levels of fasting glucose compared to the Chow AAV-GFP animals and continued to show higher glucose levels after the intraperitoneal injection of glucose compared to the Chow AAV-GFP animals. The differences are significant after 30, 60, 90 minutes and 120 minutes [Chow AAV-GFP: *n* = 6 versus HFD AAV-GFP: *n* = 5 – 0: *P*-value=0,1165; 5: *P*- value >0,9999; 15: *P*-value >0,9999; 30: *P*-value <0,0001; 60: *P*-value <0,0001; 90: *P*-value <0,0001; 120:

P-value=0,0020] **(Figure 1.22 – A).** These values demonstrate the hyperglycemia present in the obese phenotype achieved with the HFD diet. It was also possible to observe that the blood glucose levels in the Chow AAV-GFP animals start to decrease at 15 minutes of the test after the intraperitoneal injection, however in the HFD AAV-GFP animals the blood glucose levels only start to decrease after 30 minutes after intraperitoneal injection.
In order to study the impact of HFD feeding in the insulin sensibility, an ITT was performed in the 12th week **(Figure 1.22 – B).** Briefly, the animals were subjected to a starvation period for 4 hours, and after fasting blood glucose levels were measured (t=0 min). After, the animals were injected intraperitoneally with a 4mg/ml insulin solution and the blood glucose levels were measured at 5,15,30, 60, 90 and 120 min after the injection. The HFD AAV-GFP animals showed significant high glucose levels since time 0 min, and 5 minutes after the insulin injection already showed a difference in the glucose levels comparatively to the Chow AAV-GFP animals. During the test, HFD AAV-GFP animals continue to presented higher levels of glucose with significant difference in the 60 minutes after the insulin injection, comparatively to the Chow AAV-GFP animals [Chow AAV-GFP n = 6 versus HFD AAV-GFP n = 5 – 0: *P*-value=0,0214; 5: *P*-value >0,9999; 15: *P*-value=0,1686; 30: *P*-value=0,6929; 60: *P*-value=0,0174; 90: *P*-value=0,2317; 120: *P*-value=0,0548] **(Figure 1.22 – B).** It was possible to observe that the blood glucose levels started to decrease at 5 minutes after the insulin injection in the two groups of animals (Chow AAV-GFP and HFD AAV-GFP), however the glucose levels of the HFDAAV-GFP animals remained significantly higher, especially at 60 minutes after the insulin injection.
Overall, these data suggest that HFD feeding induces hyperglycemia, reduction of glucose tolerance and of insulin sensitivity in C57BL/6J wild-type mice (Chow AAV-GFP and HFD AAV-GFP).

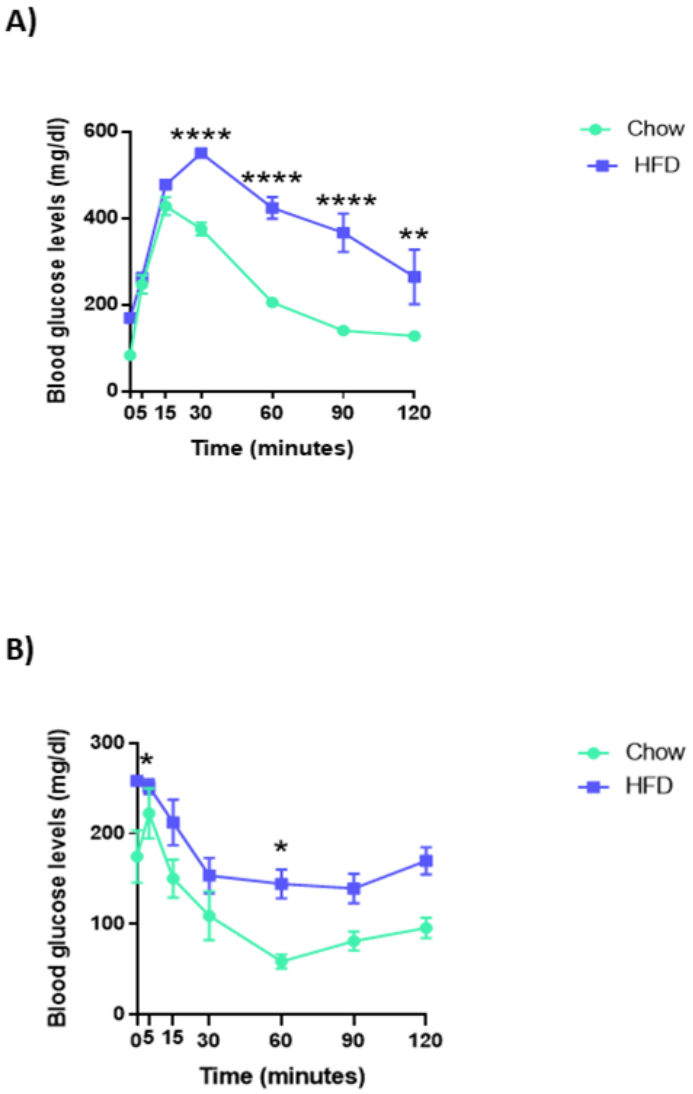


Figure 1.22. The diet HFD promotes hyperglycemia and a reduction of insulin sensitivity of C57BL/6J wild type mice. (A) The HFD AAV-GFP animals showed higher fasting blood glucose levels since time 0 and throughout the GTT test, compared to the Chow AAV-GP animals. The differences are significant after 30, 60, 90 and 120 minutes. **(B**) The HFD AAV-GFP animals showed significant high glucose levels since time 0 min, and 5 minutes after the insulin injection already showed a difference in the glucose levels comparatively to the Chow AAV-GFP animals. HFD AAV-GFP animals continue to present higher levels of glucose with a significant difference even in the 60 minutes after the insulin injection comparatively to the Chow AAV-GFP animals. Data were expressed as the mean ± SEM. [*P*-value < 0,05 (*), P-value < 0,01 (**), *P*-value < 0,001 (***), P-value < 0,0001 (****) - Two-way ANOVA with Bonferroni's multiple comparisons test: A-B.

4. Stereotaxic injection (*AAVGFP*) did not modify the behavior activity of C57BL/6J mice; however, HFD diet significantly modifies the grooming behavior activity of animals in daytime period.

The animals were subjected to an open field behavior test in the 12th week in order to study the impact of AAV-GFP expression and the impact of diets (Chow and HFD) in the motor and anxiety-like behavior. The open field behavior test consisted in placing the mouse in a wall-closed box sufficient high to prevent escaping, and the mouse motor activity was recorded (Seibenhener and Wooten, 2015) for 10 min in day time period (*n* = 11) and 10 min in the night-time period (*n* = 11). The following parameters were scored: total distance, mean speed, time spend immobile, immobile episodes, number of line crossings, number and time of rearing, number and time of grooming, number of entries and time spend in the middle.

In day-time period, the HFD AAV-GFP animals did not showed significant alteration in total distance traveled comparatively to the Chow AAV-GFP animals [Chow AAV-GFP (28,47 ± 2,417); *n* = 6 versus HFD AAV-GFP (29,65 ± 6,99); *n* = 5 – *P*-value=0,8671] **(Figure 1.23 – A)**, and, also the HFD AAV-GFP animals did not showed significant alteration in the mean speed comparatively to the Chow AAV-GFP animals [Chow AAV-GFP (0,0475 ± 0,004064); *n* = 6 versus HFD AAV-GFP (0,0494 ± 0,01167); *n* = 5 – *P*-value=0,8719] **(Figure 1.23 – B)**. In day-time period HFD AAV-GFP animals did not showed significant alteration comparatively to the Chow AAV-GFP animals of number of immobile episodes [Chow (40 ± 2,422); *n* = 6 versus HFD AVV-GFP (40 ± 4,97); *n* = 5 – *P*-value >0,9999] **(Figure 1.23 – C),** and also did not showed a significant alteration comparatively to the Chow AAV-GFP animals in the time immobile episodes relatively to Chow AAV-GFP animals [Chow AAV-GFP (146,6 ± 9,996); *n* = 5 versus HFD AAV-GFP (214,6 ± 46,71); *n* = 5 – *P*-value=0,1924] **(Figure 1.23 – D).** In day time period, HFD AAV-GFP animals showed a slight increase in the number of crossing lines comparatively to the Chow AAV-GFP animals; however, it was not significant statistically [Chow AAV-GFP (199,2 ± 23,95); *n* = 6 versus HFD AAV-GFP (234,6 ± 39,85); *n* = 5 – P-value=0,1924] **(Figure 1.23 – E).** However, HFD AAV-GFP animals did not showed significant alteration comparatively to the Chow AAV-GFP animals in the numbers of entries in the middle zone [Chow AAV-GFP (19,17 ± 3,609); *n* = 6 versus HFD AAV-GFP (26,8 ± 5,757); *n* = 5 – *P*-value=0,2739] **(Figure 1.23 – F)**. In daytime

period, the HFD AAV-GFP animals showed an increase of time spent in the middle of the arena comparatively to the Chow AAV-GFP animals; however, it was not significant statistically [Chow AAV-GFP (23,77 ± 3,848); *n* = 6 versus HFD AAV-GFP (32,56 ± 8,762); *n* = 5 – *P*- value=0,3531] **(Figure 1.23 – G).**

Importantly, in day-time period the Chow AAVGFP animals showed a significant increase in number of grooming´s comparatively to the HFD AAV-GFP animals [Chow AAV-GFP (16,17 ± 2,482); *n* = 6 versus HFD AAV-GFP (9,2 ± 2,518); *n* = 5 – *P*-value=0,0823] **(Figure 1.24 - H**), and also a significant increase in the time of grooming comparatively to the HFD AAV-GFP animals [Chow AAV-GFP (40,9 ± 4,238); *n* = 6 versus HFD AAV-GFP (22,18 ± 1,547); *n* = 4 – P-value=0,0088] **(Figure 1.24 – I).** In day-time period the HFD AAV-GFP animals did not show a significant difference in the numbers of rearing comparatively to the Chow AAVGFP animals [Chow (46,17 ± 6,258); *n* = 6 versus HFD AAV-GFP (46,2 ± 9,249); *n* = 5 *P*-value=0,9976] **(Figure 1.24 – J)**, neither in the time of rearing comparatively to the Chow AAVGFP animals [Chow AAV-GFP (34,63 ± 4,717) n=6 versus HFD AAV-GFP (34,72 ± 7,929); *n* = 5 – *P*-value=0,9924] **(Figure 1.24 – L).**

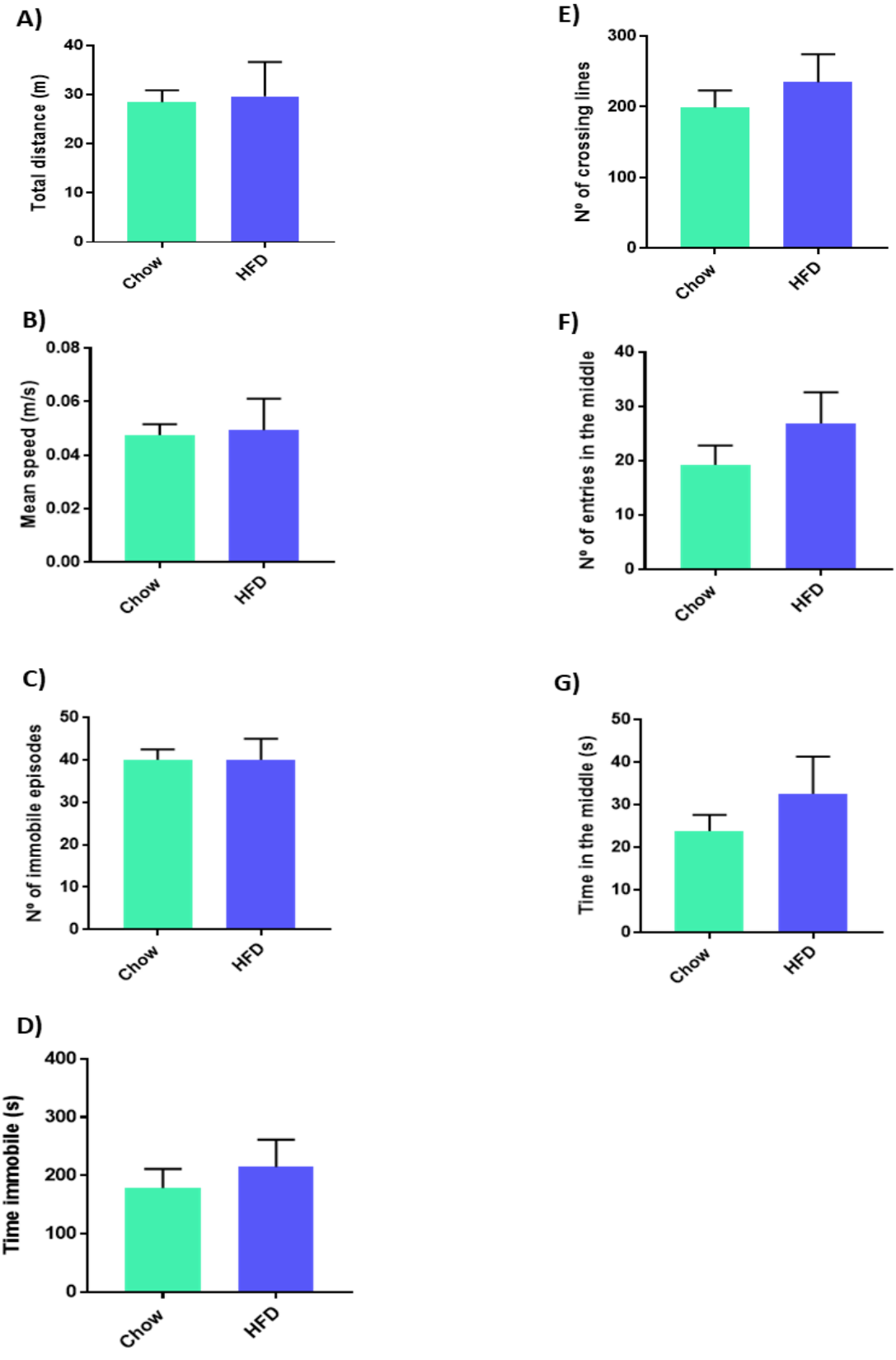

A)
Total distance (m)
40
30
20
10
0
Chow
HFD
B)
Mean speed (m/s)
0.08
0.06
0.04
0.02
0.00
Chow
HFD
C)
Nº of immobile episodes
50
40
30
20
10
0
Chow
HFD
D)
Time immobile (s)
400
300
200
100
0
Chow
HFD
E)
Nº of crossing lines
300
200
100
0
Chow
HFD
F)
Nº of entries in the middle
40
30
20
10
0
Chow
HFD
G)
Time in the middle (s)
50
40
30
20
10
0
Chow
HFD

Figure 1.23. In the daytime, HFD did not significantly modify the total distance traveled, mean speed, number of immobile episodes and time immobile of the animals. (A) The HFD AAV-GFP animals did not showed a significant alteration in total distance traveled comparatively to the Chow AAVGFP animals**. (B)** The HFD AAV-GFP animals did not showed significant alterations in the mean speed comparatively to the Chow AAV-GFP animals. **(C)** The HFD AAV-GFP animals did not showed a significant alteration of the number of immobile episodes comparatively to the Chow AAV-GFP animals. **(D)** The HFD AAV-GFP animals showed a slight increase in the time immobile episodes comparatively to the Chow AAV-GFP animals; however, it was not significant statistically. **(E)** The HFD AAV-GFP animals showed a slight increase in the number of crossing lines comparatively to the Chow AAV-GFP animals; however, it was not significant statistically. **(F)** The HFD AAV-GFP animals showed an alteration comparatively to the Chow AAV-GFP animals in the numbers of entries in the middle zone; however, it was not significant statistically. **(G)** The HFD AAV-GFP animals showed an increase of time spend in the middle comparatively to the Chow AAV-GFP animals; however, it was not significant statistically. Data were expressed as the mean ± SEM - Unpaired Student's t-test.

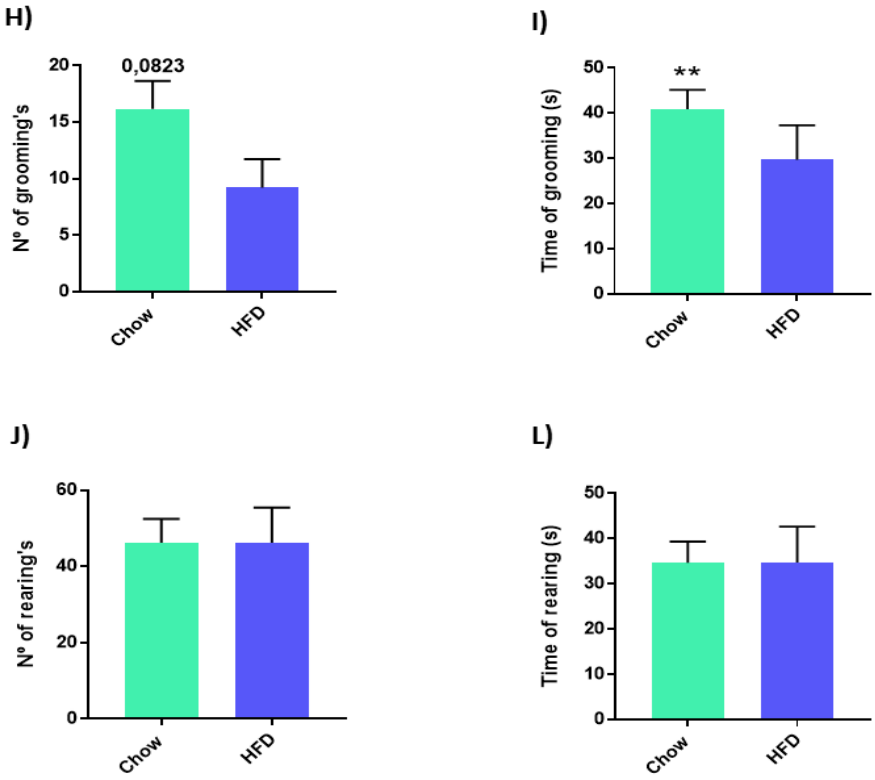


Figure 1.24. In the daytime, HFD diet significantly modifies the grooming behavior activity of the animals. (H) The Chow AAV-GFP animals showed an increase in the number of grooming's comparatively to the HFD AAV-GFP animals; however, it was not statistically significant. **(I)** The Chow AAV-GFP animals exhibit a statistically significant increase in the time of grooming's comparatively to HFD AAV-GFP animals. **(J)** The Chow AAV-GFP animals did not exhibited alterations in the numbers of rearing´s comparatively to HFD AAV-GFP. **(H)** The Chow AAV-GFP animals did not exhibit a significative alteration in the time of rearing comparatively to the HFD AAV-GFP. Data were expressed as the mean ± SEM. [P-value < 0,01 (**) - Unpaired Student's t-test.

In the night-time period, there was no significant difference in the total distance traveled between the HFD AAV-GFP and Chow AAV-GFP animals [Chow AAV-GFP (22,54 ± 2,699); *n* = 6 versus HFD AAV-GFP (21,77 ± 3,112); *n* = 5 – P- value=0,8550] **(Figure 1.25 – A)**, and, also there was no significant difference in mean speed comparatively between the HFD AAV-GFP and the Chow AAV-GFP animals [Chow AAV-GFP (0,0375 ± 0,004581); *n* = 6 versus HFD AAV-GFP (0,0364 ± 0,005134); *n* = 5 – P-value=8762] **(Figure 1.25 – B)**. In the night-time period, HFD AAV-GFP animals showed a slight increase in the number of immobile episodes comparatively to the Chow AAV-GFP, however it was not significant [Chow AAV-GFP (39,67 ± 4,58); *n* = 6 versus

HFD AAV-GFP (47,4 ± 2,657); *n* = 5 – P-value=0,2008] **(Figure 1.25 – C)**, and, also HFD AAV-GFP animals showed more time in the time immobile comparatively to Chow AAV-GFP animals, however it was not significant statistically [Chow AAV-GFP (187,8 ± 35,78); *n* = 6 versus HFD AAV-GFP (266,2 ± 32,21); *n* = 5 – *P*-value=0,1451] **(Figure 1.25 – D)**. In the night-time period, the Chow AAV-GFP did not showed significant alteration in the number of crossing lines comparatively to the HFD AAV-GFP animals [Chow AAV-GFP (205 ± 26,51) *n* = 6 versus HFD AAV-GFP (182,6 ± 24,75); *n* = 5 – P-value=0,5583] **(Figure 1.25 – E).** In the night-time period, the HFD AAV-GFP animals showed more alteration in the numbers of entries in the middle zone comparatively to the Chow AAV-GFP animals, however it was not significant statistically [Chow AAV-GFP (19,17 ± 3,609); *n* = 6 versus HFD AAV-GFP (26,8 ± 5,757); *n* = 5 – P-value=0,2739] **(Figure 1.25 – F).** In the night-time period, the HFD AAV-GFP showed an increase in the time spent in the middle of the arena comparatively to the Chow AAV-GFP animals, however It was not significant statistically [Chow AAV-GFP (29,33 ± 6,115); *n* = 6 versus HFD AAV-GFP (62,94 ± 20,41) *n* = 5 – P-value=0,1209] **(Figure 1.25 –G).** In the night-time period, the Chow AAV-GFP presented more numbers of grooming's comparatively to the HFD AAV-GFP animals, although, it was not statistically significative [Chow AAV-GFP (8,167 ± 0,7923); *n* = 6 versus HFD AAV-GFP (5,8 ± 1,828) *n* = 5 – *P*-value=0,2369] **(Figure 1.26 – H).** In the night-time period, the HFD AAV-GFP chowed increased in time of grooming's comparatively to the Chow AAV-GFP animals, although, it was not statistically significative [Chow AAV-GFP (18,08 ± 1,765); *n* = 6 versus HFD A AV-GFP (21,6 ± 5,936); *n* = 5 – P-value=0,5527] **(Figure 1.26 – I).** In the night-time period, there was no difference in the numbers of rearing between the HFD AAV-GFP and Chow AAV-GFP animals [Chow AAV-GFP (43,83 ± 5,269); *n* = 6 versus HFD AAV-GFP (43 ± 10,88); *n* = 5 – P-value=0,9434] **(Figure 1.26 – J).** In the night-time period, the HFD AAV-GFP animals did not showed modifications comparatively to the Chow AAV-GFP animals [Chow AAV-GFP (37,18 ± 3,792) *n* = 6 versus HFD AAV-GFP (26,33 ± 3,57); *n* = 4 – P-value=0,0846] **(Figure 1.26 – L).**

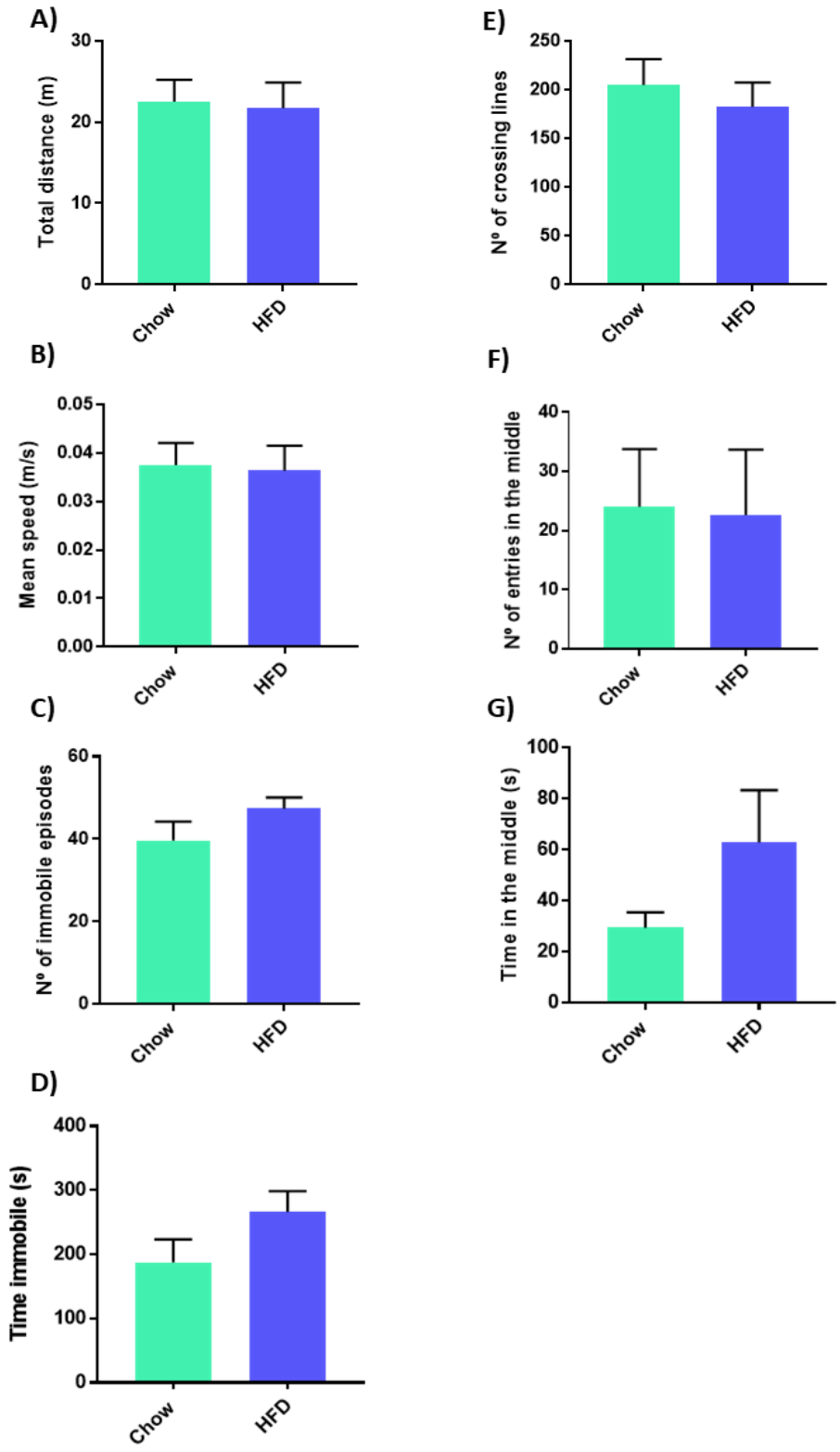

A)
Total distance (m)
30
20
10
0
Chow
HFD
B)
Mean speed (m/s)
0.05
0.04
0.03
0.02
0.01
0.00
Chow
HFD
C)
Nº of immobile episodes
60
40
20
0
Chow
HFD
D)
Time immobile (s)
400
300
200
100
0
Chow
HFD
E)
Nº of crossing lines
250
200
150
100
50
0
Chow
HFD
F)
Nº of entries in the middle
40
30
20
10
0
Chow
HFD
G)
Time in the middle (s)
100
80
60
40
20
0
Chow
HFD

Figure 1.25. In the night-time period, HFD did not modify the total distance traveled, mean speed, number of immobile episodes and time immobile of the animals. (A) The HFD AAV-GFP animals did not showed significant alteration in total distance traveled comparatively to the Chow AAV-GFP animals**. (B)** The HFD AAV-GFP animals did not showed significant alteration in the mean speed comparatively to the Chow AAV-GFP animals. **(C)** The HFD AAV-GFP animals showed an increase comparatively to the Chow AAV-GFP animals of numbers of immobile episodes; however, it was not statistically significant. **(D)** The HFD AAV-GFP animals showed an increase in the time immobile episodes comparatively to the Chow AAV-GFP; however, it was not statistically significant. **(E)** The Chow AAV-GFP animals showed an alteration in the number of crossing lines comparatively to the HFD AAV-GFP animals; however, it was not statistically significant. **(F)** The HFD AAV-GFP animals did not showed significant alteration comparatively to the Chow AAV-GFP animals in the numbers of entries in the middle zone**. (G)** The HFD AAV-GFP animals showed an increase of time spend in the middle comparatively to the Chow AAV-GFP animals; however, it was not statistically significant. Data were expressed as the mean ± SEM. [*P*-value < 0,05 (*), P-value < 0,01 (**), *P*-value < 0,001 (***), P-value < 0,0001 (****) - Unpaired Student's t-test.

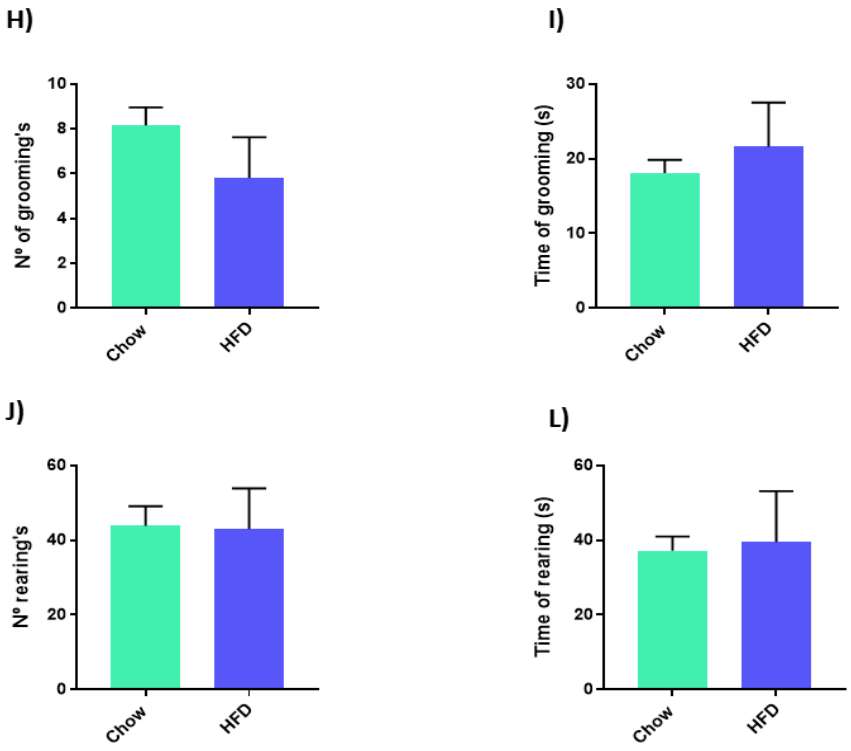


Figure 1.26. In the night-time period, HFD diet continue modifies the grooming behavior activity of C57BL/6J (HFD AAV-GFP). (A) The Chow AAV-GFP animals showed a slight decrease in the number of grooming's comparatively to the HFD AAV-GFP animals; however, it was not statistically significant. **(I)** The Chow AAV-GFP animals exhibit a decrease in the time of grooming's comparatively to HFD AAV-GFP animals; however, it was not statistically significant. **(J)** The Chow AAV-GFP animals did not exhibited alterations in the numbers of rearing´s comparatively to HFD AAV-GFP. **(H)** The Chow AAV-GFP animals did not exhibit a significative alteration in the time of rearing comparatively to the HFD AAV-GFP. Data were expressed as the mean ± SEM. [*P*-value < 0,05 (*), P-value < 0,01 (**), *P*-value < 0,001 (***), P-value < 0,0001 (****) - Unpaired Student's t-test.

5. HFD induces an increase in WAT weight and causes hypertrophy in white adipocyte of C57BL/6J mice.

HFD induces obesity and obesity-associated metabolic complications (Garg *et al.*, 2011). The development of obesity requires expanding adipose tissue by hyperplasia (cell number increase), hypertrophy (cell size increase) or a combination of both. C57BL/6 mice demonstrates that genetics and diet synergistically regulate adipocyte hypertrophy and hyperplasia (Weisberg *et al.*, 2003; Jo *et al.*, 2009). The expansion of WAT and adipocyte hypertrophy are generally associated with adipose inflammation, and suppression of this inflammation has been shown to generate benefits in diet-induced metabolic disorders (Gao, Ma and Liu, 2013). Animals fed with HFD (HFD AAV-GFP) gradually gained more body weight and fat mass compared to those fed the Chow diet (Chow AAV-GFP) [Chow AAV-GFP (1,398 ± 0,7881); n = 6 versus HFD AAV-GFP (22,31 ± 4,061), n = 5 – P-value=0,0004] **(Figure 1.27– A and Figure 1.27– B**). After sacrificing the animals, we observed a notable difference in the amount of body fat tissue present. In HFD AAV-GFP animals it was even difficult to see other organs, due to the marked expansion of WAT **(Figure 1.27 –C)**. WAT predominated in the abdominal cavity of the mice. At the end of the experiment, 12 weeks, the weight of WAT was in average ~ 0.66 g in the Chow AAV-GFP animals and 3.66 g in the HFD AAV-GFP animals [Chow AAV-GFP (0,5417 ± 0,08276); n = 6 versus HFD AAV-GFP (1,953 ± 0,5017); n = 5- P- value=0,0264] (**Figure 1.27– H**). The histological examination of WAT showed that in the animals HFD AAV-GFP, there was a substantial increase in the area and size of the adipocytes, inducing a significant level of hypertrophy of WAT comparatively to Chow AAV-GFP animals at the end of the study [Chow AAV-GFP (2239 ± 18,68); n = 6 versus HFD AAV-GFP (8592 ± 860,4); n = 4 – P-value <0,0001] **(Figure 1.27– D, 1.27–E, 1.27– F, 1.27– G and 1.27– I).** Collectively, these data demonstrate that HFD seems to promote pathological remodeling in WAT.

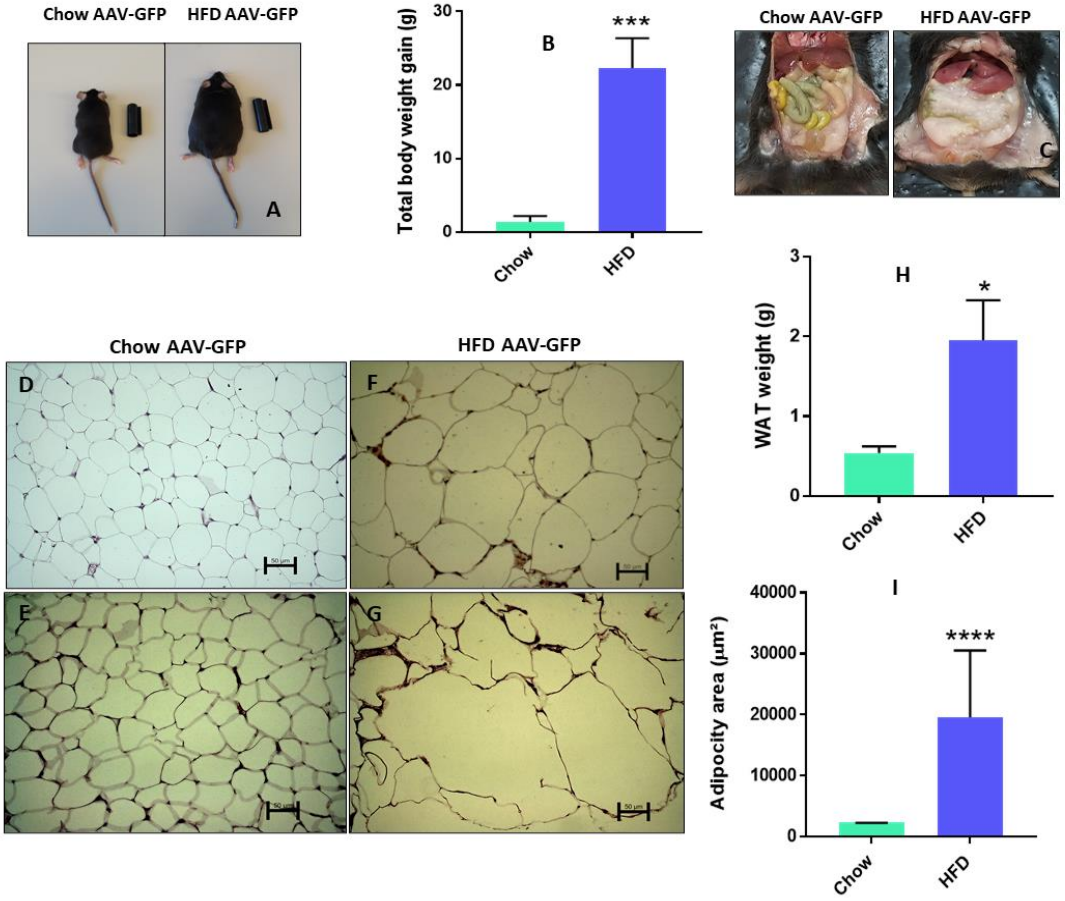


Figure 1.27. HFD induced body weight gain, an increase in WAT weight and white adipocyte hypertrophy in C57BL/6J mice. (A) The notable difference in the total body weight gain of the HFD AAV-GFP animals comparatively to the Chow AAV-GFP animals at the end of the 12 weeks of the study. **(B)** HFD AAV-GFP animals presented a statistically significant increase on total BW gain comparatively to the Chow AAV-GFP mice. **(C)** HFD AAV-GFP animals showed a substantial increase of fat mass (WAT). The WAT samples were histologically processed, and the hematoxylin-eosin (H&E) staining was performed. **(D and E)** The Chow AAV-GFP animals showed a normal phenotype of the adipocytes size. **(F and G)** HFD AVV-GFP animals showed a strong increase of the adipocytes size, comparatively to the Chow AAV-GFP animals. **(H)** The HFD AAV-GFP animals presented a statistically significant an increase in WAT weight, comparatively to the Chow AAV-GFP animals. **(I)** The HFD AAV-GFP animals presented a statistically significant strong increase of adipocytes area comparatively to the Chow AAV-GFP animals. **Magnification: × 20. HE stains. Bar: 50µm**. Data were expressed as the mean ± SEM [*P*-value < 0,05 (*), P-value < 0,01 (**), *P*-value < 0,001 (***), P-value < 0,0001 (****) - Unpaired Student's t-test.

6. HFD causes hypertrophy and whitening of BAT of C57BL/6J mice.

BAT is a highly vascularized tissue specialized in maintaining body temperature through the consumption of lipids and glucose. Recent studies demonstrate that BAT plays important roles in regulating glucose and triglyceride metabolism (Bartelt et al., 2011; Gunawardana and Piston, 2012). The BAT samples were histologically processed, and H&E staining was performed for posterior acquisition of BAT sections images. In this context, our data showed that HFD promoted substantial changes in BAT morphology, which could subsequently affect the metabolism of C57BL/6J mice **(Figure 1.28**). HFD progressively induced fat deposition in the BAT, resulting in its pathological remodeling and substantial expansion. This event called "BAT whitening" is evident when comparing the histological morphology of BAT in the Chow AAV-GFP animals compared to the HFD AAV-GFP animals. The Chow AAV-GFP showed multilocular adipocytes comparatively to the HFD AAV-GFP animals **(Figure 1.28 – A and 1.28 – B).** The HFD AAV-GFP animals showed a strong increase of lipid droplets in adipocytes, approaching the morphology of unilocular white adipocytes **(Figure 1.28 – C and 1.28 – D)**. Overall, this set of results suggested that HFD induces BAT bleaching and substantially changed the physiology and phenotype of BAT.

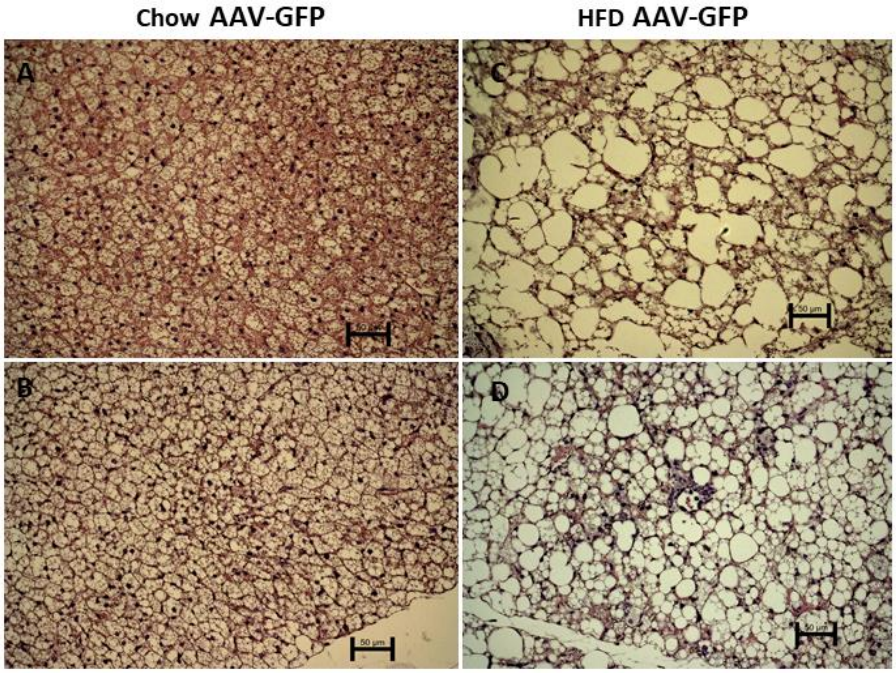


Figure 1.28. HFD induces hypertrophy of adipocytes of BAT and promotes "BAT whitening". The BAT samples were histologically processed, and H&E staining was performed. **(A and B)** The Chow AAV-GFP animals showed the characteristic phenotype of brown adipocytes: multilocular (multiple small lipid droplets), large number of mitochondria and oval central nucleus. **(C and D)** The HFD AAV-GFP animals showed a stronger increase of lipids droplets size approaching the morphology of unilocular white adipocytes, suggesting the "BAT whitening". **Magnification: × 20. HE stains. Bar: 50µm.**

7. HFD induces an increase on liver weight and hepatic steatosis in C57BL/6J mice. Obesity is usually related to the ectopic deposition of fat in the liver. The incidence of NAFLD (steatosis) is in accordance with the increasing prevalence of metabolic syndrome and obesity (Divella et al., 2019). In the 12th week of the study, the Chow AAV-GFP and HFD AAV-GFP animals were sacrificed and the liver were collected and weighted. The liver samples were histologically processed, and the H&E staining was performed for acquisition and analysis of the liver sections images. The Chow AAV-GFP animals showed hepatocytes with a single nucleus and cytoplasm of normal hepatocytes (as indicated by small arrow) **(Figure 1.29 – A and 1.29 – B).** The HFD AAV-GFP animals showed a strong increase in lipids droplets in the hepatocytes. Our images showed lobular inflammation, hepatocyte ballooning, fibrosis, vacuoles progressively

increased and macro vesicular steatosis by HFD in the liver of the HFD AAV-GFP animals, suggesting deposition of liver fat (steatosis) [Chow AAV-GFP (1,333 ± 0,2108); *n* = 6 versus HFD AAV-GFP (3 ± 0); *n* = 5 -*P*-value <0,0001] **(Figure 1.29 – E) (Figure 1.29 – C and 1.29 – D)**. Moreover, the HFD AAV-GFP animals showed a significant an increase in the liver weight comparatively to the Chow AAV-GFP animals [Chow AAV-GFP (1,143 ± 0,08301); *n* = 6 versus HFD AAV-GFP (1,687 ± 0,2531); *n* = 5 -P-value=0,0833] **(Figure 1.29 – F)**. Collectively, these results suggested that HFD induces an increase in liver weight and hepatic steatosis.

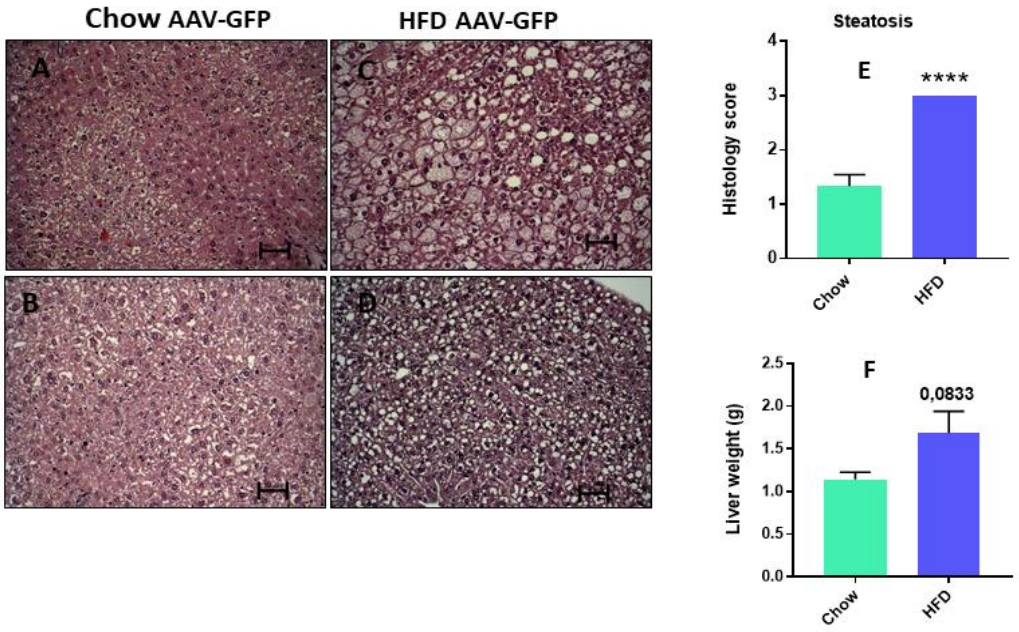


Figure 1.29. HFD induces an increase on liver weight and hepatic steatosis.

(A and B) The Chow AAV-GFP animals showed a characteristic phenotype of hepatocytes in liver; with a single nucleus and cytoplasm of normal hepatocytes comparatively to the HFD AAV-GFP animals. **(C and D)** The HFD AAV-GFP animals showed lobular inflammation, hepatocyte ballooning, fibrosis and macro vesicular steatosis, which is characterized by big lipids droplets in hepatocytes. **(E)** The HFD AAV-GFP presented a statistically significant increase in the histology score for steatosis comparatively to the Chow AAV-GFP animals. **(F)** The HFD AAV-GFP animals presented an increase in the liver weight comparatively to the Chow AAV-GFP animals; however, it was not significative statistically. **Magnification: × 20. HE stains. Bar: 50µm**. Data were expressed as the mean ± SEM [*P*-value < 0,05 (*), P-value < 0,01 (**), *P*-value < 0,001 (***), P-value < 0,0001 (****) - Unpaired Student's t-test.

8. HFD induced insulin resistance, hyperinsulinemia and pancreatic islet hypertrophy.

Adipose inflammation and ectopic lipid deposition are the main factors that contribute to impaired glucose metabolism in obesity. As previously reported in this study, ITT and GTT tests were performed to demonstrate that HFD negatively affects the C57BL/6J mice glucose homeostasis (**Figure 1.30 – B)**. This decrease in glucose homeostasis results mainly from insulin resistance, as evidenced by the reduced sensitivity to insulin administration in ITT (**Figure 1.30 – B).** Type 2 diabetes is characterized by impaired insulin secretion by pancreatic β cells. Quantifying the area of the islets is important for a detailed understanding of the histopathology of pancreatic islets (Yamazaki *et al.*, 2009). A substantial dysfunction in the physiology of the pancreas was observed in HFD obese mice (Mingming Gao et al., 2015). The HFD AAV-GFP animals presented islet hypertrophy, increased area of β / islet cells and mass content of islets and β cells in the pancreas comparatively to the Chow AAV-GFP animals [Chow (3649 ± 1152); n = 5 versus HFD AAV-GFP (37771 ± 11080); n=5- P-value=0.0155] (**Figure 1.30 – C and 1.30 – D) (Figure 1.30 – E).** The Chow AAV-GFP animals presented a non-hypertrophic conformation of β pancreatic islet cells **(Figure 1.30 – A and 1.30 – B).** After quantifying the area, our results suggest that the hyperinsulinemia presented in the HFD AAV-GFP animals was associated with pancreatic β islet hypertrophy, gradually exacerbated **(Figure 1.30 – D)**. Overall, these data suggest that HFD feeding impairs glucose homeostasis by inducing insulin resistance, which, consequently, gives rise to hyperinsulinemia and pancreatic islet hypertrophy.

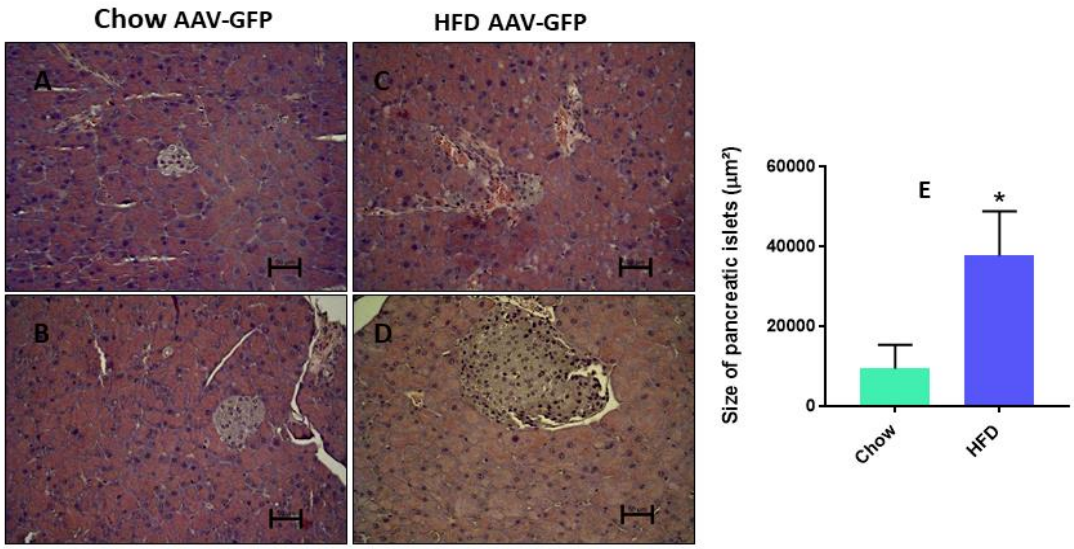


Figure 1.30. HFD impaired glucose homeostasis and induced an increase in area of β/islet cells in pancreas.

(A and B) The Chow AAV-GFP showed a normal islet of Langerhans and did not show an increase in the area of β / islet cells in the pancreas comparatively to the HFD AAV-GFP animals. **(C and D)** The HFD AAV-GFP animals presented islet hypertrophy, an increased area of β / islet cells and mass content of islets and β cells in the pancreas comparatively to the Chow AAV-GFP animals. **(E)** The HFD AAV-GFP animals showed a statistically significant increase in the area of β/ islet cells in the pancreas compared to The Chow AAV-GFP animals. **Magnification: × 20. HE stains. Bar: 50µm.** Data were expressed as the mean ± SEM [*P*-value < 0,05 (*), P-value < 0,01 (**), *P*-value < 0,001 (***), P-value < 0,0001 (****) - Unpaired Student's t-test.

9. HFD induces myosteatosis in skeletal muscles.

Nutrient overload leads to impaired muscle oxidative capacity and insulin sensitivity (Zoico et al., 2013). The pancreas samples were histologically processed, and the H&E staining was performed for acquisition and analysis of the liver sections images. Our results demonstrated that there was a strong difference in the analysis of the histological and microscopic image of the skeletal muscle tissue of animals fed with HFD (HFD AAV-GFP) in relation to the animals Chow AAV-GFP. There is substantial fat interspersed in the fascicles and muscle fibers, and a substantial infiltration of fat and some leakage of fat in the microscopic image **(Figure 1.31 – C and 1.31 – D)**. Chow AAV-GFP animals did not show any changes in skeletal muscle fibers comparatively to

the HFD AAV-GFP animals **(Figure 1.31 – A and 1.31 – B)**. Collectively, these results demonstrate that there are adaptive responses in the body of C5CLB/6J mice fed a high-fat diet, physiological changes in various organs, including skeletal muscle

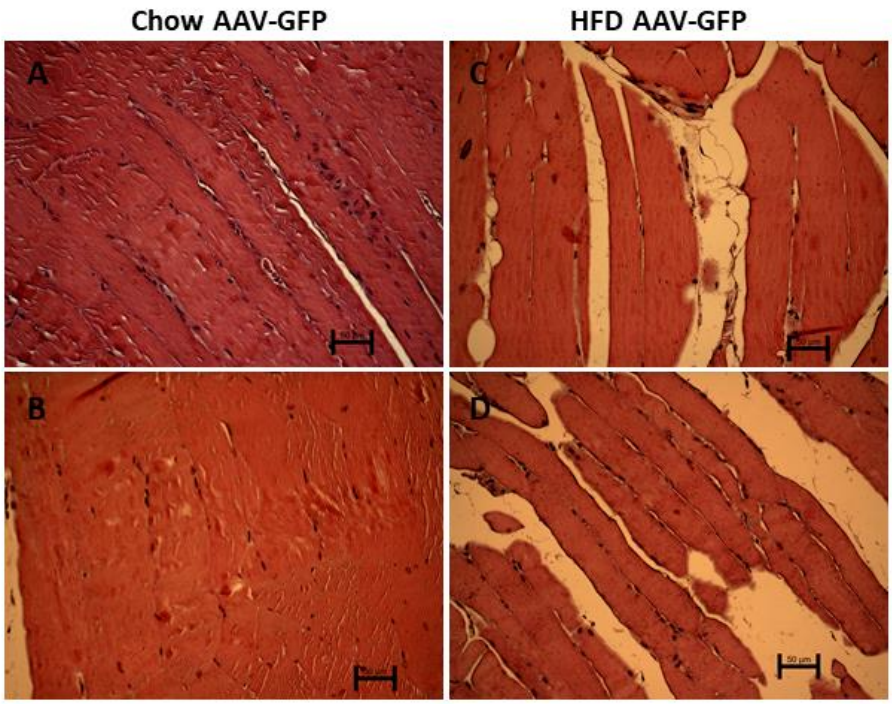


Figure 1.31. HFD induces skeletal muscle lipid accumulation (A and B) The Chow AAV-GFP animals did not showed a lipid accumulation in skeletal muscle comparatively to the HFD AAV-GFP animals. **(C and D)** The HFD AAV-GFP animals presented a stronger infiltration of fat interspersed in the fascicles and muscle fibers of skeletal muscle. **Magnification: × 20. HE stains. Bar: 50µm.**

CHAPTER 5 - Discussion

Discussion

Obesity and overweight are major public health issues with a major impact in our society. Obesity can be described as a multifactorial disorder of energy balance and represents one of the single most important modifiable risk factors for the development of several metabolic diseases such as, type 2 diabetes mellitus, metabolic syndrome, many types of cancer and neurodegenerative disorders (Eckel et al., 2011; Ashrafian et al., 2013; Bluher, 2019). Obesity, which leads to increased body weight, results from excessive fat accumulation and it can be accompanied by the development of high blood pressure, high cholesterol, insulin resistance and in addition, to substantial alterations of cholesterol homeostasis in the brain (Guillemot-Legris et al., 2017). Obesity also causes a central inflammatory process in the brain, more specifically in the hypothalamus. The hypothalamus is a key region in the brain controlling important body functions, such as heart rate and blood pressure, peripheral glucose and lipid energy homeostasis, as well as regulating sleep and circadian rhythms and aging (Saper et al., 2005; Valdearcos et al., 2015; Rahmouni et al., 2016; Kim et al., 2018).

The consumption of an HFD diet, which leads to obesity causes an inflammatory process in the hypothalamus and consequently appears to be a primary driving force behind the obesity phenotype in C57BL/6J mice (McLean et al., 2019), which were used in this study. This is the most used animal model in diet-induced obesity studies because it mimics the most prevalent cause of obesity in humans. Furthermore, in these animals it is observed hyperphagia, hyperglycemia and hyperinsulinemia (Buettner et al., 2007). In this study, our main objective was to control the effect of silencing *Cyp46a1* on the hypothalamus, through the stereotaxic injection of a control gene (GFP) mediated by *AAVs*, both in control mice (Chow AAV-GFP) and in those fed with HFD (HFD AAV-GFP). Our results showed that the HFD AAV-GFP presented an obese phenotype at the end of the study (12 weeks). Recent studies, showed that the HFD diet induces an inflammatory process in the hypothalamus (arcuate nucleus), developing the obesity phenotype in C57BL/6J mice, while the Chow diet did not cause significant changes in the metabolism of the C57BL/6J mice and did not promote obesity (Jais and Brüning, 2017). These results are similar to the results presented in non-injected animals (HFD) of a previous study of our laboratory (Pereira, 2019). In this sense, our study suggested that stereotaxic injections did not cause a compromising inflammation in the hypothalamus

(arcuate nucleus) that could have a relevant impact on the metabolism of the entire body of mice (C57BL/6J), in addition to suggesting that after stereotaxic surgery, there was no change in the phenotype of Chow AAV-GFP and HFD AAV-GFP animals. Comparatively to the previous study of silencing the *Cyp46a1* gene, our results showed a similar increase in total and cumulative body weight gain and an increase in food intake of wild type C57BL/6J (HFD Non-injected). Importantly, with these results we clarify that the phenotype obtained with the administration of the Chow diet and the HFD diet was not influenced by the surgical procedure (stereotaxic surgery). Finally, these results of this important control study, shows that the results obtained by silencing the *Cyp46a1* gene in the hypothalamus (Pereira, 2019); such as: weight gain in C57BL/6J mice fed both HFD and Chow diets could be explained by the silencing of the *Cyp46a1* gene in the arcuate nucleus.

Obesity is directly associated with several metabolic disorders and disorders, including chronic low-grade inflammation and insulin resistance, which are causally related to the development and progression of type 2 diabetes (Wellen et al., 2003; Thaler et al., 2010). In rodents, consumption of HFD predisposes to insulin resistance and low-grade inflammation (Irani et al., 2007; Gregor et al., 2011; Irani et al., 2007). The consumption of HFD alters the hypothalamic responsiveness to leptin and insulin, leading to the dysregulation of the control of energy homeostasis. In addition, these two hormones are anorexigenic and considered key regulators of energy homeostasis (Könner et al., 2009, Belgardt et al., 2010). Importantly, HFD deregulates hypothalamic neuronal circuits, known for finely adapting the hypothalamic response to the body's energy needs, leading to body weight gain, obesity and DM2 (Horvath et al., 2006; Kirk et al., 2009). Hypothalamic inflammation develops acutely within a few days after consuming HFD, especially in the ARC of the hypothalamus in association with insulin / leptin resistance and a positive regulation of neuronal injury markers (De Souza et al., 2005; Milanski et al., 2009; Thaler et al., 2012). The ARC contains two distinct functionally antagonistic neuronal populations, the orexigenic neurons that express the agouti-related peptide (AgRP) and the neuropeptide Y (NPY) and the anorexic neurons that include cocaine and amphetamine (CART) and transcription regulated neurons opiomelanocortin (POMC) neurons (Banks et al., 2004; Lenard et al, 2008). Previous studies documented that mice fed with HFD show a significant reduction of POMC mRNA expression (-55%) in the ARC, comparatively to mice fed with Chow (Lin, Storlien and Huang 2000). It was also

suggested a counter-regulatory mechanism or consequence of the circulation Lep levels increase (Beck 2006). Hypothalamic inflammation is correlated with a reduction in POMC and AgRP/NPY neurons responsiveness to insulin and Lep, which also contribute to dysfunctions in the insulin signaling pathway and IR (Valdercos et al., 2015). The anorexigenic effect of insulin on the hypothalamus is notable, where insulin modulates food intake and glucose homeostasis (Könner et al., 2009; Belgardt et al., 2010). Insulin crosses the blood-brain barrier in a receptor-dependent manner to reach the hypothalamus (Banks et al., 2004). In the hypothalamus, insulin receptors (IR) are highly expressed in the neurons POMC / CART and NPY / AgRP (Cone et al., 2001; Lenard et al., 2008). It has been also reported that insulin, through its action on hypothalamic ARC neurons, regulates hepatic glucose production (Obici et al., 2002; Könner et al., 2007), glycogen synthesis (Perrin et al., 2004), and fat metabolism (Koch et al., 2008). Relevant studies demonstrated that impaired hypothalamic insulin action can result from impaired transport through the BBB, thus reducing hypothalamic insulin uptake (Banks et al., 1997; Urayama et al., 2008) or that insulin resistance can be a consequence of impaired hypothalamic insulin signaling. This can be attributed to the HFD-induced hypothalamic overload of the cytokine signaling suppressor (SOCS3), protein tyrosine phosphatase-1B (PTP-1B) and protein kinase C, all shown to obstruct hypothalamic insulin signaling pathways (Bjørbaek et al., 1999; Zabolotny et al., 2008). In this study, we confirmed that HFD induces insulin resistance and substantial changes in blood glucose in C57BL/6J (HFD AAV-GFP) mice, as well as the results obtained in non-injected animals (HFD) from the previous study (Pereira, 2019). Decreased glucose tolerance and insulin resistance are the most important features in type 2 diabetes mellitus and metabolic syndrome (Lang et al., 2019). Thus, a reliable induction of impaired glucose tolerance is an essential feature of hypercaloric diets used in animal studies (Buettner et al., 2007). In the present study, hyperglycemia and impaired glucose tolerance were found in the group of animals fed the HFD within twelve weeks of feeding. The HFD AAV-GFP animals showed an increase in fasting glucose values. In addition, decreased insulin sensitivity was observed in the HFD AAV-GFP animals. Our results showed a consistent significant difference between the HFD AAV-GFP group and the Chow AAV-GFP group in terms of fasting blood glucose, GTT, and ITT results. According to these results, both the Chow AAV-GFP and HFD AAV-GFP groups can be considered comparable and reliable control groups for an injection of viral vectors in the hypothalamus. In the previous study of our laboratory it was observed that both the injected Chow AAV-shCyp46a1 and HFD

AAV-shCyp46a1 animals showed a substantial decrease in the levels of glucose tolerance and insulin sensitivity (Pereira, 2019). Altogether, these data point that, in the Chow *AAV-shCyp46a*1 animals, the impact of silencing *Cyp46a1* gene appears to mimic an HFD effect, whereas in HFD *AAV5-shCyp46a1* mice this silencing exacerbate the phenotype of obesity (Pereira, 2019).

Regarding insulin resistance and all generalized low-grade chronic inflammation (peripheral and central inflammation) presented in the obesity phenotype; several metabolic dysfunctions were generated that work in a progressive "vicious cycle". Metabolic dysregulation profoundly affects the physiology of metabolic tissues, such as: adipose tissue (WAT and BAT), liver, pancreas, muscles, among others. This complex and cyclic inflammatory process begins to affect adipose tissue and subsequently affects all other peripheral organs (Laurentius et al., 2019). Inflammation of adipose tissue, WAT hypertrophy, and BAT “whitening” are some of the effects of obesity and the HFD diet (Gomez Hernandez et al., 2016). HFD-induced obesity showed an accumulation of macrophages in adipose tissue and liver, as well as an increase in TNF-α in plasma serum (Sidles et al., 2019). This pathological remodeling in the adipose tissue dysregulate the physiological responses that maintain sensitivity to leptin and insulin, in addition to activating immune system responses (Chen et al., 2015). WAT is a complex organ and has primary roles in energy homeostasis control. Adipocytes not only act as a reservoir for energy storage and use, but also sense energy demands and secrete paracrine factors to regulate other metabolic tissues. In a high energy state, for example, leptin is secreted from adipocytes to reduce food intake centrally and increase energy expenditure (Scherer, 2006). However, in obesity, WAT may become severely dysfunctional and not expand properly to store the energy excess. This induces ectopic fat deposition in other tissues that regulates glucose homeostasis, an event commonly defined as “lipotoxicity”. This mechanism leads to systemic IR and an increased risk of T2D (Rutkowski et al., 2015; Reilly et al., 2017). Numerous deleterious effects have been associated with the pathologic expansion of WAT, including inflammation, fibrosis, hypoxia, altered adipokines secretion, and mitochondrial dysfunction (Kusminski et al., 2016). HFD-induced obesity promotes adipocytes to expand its size and number to compensate the need for increased lipid storage (Reilly et al., 2017). When the storage capacity of WAT, the largest adipose tissue depot, is exceeded, further caloric overload leads to the fat accumulation in ectopic tissues (liver, skeletal muscle, and heart) as well as in the visceral

deposits. The ectopic fat accumulation in the pancreas, for example, contributes to β-cell dysfunction (Singh et al., 2017). In this study, our results showed a remarkable increase on WAT weight accompanied by hypertrophy of adipocytes in HFD AAV-GFP animals comparatively to Chow AAV-GFP animals, which did not show a considerable increase in WAT weight or hypertrophy of adipocytes. These results confirm the previous data obtained in the silencing study of the *Cyp46a1* gene for the non-injected animals.

Unlike WAT, which has the main function of storing energy, BAT is specialized in dissipating energy to produce heat, thus regulating body temperature by thermogenesis (Dominguez et al., 2019). In obesity models, BAT showed higher levels of inflammation (macrophages and T-cell infiltration); stress in the endoplasmic reticulum, oxidative damage and worsening of mitochondrial respiratory activity (Alcala et al., 2017). Diet-induced obesity is also associated with decreased expression of UCP1 in BAT (Lee & Cowan, 2013). In this study, HFD AAV-GFP animals showed a strong increase of lipid droplets size in adipocytes, approaching the morphology of unilocular white adipocytes, suggesting BAT "whitening". This data is in line with the results in HFD animals (Non-injected) of the study of silencing *Cyp46a1* in hypothalamus. The AAV-*shCyp46a1* and the Chow AAV-*shCyp46a1* also showed an increase in lipid droplets size (Pereira, 2019).

Liver plays a key role in maintaining hepatic fat homeostasis and energy balance through multiple metabolic pathways (e.g., de novo lipogenesis, fatty acid uptake, fatty acid oxidation, and triacylglycerol export). HFD causes an imbalance between these processes that could result in abnormal hepatic lipid accumulation (Nguyen et al., 2008; Yaligar et al., 2014), commonly referred to as NAFLD (non-alcoholic fatty liver disease). The presence of saturated fat in the liver, in turn, causes insulin resistance in this organ (Neschen et al., 2005; Nagle et al., 2007). In HFD-induced obesity, adipose tissue is highly lipolytic and, according to the "portal hypothesis", the liver would be directly exposed to increased levels of FFAs and inflammatory factors released from fat into the portal circulation (Nagle et al., 2007; Kabir et al., 2005). Lipids accumulate as lipid droplets in the cytoplasm; however, glycerols themselves do not damage the cells, but rather the imbalance between the above-described metabolic pathways that leads to intermediate toxic lipid synthesis (e.g., diacylglycerol and ceramides) (Carr 2014; Hammarstedt et al., 2018). This chronic low-grade inflammation also promotes toxic intermediates accumulation in liver by increasing fatty acid uptake and triglyceride

synthesis and reducing fatty acid oxidation (Liu et al., 2014). In this study, HFD AAV-GFP animals showed an increase on liver weight and hepatic steatosis, comparatively to the Chow AAV-GFP. This data confirmed the results observed for non-injected HFD animals for the study of silencing *Cyp46a1* (Pereira, 2019). As already mentioned, obese animals have fasting hyperinsulinemia, insulin resistance in the tissues and an enormous accumulation of fat in the tissues (Hirata 1997; Auberval et al., 2014). Oxidative stress in metabolic tissues has emerged as a universal feature of the metabolic syndrome and its comorbidities (Auberval et al., 2014). In addition, fat-loaded adipocytes and myocytes are resistant to the effects of insulin signaling, leading to the storage of lipids and triglycerides in several tissues, including the pancreas and liver (Guichard et al., 2008). Notably, chronic adipose inflammation and ectopic lipid deposition in the liver and brown fat were accompanied by glucose intolerance and insulin resistance, which was correlated by hyperinsulinemia and pancreatic islet hypertrophy (Ribeiro et al., 2012; Auberval et al., 2014). The ectopic fat accumulation in the pancreas, for example, contributes to β-cell dysfunction (Singh et al., 2017). Substantial dysfunction in the physiology of the pancreas was observed in HFD obese mice (Mingming Gao et al., 2015). In this study, HFD AAV-GFP animals showed islet hypertrophy, increased area of β / islet cells and mass content of islets and β cells in the pancreas. This data is in line with the results for the HFD non-injected animals in the study of the *Cyp46a1* silencing (Pereira, 2019).

Skeletal muscle, as a metabolic organ, is one of the main tissues responsible for whole-body glucose homeostasis and lipids use. HFD causes skeletal muscle lipid accumulation. Myosteatosis (also known as ectopic skeletal muscle adiposity) represents the fat infiltration within myocytes (intramyocellular fat) and within the fascia surrounding skeletal muscle (intermuscular fat) (Miljkovic et al., 2010). Increased skeletal muscle lipid content has long been considered important to induce whole-body IR in HFD obese mice (Collino et al., 2014; Longo et al., 2019). In this study, HFD AAV-GFP animals showed a skeletal muscle with severe fat infiltration.

To better understand the impact of the HFD diet and the impact of stereotaxic surgery (AAV-GFP) on the animals we performed a daytime and nighttime open field behavior test (10 minutes each). The open field behavior test consists is a simple sensorimotor test used to determine general levels of activity, gross locomotor activity, exploration habits in rodents. The assessment takes place in a square transparent acrylic box. The animal is

placed in center of the arena and can move freely for 10 minutes while being recorded by a suspended camera. The footage is then analyzed by an automated tracking system (Any-maze) for the following parameters: distance moved, speed and time spent in predefined zones. Self-grooming in animals is an innate behavior that is involved in hygiene maintenance and other physiologically important processes, including thermoregulation, social communication and de-arousal (Kalueff et al., 2010). In our study, we consider grooming to be a behavior that indicates naturalness and spontaneity in the mice, being a movement that indicates non-stress. On the other hand, the rearing behavior provide an additional measure of anxiety exploratory behavior (Sturman et al., 2017). When the mice spontaneously prefer the periphery zone of the open field box to the middle zone, they display an anxiety-like behavior (Prut and Belzung, 2003). Metabolic disturbances can substantially alter the circadian rhythm in mice (Coomans et al., 2015). HFD feeding can increase daytime activity and the concomitant feeding during the light/inactive phases. Daytime feeding in mice or general feeding in metabolic resting phase, such as shift workers in human studies, promotes several metabolic alterations like obesity and insulin resistance (Mukherji et al., 2015; Opperhuizen et al., 2016). The results obtained in our study are coherent with these observations. The HFD AAV-GFP animals generally showed a behavior more active in the day-time period than the animals Chow AAV-GFP; however, in many parameters such as: total distance traveled, numbers of immobile episodes, number of crossing lines and time spend in the middle there was no significant difference between the two groups. Our hypothesis is that, despite the observable difference between the groups' behavior, the statistical analysis was not significant due to the low number of animals in the HFD AAV-GFP group. In this group we removed from the analyses two females that remained resistant to manifesting weight gain, even after 12 weeks of HFD. These two animals will be studied in detail in the future to understand the reason for not gaining weight since all other animals (females and males) fed by HFD showed high and gradual weight gain during the study. Importantly, in this study, HFD diet significantly modifies the grooming behavior activity of C57BL/6J (HFD AAV-GFP). In the daytime, the HFD AAV-GFP animals showed a significant decrease in the number of grooming's comparatively to the Chow AAV-GFP animals, suggesting an increase in the level of stress and anxiety. This result is in line with the data obtained in study of the *Cyp46a1* silencing. In that study, the HFD (non-injected) animals showed an increase in anxiety behavior and decrease in the number of grooming's comparatively to Chow animals. Moreover, the HFD *AAV-shCyp46a1* and Chow *AAV-shCyp46a1* animals

showed a phenotype of obesity in that previous study. These two groups of animals showed decrease in the number of grooming's at day-time period (Pereira, 2019). In the night-time period, HFD AAV-GFP animals were also more active and stressed in general. The Chow AAV-GFP animals showed an increase in number of grooming's at nigh-time period. This parameter showed that Chow AAV-GFP shows less anxious behavior than HFD AAV-GFP animals. The high-fat diet alters behavioral activity, eating behavior, and induces anticipatory food activity in mice, promoting hyperphagia, increased stress levels, and marked gradual weight gain (Bake et al., 2014). Collectively, these results sequentially demonstrate the events in which HFD induces physiological changes that lead to metabolic disorders in C57BL/6J mice.

Overall, the results obtained in our study are in line with the study of the silencing of the *Cyp46a1* gene in the hypothalamus (Pereira, 2019). Our results suggest that HDF AAV-GFP and Chow AAV-GFP animals are a reliable and effective control over the impact of feeding on the phenotype achieved by C57BL/6J mice. Moreover, it provided important evidence that stereotaxic surgery did not alter the physiology of the hypothalamus, and consequently does not alter the body metabolism of C57BL/6J mice. Besides that, the results presented here confirm that stereotaxic surgery and the GFP expression did not alter the homeostasis of the hypothalamus and, consequently, there is no change in the body metabolism of the mice. Finally, our work was very important to show that was the silencing of the *Cyp46a1* gene in the hypothalamus that led to a sever change of the phenotypes of HFD AAV-*shCyp46a1* and Chow AAV-*shCyp46a1* animals. Therefore, it contributes to emphases the importance of cholesterol homeostasis in the brain and the strong impact of this on the regulation of whole-body homeostasis.

CHAPTER 6 – Conclusion and Future Perspectives

In the present work, our main objective was to control the effect of silencing Cyp46a1 on the hypothalamus (arcuate nucleus). Our data confirm the project's hypothesis that the GFP protein does not interfere in the physiology of the hypothalamus and consequently in the metabolism of the whole body, regardless of diet. Ours results proved to be a safe effective control to validate the results obtained in the silencing project of the *Cyp46a1* gene (Pereira, 2019). The results presented here confirm that stereotaxic surgery and GFP expression did not alter the homeostasis of the hypothalamus and, consequently, there was no change in the body metabolism of the mice. Importantly, there have been some important studies on the association of CYP46A1 and different types of neurodegenerative diseases such as DA, MJD, HD and SLOS, including the mechanisms by which changes in CYP46A1 activity in the brain can be beneficial for these diseases (Mao, Li and Zhang, 2018; Nóbrega *et al.*, 2019, 2020).

We believe that this recent additional study of control over the possible effects caused by stereotaxic injections into the hypothalamus (arcuate nucleus) of the mice was very important to discard the influence of the surgical procedure and a study of overexpression of the *Cyp46a1* gene in the arcuate nucleus will also be needed to really validate whether this gene could be a target therapeutic potential for obesity and metabolic disorders, as well as assist in future therapies related to various types of neurodegenerative diseases or SCAs (Spinocerebellar ataxias).

As future perspectives it would be important to:

- Confirm the silencing of Cyp46a1 mouse gene in the hypothalamus, through gene expression analysis by RT-qPCR.
- It would be interesting to perform the quantification of cholesterol content and oxysterols content in the hypothalamus by mass spectrometry.
- Immunohistochemical analysis of the hypothalamus will be also interesting.
- Gene expression analysis in the hypothalamus and in other metabolic organs

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