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The effects of age, sex and genetics on the metabolic response to high fat diet intake in mice

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SUMMARY

Obesity and its secondary complications continue to increase worldwide and constitute one of the most important health care problems in many societies. It is now clear that the underlying causes of obesity can vary and that not all individuals have similar propensities to become obese and/or to develop obesity-associated pathologies. Indeed, the development of treatment strategies and drugs to curtail this epidemic span a wide range of targets. However, much is yet to be learned regarding metabolic control and individual differences in this process.

The aim of this thesis was to analyze the effects of age, sex and genetic background on the response to a high fat diet in mice. Puberty is a period of development with multiple changes occurring both in the central nervous system and systemically. However, little is known regarding the specific response during puberty to poor nutrition. Hence, we analyzed the metabolic response of both male and female peripubertal mice to a short-term high fat diet protocol. On the other extreme, aging increases the overall propensity to weight gain and some diseases, including neurodegenerative diseases such as Alzheimer's. Hence, we employed a genetic model of Alzheimer's disease in mice, submitting male and female mice to a long-term high fat diet or low fat diet and analyzing their metabolic response. Moreover, as astrocytes are involved in both metabolic control and neuroprotection, we analyzed the response of hypothalamic astrocytes from the hypothalamus of males and females to palmitic acid, a saturated fatty acid commonly found in our diet, and to amyloid-β, which is an important component in the development of Alzheimer's disease.

The results reported here indicate that during the peripubertal/pubertal period mice are less prone to excess weight gain to short-term high fat intake compared to that reported at other developmental ages. Mice of both sexes increased their energy intake, but body weight was not affected. However, metabolic profiles were modified in a sex dependant manner with males being more affected than females.

When mice were exposed to a high fat diet during later adulthood, we found that females gained more weight and fat mass than males, which is in contrast to what is commonly reported for young adults.

Key words: Age; Sex; High fat diet; Astrocytes; Sex steroids; Alzeimer’s disease.

RESUMEN

La obesidad y sus complicaciones secundarias continúan aumentando en todo el mundo y constituyen uno de los mayores problemas de salud en nuestra sociedad. Ahora sabemos que sus causas son variadas y que cada individuo muestra diferente propensión a desarrollar obesidad y/o patologías asociadas a esta. Sin duda, el desarrollo de estrategias terapéuticas y medicamentos contra esta epidemia abarca un amplio rango de posibles dianas. Sin embargo, todavía queda mucho que aprender sobre el control del metabolismo y sus diferencias individuales.

El objetivo de esta tesis es analizar los efectos de la edad, el sexo y el trasfondo genético en la respuesta a una dieta alta en grasas en ratones. La pubertad es un período del desarrollo con múltiples cambios que ocurren tanto en el sistema nervioso central como a nivel periférico. Sin embargo, las respuestas específicas a una mala nutrición durante la pubertad no han sido estudiadas en profundidad. Por tanto, hemos analizado la respuesta metabólica de ratones de ambos sexos en edad peri-puberal, sometiéndoles a una dieta alta en grasa durante un corto período de tiempo. Por otra parte, el envejecimiento aumenta la propensión al sobrepeso y ciertas enfermedades, incluyendo trastornos neurodegenerativos como el de Alzheimer. Por ello, hemos utilizado un modelo genético de enfermedad de Alzheimer en ratones, sometiendo a machos y a hembras a una dieta alta o baja en grasas durante un período largo de tiempo para analizar su respuesta metabólica. Además, dado que los astrocitos están implicados tanto en el control metabólico como en la neuroprotección, hemos analizado la respuesta de astrocitos hipotalámicos de machos y hembras al ácido palmítico, un ácido graso saturado que se encuentra comúnmente en nuestra dieta, y al amiloide-β, un compuesto importante en el desarrollo de la enfermedad de Alzheimer.

Los resultados que aquí se exponen indican que los ratones en edad puberal/peripuberal son menos propensos a la ganancia de peso tras un período corto de dieta alta en grasas, en comparación con lo que se ha observado en otros estadios del desarrollo. Los ratones de ambos sexos aumentaron su ingesta calórica sin aumentar de peso. Sin embargo, los perfiles metabólicos se vieron

alterados de diferente manera en función del sexo, con un mayor efecto en machos que en hembras.

Cuando ratones de mediana edad fueron expuestos a una dieta alta en grasas encontramos que las hembras ganaron más peso y grasa que los machos, lo que contrasta con lo que se suele observar en adultos jóvenes.

Palabras clave: Edad; Sexo; Dieta alta en grasas; Astrocitos; Esteroides sexuales; Enfermedad de Alzheimer.

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INTRODUCTION

1. Obesity and high-fat diet intake

Overweight and obesity are important health problems in our era, as being obese has been associated with the development of type 2 diabetes, dyslipidemia, asthma, cardiovascular problems (Anteneh et al 2015, Martin-Rodriguez et al 2015) and even certain types of cancer (Hursting & Dunlap 2012) and neurological diseases like dementia (Emmerzaal et al 2015). The World Health Organization (WHO) defines obesity and overweight in adults by means of body mass index (BMI). The BMI of an individual is calculated as their body weight (in kilograms) divided by the square of their height (in meters). In adults, values over 25 kg/m^2 are considered overweight and values over 30 kg/m^2 are considered obesity.

Since 1975, obesity has tripled worldwide. In 2016, 13 % of adults and 7% of children and adolescents were obese, according to the WHO. This organization places much of the blame on an increase in high-fat diet intake and a decrease of physical activity. That is, an imbalance between the intake and expenditure of energy (Garrow 1988). These changes are often a consequence of multiple environmental and social factors, like urban development, sedentarism, working habits, food processing, education and a lack of social politics in these sectors (Who 2015).

2. Neuroendocrine control of metabolism

The hypothalamus (Cone et al 2001, Kim et al 2014a, Schneeberger et al 2014), the brain area in charge of homeostatic control, is the principal center in the central nervous system (CNS) for integration of nutritional and hormonal inputs. Here, specialized neuronal circuits process this information and send signals to higher brain regions, resulting in the modification of appetite and energy expenditure (Webber et al 2015).

2.1. Neuronal circuits

Within the hypothalamus, the arcuate nucleus contains a specialized neuronal circuit known as the melanocortin system, the major network in metabolic regulation (Cone 2006). This system is formed fundamentally by two neuronal populations exerting opposite actions. One of them is orexigenic and is characterized by the co-expression of neuropeptide Y (NPY), Agouti-related protein (AgRP) and gamma-amino

butyric acid (GABA) (Hahn et al 1998, Krashes et al 2013) (Figure 1). The other is mainly anorexigenic and expresses pro-opiomelanocortin (POMC) derived peptides, particularly α-melanocyte stimulating hormone (α-MSH), with a portion of these neurons co-expressing cocaine-and-amphetamine-regulated transcript (CART) (Balthasar et al 2004, Cowley et al 2003, Elias et al 1998). These POMC-neurons are also able to produce an orexigenic signal under certain circumstances, through alternate processing of POMC resulting in the production and release of β-endorphin (Dutia et al 2012, Grossman et al 2003, Koch et al 2015).

Hypothalamic POMC/CART neurons receive tonic inhibition from NPY/AgRP/GABA neurons (Horvath et al 1992) and excitatory signals from the ventromedial nucleus of the hypothalamus (VMH) (Sternson et al 2005). In addition, glutamatergic signals from within the arcuate nucleus stimulate both of these populations of neurons (Kiss et al 2005). Hormones such as leptin and insulin stimulate this anorexigenic neuronal population while inhibiting the orexigenic population (Horvath 2005, Schwartz et al 2000). On the other hand, ghrelin inhibits POMC/CART neurons and stimulates NPY/AgRP/GABA neurons (Cowley et al 2003, Chen et al 2017). Thus, these two important populations of neurons integrate the metabolic signals received from the circulation to determine the balance of energy need/energy excess.

The main targets of these neurons are cells expressing the melanocortin 4 receptor (MC4R) and the melanocortin 3 receptor (MC3R), which receive excitatory signals (*e.g.,* α-MSH) from POMC/CART neurons. In contrast, AgRP released from NPY/AgRP/GABA neurons acts as an inverse agonist on these same receptors, resulting in inhibitory signals and opposing effects on energy expenditure (Fan et al 1997, Ollmann et al 1997).

The actions of these neurons constitute the first regulatory response of the CNS to the energy status of the body. These neurons send projections to other hypothalamic nuclei, such as the paraventricular nucleus (PVN), dorsomedial hypothalamus (DMH) and the lateral hypothalamic area (LHA) (Elias et al 1998, Elmquist et al 1998) where, in conjunction from direct inputs of both hormones and nutrients, will determine the output of these brain areas. The signals are then further processed by other brain areas, like the solitary nucleus, amygdala and prefrontal cortex (Abizaid & Horvath 2008, Horvath 2005, Kim et al 2014a, Schneeberger et al 2014, Schwartz et al 2000). Together, these signals determine appetite and energy output.

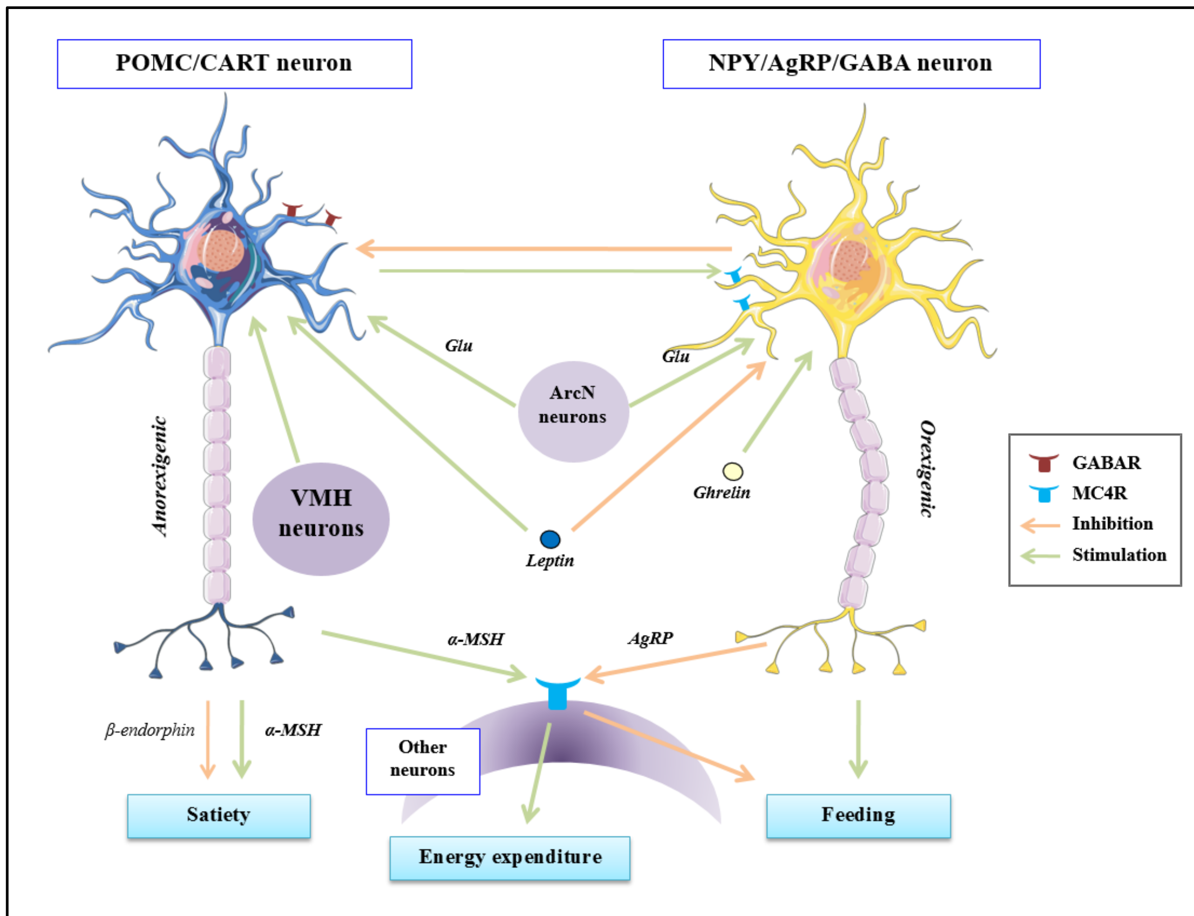

Figure 1. The two main neuronal populations forming the melanocortin system and their interactions.

2.2. Role of astrocytes and other non-neuronal cells in energy homeostasis

Neurons are not the only cell type involved in the control of metabolism within the CNS. To begin with, the epithelial cells found in the choroid plexus and lining the ventricles secrete cerebrospinal fluid (CSF) and factors involved in neurogenesis and development (Lehtinen et al 2011, Marques et al 2011, Parada et al 2008, Thouvenot et al 2006). They also exert barrier functions and express transporters for metabolites and receptors for sex steroids and leptin (Alves et al 2009, Hong-Goka & Chang 2004, Mitchell et al 2009, Quadros et al 2007, Saunders et al 2015, Spector 1989, Spector & Johanson 2006). Together with tanycytes, they form the hypothalamic neurogenic niche (Mirzadeh et al 2008).

Tanycytes are polarized cells lining the walls of the third ventricle. They have a long process projecting into the hypothalamus or the median eminence (Rodriguez et al 2005). These specialized cells are involved in the control of the substance entry into the hypothalamus, as they express specific transporters and modulate the permeability of

the blood-CSF barrier (Langlet 2014, Langlet et al 2013a, Langlet et al 2013b). Moreover, tanycytes participate in glutamate recycling, nutrient sensing and the conversion of thyroid hormones from its inactive to active form (Barrett et al 2007, Bolborea & Dale 2013, Frayling et al 2011, Nilaweera et al 2011). They are interconnected by gap junctions, which allow them to transmit coordinated signals through calcium waves (Orellana et al 2012).

Distributed throughout the CNS are microglial cells, the brain's immune system. These cells respond to brain damage, toxins and harmful conditions by changing their phenotype to a reactive form, producing cytokines, nitric oxide or reactive oxygen species (Dalmau et al 1997, Delgado et al 1998, Ginhoux et al 2013, Gonçalves et al 2012, Ling et al 1980). In addition, they regularly phagocyte cellular debris and other wastes and release gliotransmitters and metabolic factors (Aloisi 2001, Elkabes et al 1996, Gertig & Hanisch 2014). They are also involved in synaptic pruning, both during development and, at later ages, in response to specific signals (Batchelor et al 1999, Batchelor et al 2002, Zhong et al 2010).

Forming the blood-brain barrier (BBB), we find endothelial cells, pericytes and astrocytes. Endothelial cells strictly control the entry of substances from the blood to the CNS and pericytes, contractile cells surrounding the vessels, are involved in the regulation of blood flow and participate in the development and maintenance of the BBB, in addition to performing macrophage-like functions and being intricately involved in neuroinflammation (Abbott et al 2010).

Astrocytes are the most abundant cell type in the CNS. They exert multiple functions, beginning with their indispensable role in supplying physical and metabolic support to neurons. Astrocytes are also an important part of the BBB, transporting nutrients and metabolic factors and, thus, controlling what enters the CNS (Abbott et al 2006, Pellerin & Magistretti 1994). Also, they have a role in the maintenance of the barrier and the blood flow at this level (Janzer & Raff 1987, MacVicar & Newman 2015, Zonta et al 2003). These cells form a syncytium, as they are connected through gap junctions that enable the transport of some molecules -in a regulated fashion (Söhl & Willecke 2004, Theis et al 2005)- and the transmission of calcium waves (Scemes & Giaume 2006). Astrocytes store energy through glycogenesis (Cataldo & Broadwell 1986). They form part of the "tripartite synapse", as they express glutamate transporters GLT1 and GLAST and take-up glutamate from the synaptic cleft (Pines et al 1992, Schmitt et al 1997), terminating transmission and preventing excitotoxicity (Araque et

al 1999, Ransom et al 2003). Astrocytes also regulate synaptogenesis and neuronal proliferation and differentiation during development (Clarke & Barres 2013, Nedergaard et al 2003). Importantly, they respond to injuries, foreign substances and infections by producing cytokines (Aschner 1998a, Aschner 1998b). In addition, astrocytes are important in glucose transport (Morgello et al 1995) and they can change their morphology, modifying synaptic structure/inputs (Clarke & Barres 2013, Nedergaard et al 2003).

2.3. Astrocytes in metabolism and nutrient sensing

Astrocytes express glutamate transporter GLUT2, sodium glucose transporter 1 (SGLT1), glucokinase (GCK) and K_{ATP} channels, all involved in the glucose sensing process (Leloup et al 2016, Steinbusch et al 2015). Glucose enters the cell through GLUT2 and it is phosphorylated by GCK, unless it undergoes glycogenesis. Then, glucose-6-phosphate produces pyruvate, which is converted in lactate via lactate dehydrogenase. Finally, lactate exits the cells through monocarboxylate transporter (MCT) 4 or 1 and it is taken up by neurons through MCT-2 (Elizondo-Vega et al 2015). This process has been named the "astrocyte-neuron lactate shuttle" by Pellerin and Magistretti (Pellerin & Magistretti 1994). Lactate release by astrocytes also serves as a signal of energy availability to glucose-sensing neurons (Leloup et al 2016). Moreover, in response to an increase in glucose or to other factors, like neurotransmitters, astrocytes release endozepines, peptides that exert an anorexigenic effect on hypothalamic neurons (Lanfray et al 2013, Tonon et al 2013) and also have a role in unsaturated long-chain fatty acid metabolism in astrocytes (Bouyakdan et al 2015). The ability of astrocytes to take up glucose is regulated by various signals: it is increased by leptin (Fuente-Martin et al 2012), reduced by ghrelin (Fuente-Martin et al 2016) and it has been also demonstrated that insulin is important for glucose uptake by astrocytes (Garcia-Caceres et al 2016). Glucose storage in the form of glycogen is another important role of astrocytes on the management of energy availability. If needed, *i.e.*, when there is an increase in neuronal activity (Cruz & Dienel 2002), astrocytic glycogen is processed to lactate via glycogenolysis. Glycogen production in astrocytes can be increased by diverse hormones, such as insulin, insulin-like growth factor (IGF)-1 and leptin (Bosier et al 2013, Heni et al 2011, Muhič et al 2015, Sartorius et al 2012). Also,

glycogenolysis has been shown to be promoted by ghrelin in hypothalamic astrocytes (Fuente-Martin et al 2016).

Astrocytes are the main metabolizers of lipids in the CNS. Moreover, they express proteins for lipid sensing, such as fatty acid translocase CD36 and peroxisome proliferator-activated receptor gamma (PPARγ), a lipid-activated nuclear receptor that regulates transcription of several genes, some of them involved in lipid metabolism (Cristiano et al 2005, Heneka & Landreth 2007). Astrocytes are able to take up ketone bodies via MCT-1 and 4 (Bergersen et al 2002, Pierre & Pellerin 2005, Rafiki et al 2003) and also to synthesize them from fatty acids through fatty acid β-oxidation and release them to the extracellular space, where other cells use them as an energy source. This process is important in nutrient sensing because ketone bodies produced and released by astrocytes are a sign for hypothalamic neurons of an excess of fatty acids, so they act to reduce food intake (after an initial HFD-induced hyperphagia) (Carneiro et al 2016, Le Foll et al 2014, Le Foll et al 2015). In fact, it has been reported that β-oxidation in hypothalamic astrocytes increases in obese mice fed a HFD and that tanycytes prevent the entry of an excess of saturated fatty acids into the hypothalamus (Hofmann et al 2016). Lipids can enter the CNS from the bloodstream as fatty acids, through simple diffusion or with the help of different fatty acid translocases, transport and binding proteins (Kamp et al 2003, Levin 1980), or as lipoproteins. The latter process is mediated by apolipoprotein E (ApoE), which is expressed in astrocytes and tanycytes and interacts with lipoprotein receptors (Boyles et al 1985). Thyroid hormones and leptin upregulate ApoE expression, thus regulating energy balance (Roman et al 2015, Shen et al 2009, Shen et al 2008). A summary of the main interactions of astrocytes in the hypothalamus is represented in Figure 2.

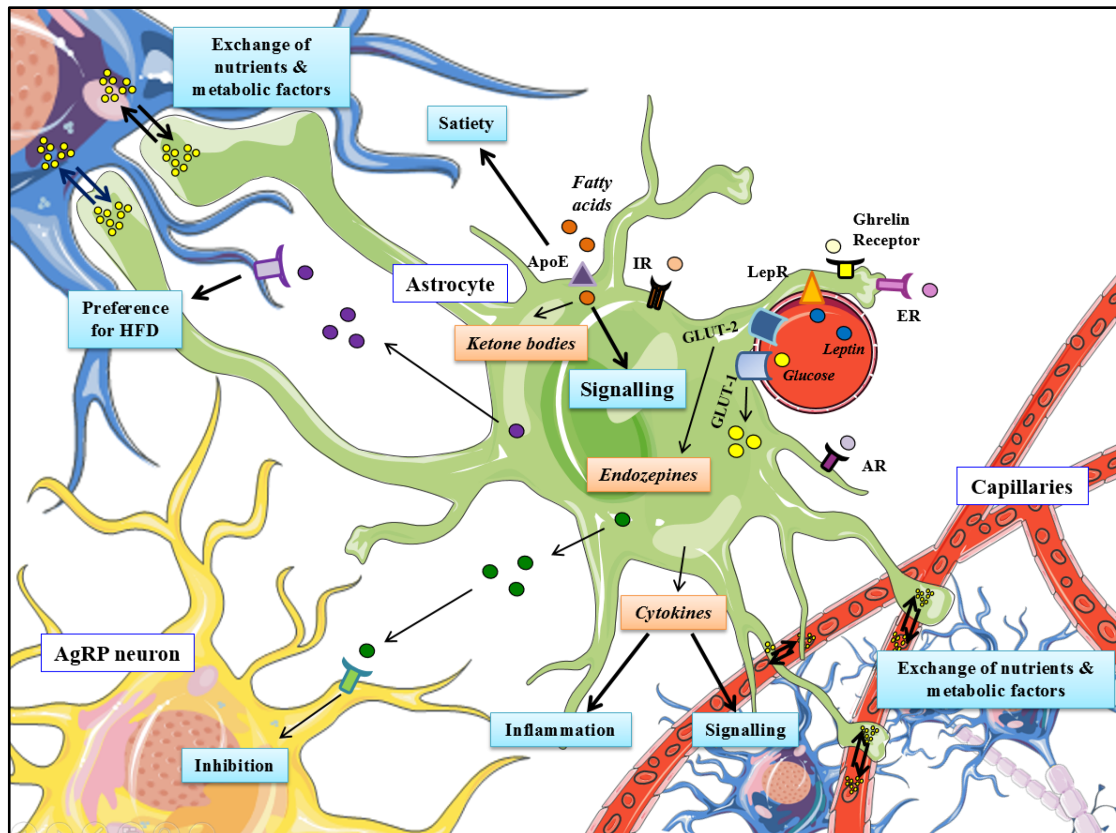

Figure 2. The complex functions of astrocytes on metabolism.

2.4. Hormones in metabolism

2.4.1. Leptin

Leptin is an anorexigenic hormone secreted principally by adipose tissue, although it can be produced also by the hypothalamus, stomach, liver, ovaries and placenta (Bado et al 1998, Hoggard et al 1997, Mantzoros 1999). When leptin reaches the hypothalamus, it inhibits NPY/AgRP/GABA neurons and stimulates POMC/CART neurons, thus decreasing food intake and increasing energy expenditure (Horvath 2005, Schwartz et al 2000, Simpson et al 2009). The leptin receptor (LepR) is also highly expressed in astrocytes (Hsuchou et al 2009) and leptin signaling in these cells is important for energy balance (Kim et al 2014b, Wang et al 2015).

Impairment of leptin expression or action leads to obesity, hyperphagia and low energy expenditure (Drel et al 2006, Farooqi et al 1999). Leptin levels in plasma correlate positively with BMI, as it is released to the bloodstream in proportion to the amount of adipose tissue (Argente et al 1997, Frederich et al 1995a), and also respond to the acute metabolic status, increasing with HFD intake and decreasing during fasting (Considine et al 1996, Friedman & Halaas 1998). In humans, leptin replacement

ameliorates the symptoms of obese leptin deficient patients, reducing adipose tissue (Farooqi et al 2002, Mantzoros et al 2011). However, obese individuals normally show elevated plasma leptin levels, implying the existence of a leptin resistance associated with weight gain (Frederich et al 1995b, Maffei et al 1995). It has been reported that HFD-induced obesity causes leptin resistance by two possible mechanisms: affecting the entry of leptin into the brain (Balland et al 2014) and reducing the central response to this hormone (Balland & Cowley 2015). Obesity, fasting and certain metabolic factors intervene in the regulation of leptin transport. Long term HFD induces leptin resistance in mice only when there are high levels of leptin in plasma (Knight et al 2010), suggesting that hyperleptinemia is one of the triggers for diet-induced leptin resistance. Also, hypothalamic inflammation linked to diet-induced obesity could be involved in leptin resistance by modifying the cellular and molecular systems that control energy homeostasis (de Git & Adan 2015). However, some studies suggest that the only response impaired in leptin resistance is the one from exogenous leptin, while the responsiveness to endogenous leptin remains intact (Flak & Myers 2016, Ottaway et al 2015).

Leptin participates in the control of peripheral glucose and lipid metabolism, promoting lipolysis and inhibiting lipogenesis in adipose tissue and liver (Frühbeck et al 1998, Hynes & Jones 2001, Kamohara et al 1997). Glucose and insulin favor the passage of leptin across the BBB (Kastin & Akerstrom 2001) and a high level of plasma triglycerides reduces it (Banks et al 2004). On the other hand, leptin decreases glucose-induced insulin secretion (Hynes & Jones 2001, Muzumdar et al 2003) and also affects the ability of hypothalamic astrocytes to transport glucose and glutamate (Fuente-Martin et al 2012).

2.4.2. Insulin

Insulin is a peptide mainly produced by the pancreas in response to high levels of glycemia. Insulin regulates glucose levels in blood by stimulating its uptake by other tissues and suppressing glucose productions by the liver (Kahn 1994). Insulin levels correlate positively with adiposity (Polonsky et al 1988). Insulin also acts as an anorexic hormone. The insulin receptor (IR) is expressed throughout the brain and is highly expressed in the arcuate nucleus (Van Houten et al 1979), where insulin activates POMC neurons and inhibits AgRP expression (Brüning et al 2000, Van Houten et al

1979), decreasing food intake and increasing energy expenditure (Qiu et al 2014, Woods et al 1979).

Astrocytic IRs have a role in the transport of insulin across the BBB, and insulin signaling in astrocytes is important in the regulation of glycemia (Garcia-Caceres et al 2016). Satiation hormones, like cholecystokinin (CCK) promote insulin transport into the brain and other hormones, like estradiol inhibit insulin functions in the brain, although not its transport (May et al 2016). The relationship and interactions between leptin and insulin need further research, as some studies have found that leptin enhances insulin sensitivity (Koch et al 2010) while others show that leptin inhibits insulin signaling in the brain (Sartorius et al 2012). In fact, these two hormones share some molecular pathways, although they have antagonic effects in some hypothalamic neurons (Xu et al 2005). Obesity-induced hyperinsulinemia leads to insulin resistance and type-2 diabetes (Moller & Flier 1991). Saturated fatty acids have been shown to be involved in insulin resistance in the hypothalamus (Benoit et al 2009) and in peripheral tissues (Boden et al 2002).

2.4.3. Ghrelin

Ghrelin is a gastrointestinal hormone that promotes food intake and is produced mainly in the stomach (Kojima et al 1999, Nakazato et al 2001). Ghrelin has also been shown to promote fat accumulation and control glucose homeostasis and energy expenditure (Müller et al 2015, Tschöp et al 2000, Wren et al 2001). During fasting, it is released and reaches the CNS, stimulating NPY/AgRP/GABA neurons and inhibiting POMC/CART neurons through its receptor, the growth hormone secretagogue receptor 1a or GHS-R1a, which is highly expressed in the hypothalamus (Kamegai et al 2001, Riediger et al 2003, Wang et al 2013, Zigman et al 2006). Ghrelin transport into the brain is enhanced by serum triglycerides, which are high during fasting, and is inhibited in obese individuals (Banks et al 2008). Also, diet-induced obesity can impair the hypothalamic response to ghrelin (Briggs et al 2010).

2.4.4. Sex steroids

Estrogens, androgens and progesterone are secreted principally by the reproductive organs (ovaries and testes), although they are also produced by other tissues such as adipose tissue (Simpson 2003) and the brain (Baulieu & Robel 1990).

The latter are called neurosteroids and can be synthesized from brain-borne cholesterol or from steroid precursors coming from the periphery, like pregnenolone, deoxycorticosterone and testosterone (Reddy 2010). The synthesis can take place in astrocytes, tanycytes, ependymal cells and oligodendrocytes (Jung-Testas & Baulieu 1998, Mensah-Nyagan et al 1999), and even in some neurons (Agís-Balboa et al 2006).

Sex steroids, mainly estrogens, are involved in the control of energy homeostasis at the hypothalamic level, reducing food intake (Butera & Czaja 1984, Czaja & Goy 1975, Gao et al 2007), increasing energy expenditure (Musatov et al 2007) and regulating the sensitivity to metabolic hormones like leptin, insulin and ghrelin (Clegg et al 2006, Clegg et al 2007). Their effect is generally anorectic (Asarian & Geary 2002, Asarian & Geary 2006, Geary et al 2001), although it varies between different neuronal populations (Smith et al 2014, Xu et al 2011). Estrogens' effects are mediated fundamentally by the nuclear estrogen receptors (ERs) α and β, especially ERα (Geary et al 2001, Liang et al 2002, Roesch 2006, Santollo et al 2010). However, estrogen responsive G-coupled membrane receptors, like GPR30, mediate some of their functions (Qiu et al 2006, Roepke et al 2010). In fact, some studies have found that the different receptors could be acting in combination (Filardo et al 2000).

The neuroprotective effects of steroids and neurosteroids have been widely demonstrated (Brotfain et al 2016, Day et al 2013, De Nicola et al 2013, Gold & Voskuhl 2009, Rahmani et al 2016, Sarkaki et al 2011, Vegeto et al 2008, Wise 2003). Aromatase, the enzyme that converts testosterone into estradiol, shows increased expression in reactive astrocytes following brain injury (Azcoitia et al 2003, García-Segura et al 1999, Saldanha et al 2009). Neuroprotection by sex steroids involves both astrocytes and microglia (Johann & Beyer 2013), as they decrease microglia reactivity (Cerciat et al 2010, Kipp et al 2007, Lewis et al 2008) and production of proinflammatory factors by astrocytes (Bruce-Keller et al 2000, Dimayuga et al 2005, Drew & Chavis 2000, Vegeto et al 2006).

3. Inflammation/gliosis in metabolism

Inflammation should not always be considered pathological, but as a protective response after an insult, necessary for tissue repair and control of homeostasis (Calder et al 2009). It is also a characteristic of obesity. However, obesity-linked inflammation is different from classical inflammation, as it lacks the rapid immune response and the

typical signs of heat, swelling, pain and redness. In contrast, inflammation associated to obesity is chronic and considered of low grade, in comparison to classical inflammatory responses in injury or infection (Gregor & Hotamisligil 2011, Medzhitov 2008).

Obese individuals, at least in adulthood, have high levels of circulating cytokines, such as TNF-α, IL-1β and IL-6 (Berg & Scherer 2005, Hotamisligil et al 1995, Hotamisligil et al 1993, Shoelson et al 2006). This increase in cytokine levels is marked in adipose tissue (Fried et al 1998) and is combined with adipocyte hypertrophy, but not adipocyte proliferation (Weisberg et al 2003). This adipocyte hypertrophy is related to T cell and macrophage infiltration into the tissue (Minihane et al 2015). Interestingly, it has been found that visceral adipose tissue is more important in the inflammatory process than subcutaneous adipose tissue (Weiss 2007).

Other peripheral tissues also involved in obesity-related inflammation are the muscle (Saghizadeh et al 1996), liver (Cai et al 2005) and pancreas (Nicol et al 2013). Importantly, inflammation associated to obesity is not only systemic, but it also occurs in the brain, and especially, the hypothalamus (Thaler et al 2012b). Besides adipose-borne cytokines that cross the BBB (Banks et al 1995, Benveniste 1998), cytokines can also be produced directly in the brain (Benveniste 1998). The activation and proliferation of astrocytes and microglia in response to an injury is known as gliosis, or reactive gliosis (Johns 2014), and frequently concurs with inflammation in the CNS. Brain inflammation can be induced by an excess of nutrients (Aljada et al 2004, Cani et al 2007, Erridge et al 2007), especially long-chain saturated fatty acids, as they accumulate in the hypothalamus after HFD intake (Posey et al 2009). These nutrients and other inflammatory signals activate the toll-like receptor (TLR) 4 pathway. TLR4 activates JNK, IKKβ and other kinases, which interfere with the insulin-signaling cascade by phosphorylating IRS-1 (Boura-Halfon & Zick 2009, Tanti & Jager 2009) and stimulate cytokine production (Gorina et al 2011). This implies that HFD can have deleterious effects by itself; that is, these inflammatory effects are not solely related to excess body weight (Clegg et al 2011, Doerner et al 2016, Gao et al 2014). For example, hypothalamic inflammation/gliosis is a rapid response to HFD intake, occurring even before a gain of weight or any sign of adipose tissue inflammation can be observed (Thaler et al 2012b). This may be related to the cell stress that saturated fatty acids cause (Diaz et al 2015, Němcová-Fürstová et al 2013). However, the quick inflammatory reaction may be protective, as some studies have suggested (Buckman et al 2014). Thus, prolonged inflammation could possibly be involved in the perpetuation

of obesity and the development of some of its secondary complications (De Souza et al 2005, Douglass et al 2017, Milanski et al 2009). Other mechanisms, such as endoplasmic reticulum stress, have been found to be involved in hypothalamic inflammation (Hotamisligil 2010, Purkayastha et al 2011, Zhang et al 2008). Also, endoplasmic reticulum stress can induce autophagy (Butler & Bahr 2006, Yorimitsu et al 2006) which is related with the inflammatory pathways (Gan et al 2017, Muriach et al 2014).

4. Sex and age differences in response to HFD intake

During many years, most studies have been performed using only male individuals, alleging difficulties in the control of the estrous cycle in rodents and homogenization of the initial conditions. However, as some studies in females demonstrated important differences in the results compared to those obtained in males, the scientific community began to further explore such differences. In a similar way, most studies of obesity and dietary changes in rodents have used young adults as a standard. But, when performing similar experiments with pups, pubescents, middle aged or older adults, the results have often changed significantly, and sex differences have not always been reported to be the same.

4.1. Sex differences in young adults

High fat diet (HFD) intake causes weight gain at all ages, from prepubescents to adults (Grove et al 2010, Mela et al 2012b, Morselli et al 2014, Oliveira et al 2015, Venancio et al 2017, Williams et al 2014a). However, there is a sexual dimorphism in the effects of dietary habits. Young female adult rodents are usually less susceptible than males to weight gain and its secondary complications (Argente-Arizón et al 2016, Estrany et al 2013, Estrany et al 2011, Grove et al 2010, Hwang et al 2010, Mela et al 2012b, Morselli et al 2014, Oliveira et al 2015, Sánchez-Garrido et al 2013). In evolutionary terms, male mammals are reported to follow a strategy of increasing energy intake in order to raise fat stores (Shi et al 2009, Wade 1972), while females are more prone to preserve body fat by reducing energy expenditure (Shi et al 2009). These different strategies may be related to the differences in the hypothalamic melanocortin system (Mauvais-Jarvis 2015). That is, young male mice have fewer POMC neurons

and lower POMC expression than females (Nohara et al 2011) and are more sensitive to insulin, while females are more leptin sensitive (Clegg et al 2003).

Another difference between the sexes lies in the use of nutrients as energy sources. In resting conditions, women convert free fatty acids (FFA) to triglycerides (TG), thus storing fat, whereas FFA undergo higher levels of oxidation in men (Uranga et al 2005). On the other hand, when more energy is needed, women tend to use lipids and men tend to use carbohydrates (Carter et al 2001, Henderson 2014, Horton et al 1995).

Finally, one of the best-known differences between the body composition of men and women is the distribution of adipose tissue: women tend to store fat in subcutaneous pads, for long-term storage, and men tend to accumulate it in visceral pads, which are more metabolically active (Enzi et al 1986, Karastergiou et al 2012, Palmer & Clegg 2015, Vague 1947). Moreover, middle-age women usually present higher levels of brown adipose tissue (Cypess et al 2009, Rodríguez-Cuenca et al 2002). In addition, the fat-borne hormones adiponectin and leptin have higher circulating levels in postpubertal women than in men (Considine et al 1996, Nishizawa et al 2002).

4.2. Sex differences at early ages

Although many of these sex differences are attributable to the effect of estrogens (Chowen et al 2018, Mauvais-Jarvis 2015), some studies have also found sex differences during the prepubertal period (Argente-Arizón et al 2016, Boukouvalas et al 2008, Krolow et al 2013), implying that sex steroids might not be the lone cause of this dimorphism. The effects of overweight, obesity and its secondary complications in children and immature animals are not directly comparable to what happens in adults (Martos-Moreno et al 2013, Pietrobelli et al 2008). These young individuals have a greater ability for tissue expansion, including adipose tissue, which could account for some of these age differences. For example, neonatal overnutrition results in increased weight gain in prepubertal male and female rats, but without showing any hypothalamic inflammation/gliosis, and this increased weight gain reappears only in adult males where signs of hypothalamic inflammation/gliosis are observed (Argente-Arizón et al 2018).

The effects of HFD intake on metabolism also differ between young adults and pubertal individuals. Puberty is defined as a critical phase of genital maturation and

development of secondary sex traits enabling sexual reproduction. It involves rapid growth, changes in body composition, fat redistribution and hormonal variations (Loomba-Albrecht & Styne 2009, Rogol 2010). Various studies have observed that early nutritional challenges induce an increase in body weight that then decreases or becomes non-existent during the pubertal period, only to reappear in adulthood (Argente-Arizón et al 2018, Habbout et al 2012, Mela et al 2012b, Stefanidis & Spencer 2012). These long-term effects of early nutritional and hormonal manipulations often do not become apparent until mid to late adulthood (Argente-Arizón et al 2018, Li et al 2013), suggesting that, during puberty and early adulthood, individuals have different susceptibilities to dietary or metabolic modifications. This raises the question as to what the responses to these kinds of challenges are when they occur specifically around pubertal onset. Secondary complications may also be affected by the timing of dietary challenges as, for example, HFD has a higher effect promoting mammary gland tumors when the intake takes places during puberty compared to adulthood (Zhu et al 2016).

4.3. Sex differences at old ages

There are multiple studies showing that females are more resistant to HFD-induced obesity and its secondary complications (Palmer & Clegg 2015, Pucci et al 2017). However, this statement has been recently challenged by other studies finding that middle-aged and old male and female mice exhibit the opposite response than their younger counterparts, with females being equally or more susceptible to the effects of poor dietary habits (Nishikawa et al 2007, Salinero et al 2018). The inversion of sex differences with age may be related to modifications in estrogen levels, which are high in young females, protecting them from the deleterious effects of obesity and metabolic challenges (Musatov et al 2007, Xu et al 2011), but diminish with age, with the consequent disappearance of their benefits (Leeners et al 2017, Nelson & Bulun 2001). Indeed, a similar change occurs in humans, with post-menopausal women being more prone to obesity and metabolic syndrome (Kapoor et al 2017). The effects of testosterone on metabolic health and body composition have also been reported (Harada 2018, Kelly & Jones 2013) and circulating levels of this sex steroid may also decline with aging (Blaya et al 2017), but this modification is not a severe as that seen in women.

5. Interaction between metabolism, age and neurodegenerative diseases

It has been reported that obesity increases the risk of dementia (Emmerzaal et al 2015) and poor dietary habits, which can lead to neuroinflammation, can also produce cognitive dysfunction (Davidson et al 2013, Granholm et al 2008, Kanoski & Davidson 2011). Moreover, the prevalence of neurodegenerative diseases has been associated to obesity. The decrease of sex steroids that comes with aging has also been also highlighted as a factor raising the risk of cognitive deterioration (Manly et al 2000, Moffat et al 2004, Paoletti et al 2004, Pike et al 2009).

The most common cause of dementia is Alzheimer's Disease, a neurodegenerative process that was first described more than a hundred years ago (Alzheimer 1907) and remains one of the major health challenges as life span continues to increase, resulting in a higher prevalence of this disease. Unfortunately, an effective treatment remains elusive. AD implies a progressive cognitive decline, with memory loss and impaired reasoning. Its more characteristic features are high cortical concentrations of neurofibrillary tangles, composed of hyper-phosphorylated tau protein, and neuritic plaques of amyloid-β peptide (Aβ) (Braak & Braak 1997). The most accepted hypothesis for AD's pathogenesis is that soluble Aβ oligomers affect synaptic functioning and unleash inflammation pathways, an imbalance in central calcium concentrations and oxidative stress, leading to cognitive dysfunction (Selkoe 2000). The Aβ peptide is a cleavage product of the amyloid protein precursor (APP) (LaFerla et al 2007). Mutations of the APP gene or in genes encoding for presenilin 1 (PS1), a protein involved in APP cleavage (Hardy & Selkoe 2002), lead to an increase of Aβ levels and could account for the inheritable, early-onset form of the disease (Lane et al 2018, Pimplikar 2009). However, most cases of AD are of late onset, in which the impairment of Aβ clearance is possibly the cause of its accumulation (Mawuenyega et al 2010). Nevertheless, there are genetic factors involved in late-onset AD, such as mutations in APOE, a gene that encodes the apolipoprotein E (ApoE), a lipid carrier (Bu 2009, Farrer et al 1997, Liu et al 2013). ApoE malfunctioning may be related to oxidative stress, synaptic loss and formation of amyloid plaques (Bell et al 2007, Hashimoto et al 2012, Miyata & Smith 1996, Sen et al 2012). The insulin signaling pathway has been shown to be affected in some AD patients (Messier & Teutenberg 2005) and high circulating glucose levels can be involved in the defective clearance of

Aβ and the formation of neurofibrillary tangles (Querfurth & LaFerla 2010). These observations indicate a possible link between AD and energy metabolism.

The complex etiology of AD makes it difficult to establish animal models that parallel the causes and progression of the disease in humans. Although AD does not appear in rodents, there are some acceptable transgenic mice models that are widely used in the study of this disease, presenting mutations in genes APP, APP and PS1, APP and APOE and APP with insulin desensitization (Esquerda-Canals et al 2017). These models have been employed in attempt to further our understanding of this devastating disease.

The incidence of AD is higher in women than in men, probably due to our different levels of sex steroid hormones (Li & Singh 2014, Moser & Pike 2016), and some observations are similar in genetic animal models. Indeed, Aβ depositions appear earlier in female than in male transgenic AD mice, as well as cognitive deficits (Henderson & Buckwalter 1994, Yang et al 2018). Understanding the interaction between aging, sex and dietary habits in neurodegenerative processes could improve the chances of finding effective treatments for them.

HYPOTHESIS

Hypothesis:

There is an intricate interaction between genetics, sex and age in the response to poor dietary habits and this underlies the differences observed in weight gain and the development of secondary complications related to excess body weight.

OBJECTIVES

The specific objectives of this thesis were:

Objective 1. To analyze the response of peripubertal animals to a short-term exposure to a HFD and determine if males and females respond differently.

Objective 2. To study the interaction between long-term HFD exposure, sex and genetic over-expression of beta-amyloid on weight gain and metabolic complications.

Objective 3. To determine the response of hypothalamic astrocytes to fatty acids, sex steroids and beta-amyloid.

MATERIAL AND METHODS

1. Experimental models

1.1. *In vivo* studies

All of the studies were designed according to the European Community Council Directive (86/609/EEC; 2010/63/UE) and NIH guidelines for animal care and complied with the Royal Decree 53/2013 pertaining to the protection of experimental animals. The studies and use of animals were approved by the animal care and use committee of the Cajal Institute (Comité de Experimentación Animal del Instituto Cajal) and the Consejeria del Medio Ambiente y Territorio (Comunidad de Madrid, Ref. PROEX 200/14).

1.1.1. Response of peripubertal animals to short-term exposure to a HFD

a) Experimental design

Mice from C57BL/6Jola strain (5 weeks-old) were purchased from Harlan Interfauna Ibérica S.A., (Barcelona, Spain). Four animals of the same sex were placed in each cage and allowed to acclimate for at least one week before the start of the experiment. During this time, they were fed a normal chow diet and water *ad libitum*. All mice were maintained at a constant temperature (21 ± 1ºC) and humidity (50 ± 1%) in a 12-hour light-dark cycle.

At post-natal day (PND) 42, day one of the study, animals were weighed and mice of each sex were randomly separated in two groups receiving either a low-fat diet (LFD; 3.76 Kcal/g, 10.2% fat; LabDiet, Sodispan Research SL, Madrid, Spain) or high-fat diet (HFD; 5.1 Kcal/g, 61.6% fat; LabDiet). The composition of each diet is shown in Table 1. Food intake during 24 hours was measured on 3 different days throughout the week of the experiment by placing a known amount of food in the cage and weighing the remaining food the following day. Mean energy intake is reported as total kilocalories consumed and the number of kilocalories consumed/ gram bodyweight.

b) Sacrifice and sample collection

On day 7, after weighing the remaining food the animals were subjected to a 12 hour fast and killed on the morning of day 8 by decapitation (Figure 3). Their hypothalamus, hippocampus, cerebellum, liver and subcutaneous and visceral fat pads were extracted. The brains and fat pads were weighed, and the fat pads frozen in dry ice.

Dissection of the brains was performed rapidly and on a cold surface before freezing in dry ice. Trunk blood was collected, allowed to clot, centrifuged (3000 rpm during 10 min at 4 °C) and the serum removed. All tissues were stored at −80 °C until processed. Glycemia was measured at the moment of sacrifice using a glucometer (Optium Xceed, Abbott Diabetes Care, Inc. CA, USA).

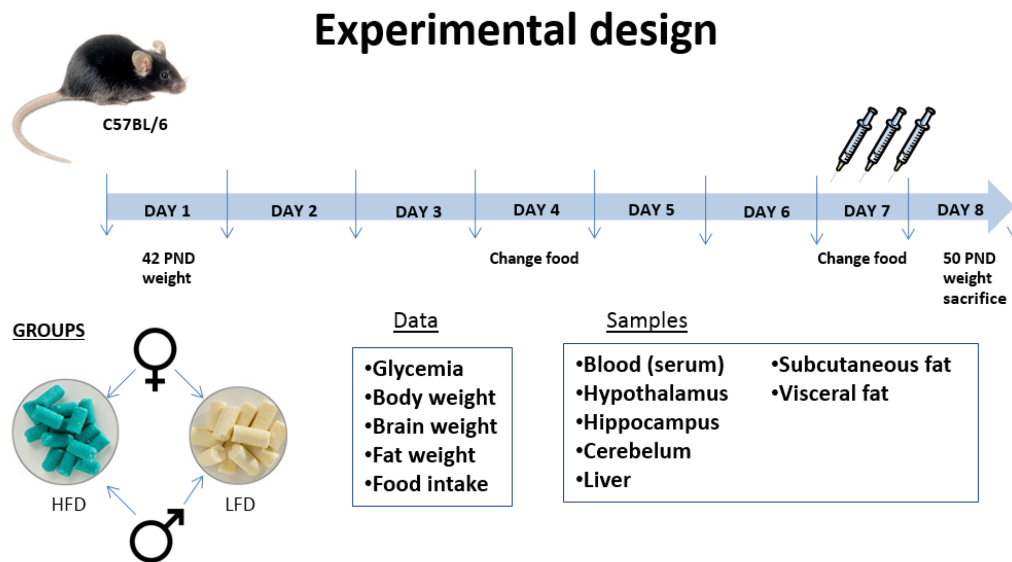

Figure 3. Experimental design for Objective 1.

1.1.2. Interaction between long-term HFD exposure, sex and genetic over-expression of beta-amyloid on weight gain and metabolic complications.

a) Experimental design

Transgenic *knock-in* heterozygous mice that over-express amyloid precursor protein (TgAPP) mice were employed for this study. These mice were created on a C57BL/6 background by heterozygous breeding of mice expressing the human APP long isoform with two mutations [Lys 670- Asn and Met 671-Leu (Swedish mutation)] under transcriptional control of the hamster prion promoter (Hsiao et al 1996). Wild type (WT) littermates were used as controls. Four animals of the same sex, but of both genotypes (WT or TgAPP) were placed in each cage and allowed to acclimate for at least one week before the start of the experiment.

During this time, they were fed a normal chow diet and water *ad libitum*. All mice were maintained at a constant temperature (23 ± 2ºC) and humidity (55 ± 1%) in a

12-hour light-dark cycle. Fifty-seven mice (11 WT males, 18 APP males, 10 WT females and 18 APP females) 7 months old were randomly separated in two groups (5 WT LFD males, 6 WT HFD males, 9 APP LFD males, 9 APP HFD males, 5 WT LFD females, 5 WT HFD females, 9 APP LFD females and 9 APP HFD females) receiving either a LFD (3.76 Kcal/g, 10.2% fat; LabDiet) or HFD (5.1 Kcal/g, 61.6% fat; LabDiet). See table 1 for composition of the diets. This resulted in 8 groups of mice: male and female WTLF, WTHF, APPLF and APPHF. The mice were maintained on these diets for 18 weeks and body weight was measured weekly.

	LFD	**HFD**
Carbohydrates:	**67.4 %**	**25.9 %**
Sugars	*33.13 %*	*8.85 %*
Fats:	**4.3 %**	**34.9 %**
Cholesterol	*18 ppm*	*301 ppm*
Saturated FA	*1.14 %*	*13.68 %*
Monounsaturated FA	*1.3 %*	*14 %*
Polyunsaturated FA	*1.59 %*	*5.15 %*
Proteins	**16.9 %**	**23.1 %**
Fiber	**4.7 %**	**6.5 %**

Table 1. Diet composition in percentage of weight (except cholesterol, which is in parts per million).

b) Sacrifice and sample collection

The mice were subjected to a 12 hour fast before sacrifice. They were weighed and sacrificed by decapitation and their hypothalamus, hippocampus, cerebellum, pituitary and subcutaneous and visceral fat pads extracted. The fat pads were weighed and frozen in dry ice. Dissection of the brains was performed rapidly on a cold surface before freezing in dry ice. Trunk blood was collected, allowed to clot, centrifuged (3000 rpm during 10 min at 4 °C) and the serum removed. All tissues were stored at −80 °C until processed. Glycemia was measured at the moment of sacrifice by using a glucometer (Optium Xceed, Abbott Diabetes Care, Inc. CA, USA). A summarized representation of the experimental design can be consulted in Figure 4.

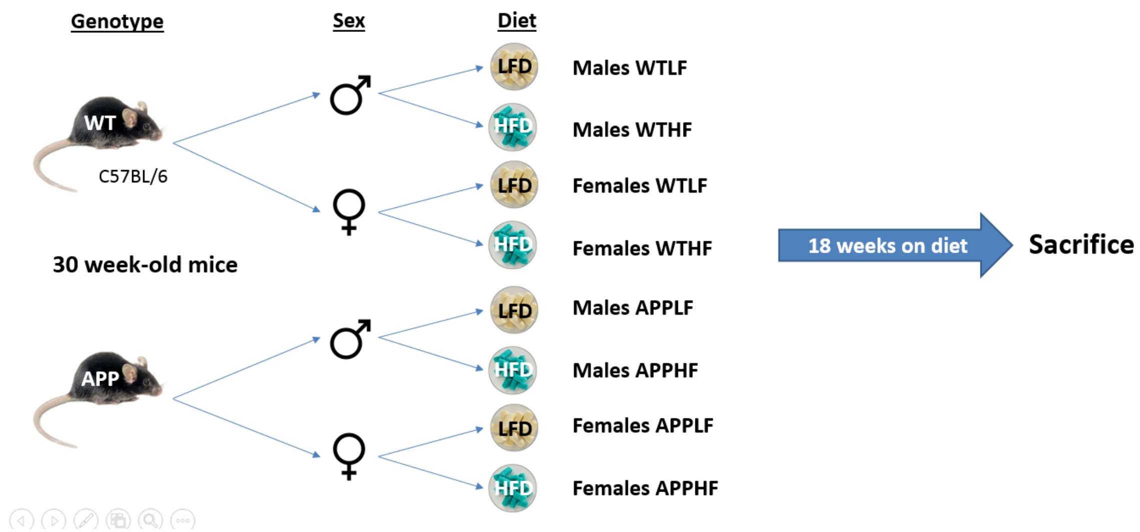

Figure 4. Experimental design for Objective 2.

1.2. *In vitro* studies

1.2.1. Primary hypothalamic astrocyte cultures

Primary cultures of hypothalamic astrocytes were prepared from post-natal day two (PND2) Wistar rat pups. Both sexes were used, and male and female cells were cultured separately after determining the animal's sex by anogenital distance. Pups were sacrificed by decapitation and the brain quickly removed and immediately placed on ice in Dulbecco's modified Eagle's medium: Nutrient mixture F-12 (DMEM/F12) supplemented with 1% penicillin/streptomycin and anti-mycotic (Ab/Am; Gibco). Hypothalami were dissected and the meninges carefully removed. Hypothalami were then triturated in the same media as indicated previously. The tissue was dissociated by first using a P1000 pipette and then pulled Pasteur pipettes several times until the suspension was clear. The suspension was then centrifuged at 1000 rpm for 7 min. The resulting pellet was resuspended in DMEM/F12 enriched with 10% fetal bovine serum (FBS) plus 1% Ab/Am solution. Cells were then seeded in 75 cm2 culture flasks (BD Falcon) containing 9 mL of DMEM/F12 plus 10% FBS and 1% Ab/Am. The cells were incubated at 37 °C and 5% CO2. After three days, the flasks were washed twice with tempered phosphate buffered saline (PBS) and fresh media was added. Media was changed three times a week and after a total of 9-10 days of incubation, when the cells reached approximately 90% confluency, flasks were placed in a 37 °C shaking

incubator (SI-300, Jeoi Tech; Medline Scientific) at 280 rpm overnight. A summarized representation of this experimental design can be seen in Figure 5.

After shaking, flasks were washed twice with PBS to remove non-attached cells. Then, 1 mL of trypsin (0.05% trypsin/EDTA solution; Biochrom AG) was added and the flasks hit together 10 times (in order to detach and harvest the astrocytes). DMEM/F12 plus 10% FBS and 1% Ab/Am was added to the flasks to stop the action of the trypsin and resuspend the cells. The suspension was centrifuged for 5 min at 1150 rpm. After centrifugation, the supernatant was discarded and the pellet resuspended in DMEM/F12 plus 10% FBS and 1% Ab/Am. Astrocytes were seeded in 60 mm or 100 mm culture plates, that had been previously treated with poly-L-lysine hydrobromide (10 µg/ml; Sigma-Aldrich), at a density of 4.35 x 10^5 or 1 x 10^6 cells/plate, respectively. Cells were then grown for 24 hours and then the media was changed to DMEM F-12 plus 1% Ab/Am (without FBS). Thus, the cells were serum starved for 24 h before the experimental treatments were added. The treatments were prepared with the same media (DMEM F-12 plus 1% Ab/Am, without FBS). In each experiment, treatments were done in triplicate; each experiment was repeated 3 to 4 times (N = 3 or 4).

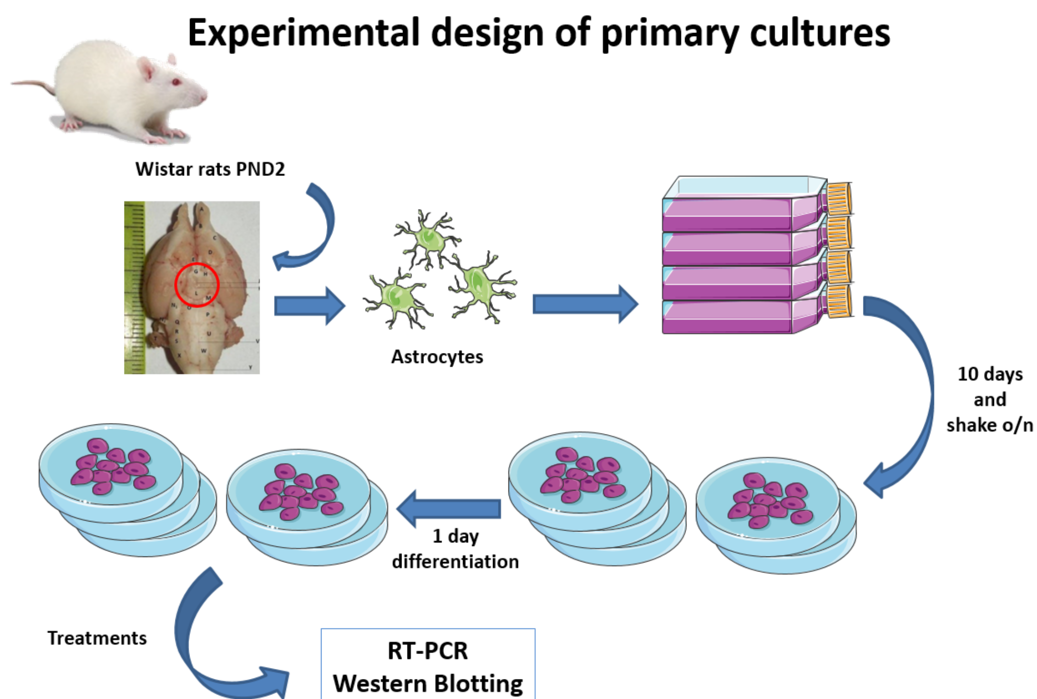

Figure 5. Experimental design of primary cultures of hypothalamic astrocytes.

1.2.2. Treatments

a) Palmitic acid

All reagents were purchased from Sigma-Aldrich, Inc. (Saint Louis, MO, USA). Palmitic acid (PA) supplemented medium was prepared according to previously published protocols (Huynh et al., 2014) and used to treat astrocytes. Stock solutions of PA (Sigma, P-5585), BSA (Sigma, A-9205) and L-carnitine (Sigma, C-0283) were prepared and sterilized by filtration with 0.45 µm filters and stored at -20°C until used. The PA stock solution was prepared at 200 mM in ethanol and vortexed until the solution was clear. Fatty acid free bovine serum albumin (BSA) was added to ensure lipid solubility in the aqueous solution The BSA stock solution was prepared at 30% and L-carnitine at 200 mM; both substances were dissolved in sterile H_2O. Palmitic acid was then conjugated with fatty acid-free BSA to act as a carrier and ensure lipid solubility in the aqueous solution. Fatty acid-albumin solutions were diluted in DMEM F-12 plus 1% Ab/Am (without FBS) to achieve the desired final fatty acid concentration. The L-carnitine (1 mM) was added to the final fatty acid solution before being added to cultures. Control plates received an equivalent amount of vehicle solution. Treatments had a duration of 24 hours.

b) Estradiol

A stock solution of 1 mg/mL of 17β-estradiol (Sigma, E-8875) was prepared in ethanol. Then, a 10^{-9} M working solution was made in DMEM F-12 plus 1% Ab/Am (without FBS) medium for treatment of cultured astrocytes.

When 17β-estradiol was used in combination with PA, astrocytes were pre-treated with 10^{-9} M β-estradiol for 3 h previous to PA addition. Once all the treatments were added, they were incubated for 24 hours.

c) Testosterone

Testosterone (Sigma, T-1500) was purchased from Sigma-Aldrich. A stock solution of 0.1 mg/mL was prepared in ethanol. Then, 10^{-8} M, 10^{-9} M and 10^{-10} M working solutions were made in DMEM F-12 plus 1% Ab/Am (without FBS) medium for treatment of cultures astrocytes. Testosterone was added to the cultures at the same time as PA and vehicle (controls) and all the plates were incubated with the treatments for 24 hours.

d) Amyloid-β

Amyloid-β protein 1-40 (SC875) was purchased from PolyPeptide (Strasbourg, France). A stock solution of 1 mg/mL was prepared in PB 0.05 M. Before treatment, the volume of stock solution needed was incubated at 37ºC for 24 hours, with occasional vortexing. This process allows the peptide to aggregate and therefore induce neurotoxicity in vitro (Iversen et al 1995). Then, 1, 5 and 20 μg/mL working solutions were made in DMEM F-12 plus 1% Ab/Am (without FBS) medium for 24 hours treatment of cultured astrocytes.

2. Techniques and protocols

2.1. Serum analysis

Circulating leptin, insulin, interleukin (IL) 6 and tumor necrosis factor α (TNFα) for both Objective 1 and Objective 2 and monocyte chemoattractant protein (MCP)-1 and total plasminogen activator inhibitor (PAI)-1 for Objective 2, were determined by multiplexed immunoassay according to the manufacturer's instructions (Millipore, Billerica, MA) in a Bio-Plex suspension array system 200 (Bio-Rad Laboratories, Hercules, CA, USA). Mean fluorescence intensity was analyzed by using Bio-Plex Manager Software 4.1. All samples were run in duplicate and within the same assay for all analyses. The minimum detectable concentrations of IL-6, insulin, leptin, MCO-1, PAI-1 Total and TNFα were 2.3, 13.0, 4.2, 4.9, 4.0 and 5.3 pg/ml, respectively. The intra-assay coefficients of variation were 5 % for all analytes except for TNFα, which was 4 %. The inter-assay coefficients of variation of IL-6, insulin, leptin, MCO-1, PAI-1 Total and TNFα were 11, 11, 10, 10, 15 and 20 %, respectively.

Circulating triglycerides (Spinreact S.A., Sant Esteve de Bas, Spain) and non-esterified fatty acids (NEFA; Wako, Neuss, Germany) were measured using enzymatic colorimetric kits according to the manufacturers' instructions.

2.2. RNA and protein isolation

Total mRNA was isolated from non-adipose tissues or astrocyte cultures by using an RNeasy® Plus Mini Kit (Qiagen, Hilden, Germany). For adipose tissue, a RNeasy® Lipid Tissue Mini Kit (Qiagen) was used. Protein was isolated from the same

tissues by collecting the first elution from the RNeasy® Mini Spin columns and diluting 1:5 with cold acetone. It was then stored at -20 ºC for at least 30 minutes before centrifuging (3000 rpm during 10 min at 25 ºC), after which the acetone was removed and the pellet resuspended in 100 µl of 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate (CHAPS) buffer (7 mM urea, 2 M thiourea, 4% [w/v] CHAPS, and 0.5% [v/v] 1 M Tris; pH 8.8). Protein samples were stored at -20 ºC until protein quantification by the method of Bradford (Bio-Rad Laboratories). Determination of mRNA purity and concentration was performed by using a Nanodrop (Thermo Scientific, Washington, DE, USA) and samples were stored at -80 ºC until analyzed.

2.3. RT-qPCR

Using 1 µg of each RNA sample, cDNA was synthesized with a high-capacity cDNA RT kit (Applied Biosystems, Foster City, CA) and stored at -20 ºC. Quantitative RT-PCR was performed by using TaqMan Universal PCR Master Mix (Applied biosystems) and TaqMan Gene Expression Assay-on-demand kits to analyze neuropeptides and receptors involved in metabolic control (Table 2). All samples were run in duplicate. Various housekeeping genes were tested and those that did not vary between experimental groups were chosen to normalize the data (Table 2). The ΔΔCT method was used to determine relative expression levels and for statistical analysis. All data are expressed as % control group.

Gene	Reference	Animal
GFAP	Mm01253033_g1	Mouse
	Rn00566603_m1	Rat
DDIT-3	Mm01135937_g1	Mouse
	Rn00492098_g1	Rat
IL-6	Mm00446190_m1	Mouse
	Rn01410330_m1	Rat
IL-1β	Mm01336189_m1	Mouse
TNFα	Mm00443260_g1	Mouse
POMC	Mm00435874_m1	Mouse
NPY	Mm03048253_m1	Mouse
AgRP	Mm00475829_g1	Mouse
Leptin	Mm00434759_m1	Mouse
LepR	Mm00440181_m1	Mouse
InsR	Mm01211875_m1	Mouse
IκBKβ	Mm01222247_m1	Mouse
StAR	Rn00580695_m1	Rat
TSPO	Rn00560892_m1	Rat
Aromatase	Rn00567222_m1	Rat
18S	Mm03928990_g1	Mouse
	Rn01428915_g1	Rat
Pgk1	Mm00435617_m1	Mouse
	Rn00821429_g1	Rat
Rpl13a	Rn00821946_g1	Rat
Ppia	Mm02342430_g1	Mouse
Actin beta	Mm00607939_s1	Mouse
	Rn00667869_m1	Rat
GAPDH	Mm99999915_g1	Mouse
	Rn99999916_s1	Rat

Table 2. List of genes analyzed by qRT-PCR.

2.4. Western blotting

For Western blotting, equal quantities of protein of each sample were resolved on an SDS- acrylamide gel under denaturing conditions (see Table 2 for protein quantities and acrylamide concentrations). Proteins were then transferred to a polyvinylidine difluoride membrane (Bio-Rad, Hercules, CA, USA). Transfer efficiency was determined by Ponceau red dyeing. Membranes were blocked with Tris-buffered saline (TBS) containing 5% (w/v) non-fat milk or bovine serum albumin (phosphorylated proteins) and incubated with the appropriate primary antibody and concentration overnight at 4 ºC under agitation. The antibodies and their concentrations used are listed in Table 3.

The following day after washing, membranes were incubated with the secondary antibody conjugated with peroxidase (Pierce Biotechnology, 1:1000 or 1:2000). Peroxidase activity was visualized by using chemiluminescence and quantified by densitometry using an Image-Quant LAS4000 mini TL Software (GE Healthcare Europe GmbH, Spain). All blots were rehybridized with actin to adjust for loading and then normalized to % control group values on each gel.

Antigen	Host	Company	Dilution
Actin, pan Ab-5	Mouse	Thermo Fisher Scientific	1:1000
GAPDH	Rabbit	AnaSpec	1:1000
GFAP	Mouse	Sigma-Aldrich	1:1000
Vimentin	Rabbit	Sigma-Aldrich	1:1000
IBA-1	Rabbit	Wako	1:1000
JNK (F-3)	Mouse	Santa Cruz Biotechnology	1:500
pJNK	Mouse	Promega	1:500
SOD	Rabbit	Sigma-Aldrich	1:500
Hsp-70	Rabbit	Enzo	1:1000
pNFκB	Rabbit	Cell Signalling	1:500
NFκB	Mouse	Thermo Fisher Scientific	1:500
pAKT	Mouse	Cell Signalling	1:500
AKT	Goat	Santa Cruz Biotechnology	1:500
pIκBα	Mouse	Cell Signalling	1:500
pIRS1	Mouse	Cell Signalling	1:500
IRS1	Rabbit	Sigma Aldrich	1:500

Table 3. List of antibodies used in Western Blots.

2.5. Estimation of the number of cells

To determine cell viability in response to amyloid-β treatment in primary hypothalamic astrocyte cultures we used the crystal violet dye elution method. The dye binds to the nuclei of cells and the amount of dye is quantified by solubilizing it and using a spectrophotometer. The absorbance is proportional to the number of cells.

Cells were grown in 24-well plates. After washing twice with PBS, cells were incubated 10 min at RT with 1 mL of 1% glutaraldehyde for fixation. Then, the cells were washed again twice with PBS and 1 mL of 0.1% crystal violet solution was added to each well and incubated at RT for 20 min. The solution was then removed and the cells rinsed under running tap water for 20 min. To dry the plates, they were left at RT for at least 2 hours. Finally, the dye was dissolved with 10% acetic acid, mixed well and 0.2 mL added to a 96-well plate to read the absorbance at 590 nm. The number of cells was calculated with the following equation: Number of cells = (265030 × absorbance) - 1950.

2.6. Analysis of adipocyte size

In the peripubertal mice from experimental design 1, visceral adipose tissue was imbedded in paraffin, cut on a sliding microtome (4 µm) and processed for hematoxylin and eosin staining. Sections were visualized by using a light microscope and a digital camera and Image-Pro Plus software (version 5.0; Media Cybernetics, Silver Spring, MD) were used to capture images (20X). Four images/slice and 4 slices/animal of 6 animals/experimental group were analyzed by using Adiposoft, a program of ImageJ designed for the analysis of adipocytes. The mean cell size and the mean number of cells/image of each animal were used for the statistical analysis.

2.7. Levels of amyloid-β in the hypothalamus

In mice from experimental design 2, amyloid-β peptide-42 detection in the hypothalamus was performed using a Mouse Aß42 ELISA kit (Invitrogen, Carlsbad, CA, USA, KMB3441) on hypothalamic protein samples diluted 1:10, following the manufacturer's instructions.

2.8. Statistical analysis

The programs SPSS (SPSS Inc., Chicago, IL, USA) and GraphPad Prism (GraphPad Software, Inc., CA) were used for data analysis. A three-way or two-way ANOVA was performed, depending on the experimental design, followed by one-way analysis if appropriate. Scheffé's f test was used as a post-hoc test to determine whether

specific differences existed between the experimental groups. For energy intake over time, repeated measures ANOVA was used. If data were non-parametric, a Kruskal-Wallis test with Dunn's pair test was performed. Two-tailed Student's t test was performed on data from primary hypothalamic cultures. All data are presented as mean ± SEM. The results were considered statistically significant at $p < 0.05$. The p-values in the figures represent the results of the one-way ANOVA, the Student's t test after factor separation or the Kruskal-Wallis test.

RESULTS

1. Sex differences in the response to HFD intake of peripubertal mice

1.1. Sex differences in the metabolic response to HFD

Males weighed more than females at the beginning of the experiment ($F_{(1,48)}$ = 153.7, $p < 0.0005$) with no difference between the starting weight of the different dietary groups in each sex (Males: LFD 22.3 ± 0.4 g, HFD 21.7 ± 0.4 g; Females: LFD 17.3 ± 0.4 g, HFD: 17.5 ± 0.3 g). At the moment of sacrifice, males of both groups continued to weigh more than the corresponding female groups ($F_{(3,48)}$ = 51.7, $p <$ 0.0005), with no effect of diet (Fig. 6A). Weight change was lower in females than in males with both diets (Kruskal-Wallis = 31.97, p < 0.0001) (Fig. 6B).

Mean energy intake was affected by diet ($F_{(1,12)}$ = 10.1, $p < 0.02$). Mice fed with a HFD ingested more kilocalories than those fed a LFD, regardless of sex ($F_{(3,11)}$ = 9.4; p = 0.005; Fig. 6C). Food intake was divided into two periods, one comprising the first three days of diet and the other the remaining days (4-8), to determine if dietary change resulted in a rapid hyperphagic period. There was an effect of time on energy intake ($F_{(3,8)}$ = 48.5, p<0.0001, with energy intake being greater during the first 3 days after dietary change in all groups. There was no difference between experimental groups during the first 3 days after dietary change; however, during the subsequent period there was a significant effect of diet ($F_{(1,3)}$ = 16.6, p<0.004) with mice on a HFD having a greater energy intake than those on a LFD regardless of sex. (Fig. 6E).

Energy efficiency, which was calculated by dividing mean weight gain by mean food intake, was affected by sex ($F_{(1,11)}$ = 15.8, $p < 0.005$) and a significant difference between HFD males and females ($p < 0.04$). Females have lower energy efficiency than males ($F_{(3,11)}$ = 5.3, $p < 0.03$; Fig. 6D).

The weights of subcutaneous and visceral fat pads were normalized to body weight and are expressed as a percentage. The percentage of subcutaneous (Males LFD: 0.9 ± 0.2, Males HFD: 1.2 ± 0.2, Females LFD: 0.9 ± 0.2, Females HFD: 0.9 ± 0.1 %) or visceral (Males LFD: 1.2 ± 0.2, Males HFD: 1.5 ± 0.1, Females LFD: 1.2 ± 0.2, Females HFD: 1.1 ± 0.3 %) adipose tissue did not differ between any of the groups.

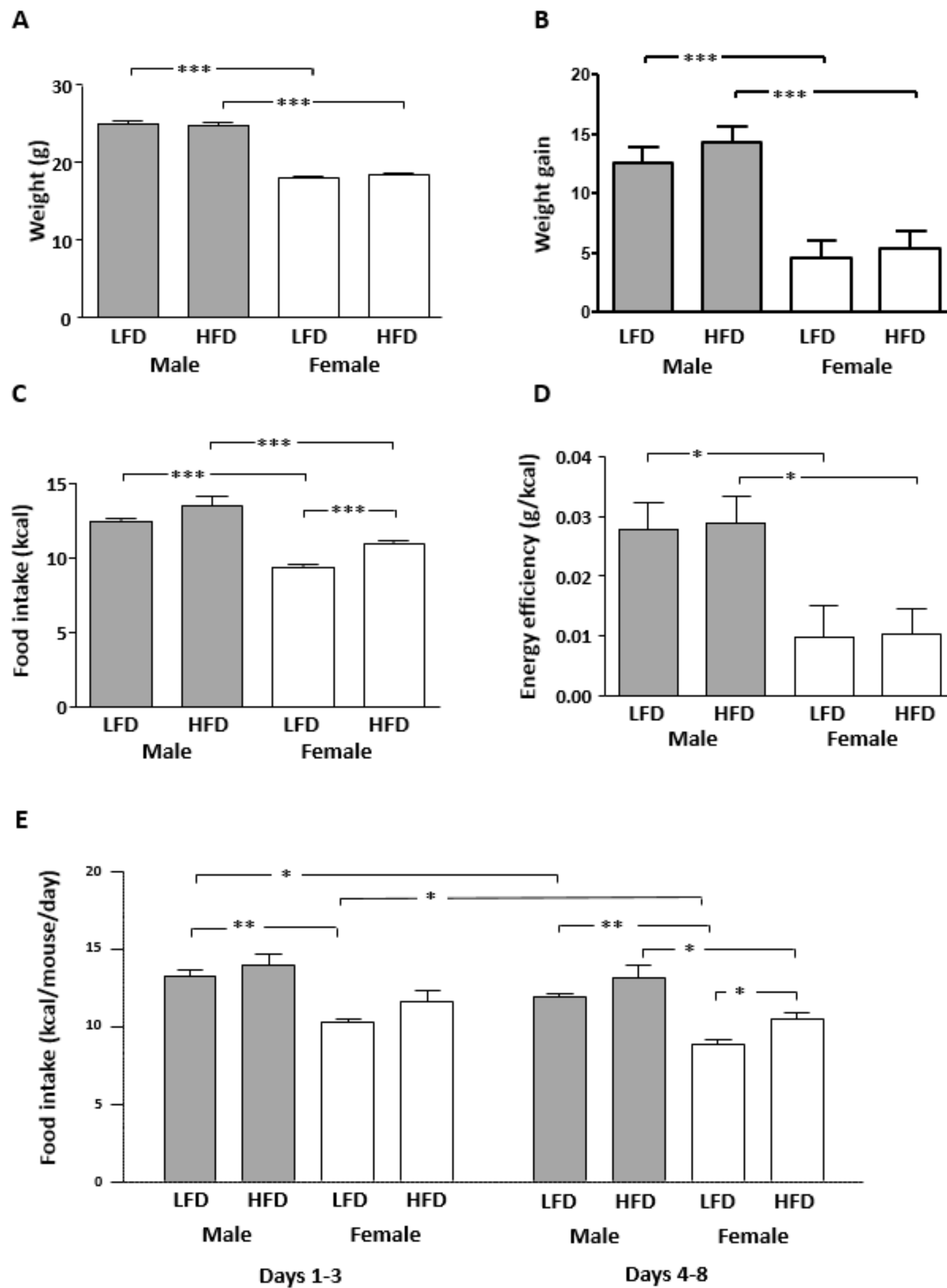

Figure 6. A: Body weight in grams. B: Change in body weight. C: Food intake in kilocalories. D: Energy efficiency in g/kcal. E: Food intake in kilocalories per mouse per day. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

1.2. Sex differences in serum levels of glucose, metabolic factors and inflammatory markers

Glycemia ($F_{(3,22)} = 5.0$, $p < 0.02$) was affected by sex ($F_{(1,22)} = 5.5$, $p < 0.04$) and diet ($F_{(1,22)} = 5.6$, $p < 0.03$). Males had higher glycemia on a HFD than those on a LFD

(split by sex: $F_{(1,9)} = 16.3$, $p < 0.005$) and higher levels than females on a HFD (split by diet: $F_{(1,11)} = 19.5$, $p < 0.001$; Fig. 7A).

There was an overall effect of sex ($F_{(1,22)} = 17.9$, $p < 0.002$) on serum insulin levels, with males having higher levels than females and with this reaching significance in the HFD groups ($F_{(3,22)} = 6.6$, $p < 0.004$; Fig. 7B).

The homeostasis model assessment of insulin resistance (HOMA-IR) index, calculated multiplying insulin (IU/l) and glycemia (mmol/l) and dividing by 22.5, showed an effect of sex ($F_{(1,20)} = 14.7$, $p < 0.002$), with a higher index in HFD males than HFD females ($F_{(3,20)} = 6.8$, $p < 0.005$) (Fig. 7C).

Serum leptin levels were affected by sex ($F_{(1,22)} = 6.2$, $p < 0.03$). Males had higher levels than females regardless of diet ($F_{(3,22)} = 4.0$, $p < 0.03$). There was also an overall effect of diet ($F_{(1,22)} = 5.2$, $p < 0.04$), with mice on a HFD having higher leptin levels compared to LFD mice (Fig. 7D). There were no differences between groups in NEFA or triglyceride levels (Figs 7 E & F).

Serum levels of IL-6 were influenced by sex ($F_{(1,21)} = 16.7$, $p = 0.001$), with LFD males having significantly higher levels than females on a LFD compared to females on the same diet ($F_{(3,21)} = 5.8$, $p < 0.007$; Fig.7G). Although males on a HFD tended to have higher levels than females on a HFD, this did not reach statistical significance. There was no effect of diet in either sex. Serum levels of TNF-α (Fig. 7H) were not statistically different between groups.

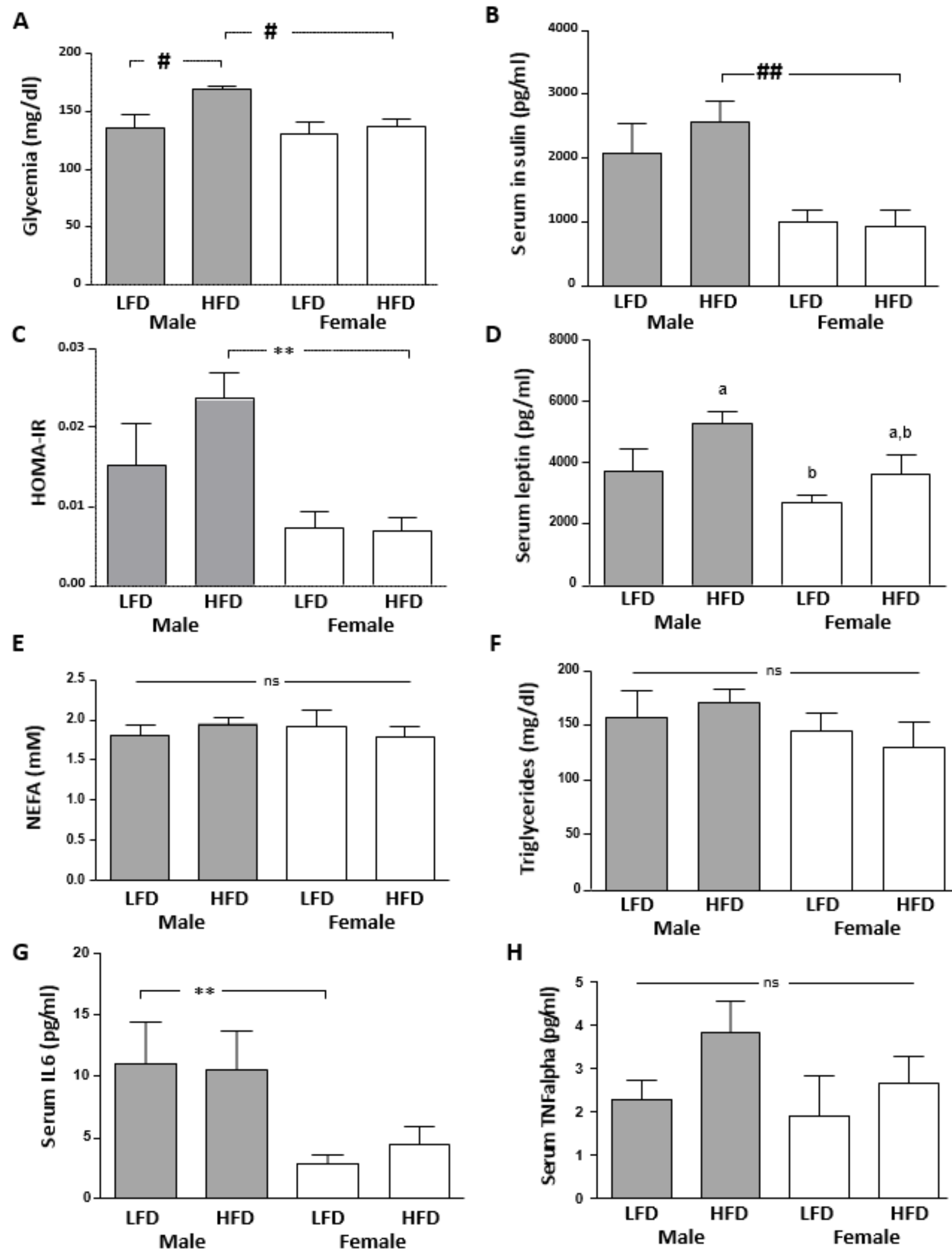

Figure 7. A: Glycemia. B: Circulating insulin. C: Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) index. D: Circulating leptin. E: Circulating Non-Esterified Fatty Acids (NEFA). F: Circulating triglycerides. G: Circulating interleukin (IL)-6. H: Circulating tumor necrosis factor (TNF)α. # p < 0.02; ## p < 0.004; ** p < 0.001; a: overall effect of sex; b: overall effect of diet; n.s.: non-significant.

1.3. Systemic expression of inflammatory markers

In VAT, there was an overall effect of HFD to induce higher levels of leptin mRNA ($F_{(1,20)}$ = 4.2, p<0.05), with males having overall higher levels than females

regardless of diet ($F_{(1,20)}$ = 5.0, p<0.04; Table 4). Expression of IL-1β was not significantly affected by diet, but males had higher levels than females (sex: $F_{(1,20)}$ = 7.5, p<0.02). There was no effect of either diet or sex on IL-6 or TNF-α mRNA levels, although males tended to have higher mRNA levels of both cytokines.

Leptin expression was affected by sex in SCAT ($F_{(1,19)}$ = 31.0, p<0.0001), but not by diet, with males having higher expression levels than females (Table 4). Males also had higher levels of IL-1β ($F_{(1,18)}$ = 20.7, p<0.0002), IL-6 ($F_{(1,18)}$ = 16.8, p<0.0005) and TNF-α ($F_{(1,19)}$ = 15.3, p<0.01) compared to females, with no effect of diet on the mRNA levels of these cytokines.

In the liver there was no effect of either diet or sex on IL-1β, IL-6 or TNF-α mRNA levels (Table 4).

	Male LFD	**Male HFD**	**Female LFD**	**Female HFD**	**P value**
VAT leptin	100 ± 24.9	172.2 ± 36.3	50.9 ± 9.9	93. 6 ± 35.4	Diet: p<0.05 Sex: p<0.04
VAT IL-1β	100 ± 24.5	97.9 ± 39.7	40.4 ± 9.8	24.8 ± 7.7	Sex: p<0.02
VAT IL-6	100 ± 21.6	130.6 ± 41.5	78.4 ± 8.0	65.0 ± 18.2	NS
VAT TNF-α	100 ± 16.2	136.1 ± 23.7	93.1 ± 17.3	77.6 ± 15.4	NS
SCAT leptin	100 ± 14.1	105.0 ± 21.3	22.0 ± 5.3	29.7 ± 12.4	Sex: p<0.0001
SCAT IL-1β	100 ± 14.3	74.7 ± 21.8	23.0 ± 8.0	30.5 ± 6.0	Sex: p<0.0002
SCAT IL-6	100 ± 28.3	109.7 ± 15.3	28.8 ± 5.2	50.5 ± 10.7	Sex: p<0.001
SCAT TNF-α	100 ± 19.6	83.3 ± 27.9	17.5 ± 5.1	38.7 ± 5.8	Sex: p<0.01
Liver IL-1β	100 ± 23.3	60.0 ± 12.2	101.9 ± 14.6	130.2 ± 31.2	NS
Liver IL-6	100 ± 16.1	75.6 ± 17.6	125.8 ± 22.1	115.2 ± 14.3	NS
Liver TNF-α	100 ± 12.5	86.7 ± 10.2	140.3 ± 34.7	103.0 ± 12.4	NS

Table 4. Inflammatory and gliosis markers in visceral adipose tissue (VAT), subcutaneous adipose tissue (SCAT), and liver of males and females on a low fat diet (LFD) or high fat diet (HFD). All data are presented as the percentage of the results of the male LFD group. N = 6. NS: non-significant.

1.4. VAT adipocyte morphology analysis

There was an effect of diet on adipocyte size in VAT ($F_{(1,8)} = 8.4$, $p<0.02$), with the mean circumference being significantly smaller in females on the HFD ($F_{(3,8)} = 5.3$, $p<0.03$), compared to females on the LFD, as well as to males on the HFD (Fig. 8 A & B). The reduction in size was inversely related to the number of adipocytes/area where there was also an effect of diet ($F_{(1,8)} = 14.5$, $p<0.005$) and sex ($F_{(1,8)} = 10.1$, $p<0.02$), with an interaction between these factors ($F_{(1,8)} = 6.7$, $p<0.03$). Females on the HFD had a greater number of adipocytes/area compared to females on the LFD and males on the HFD ($F_{(3,8)} = 10.5$, $p<0.004$; Fig. 8C).

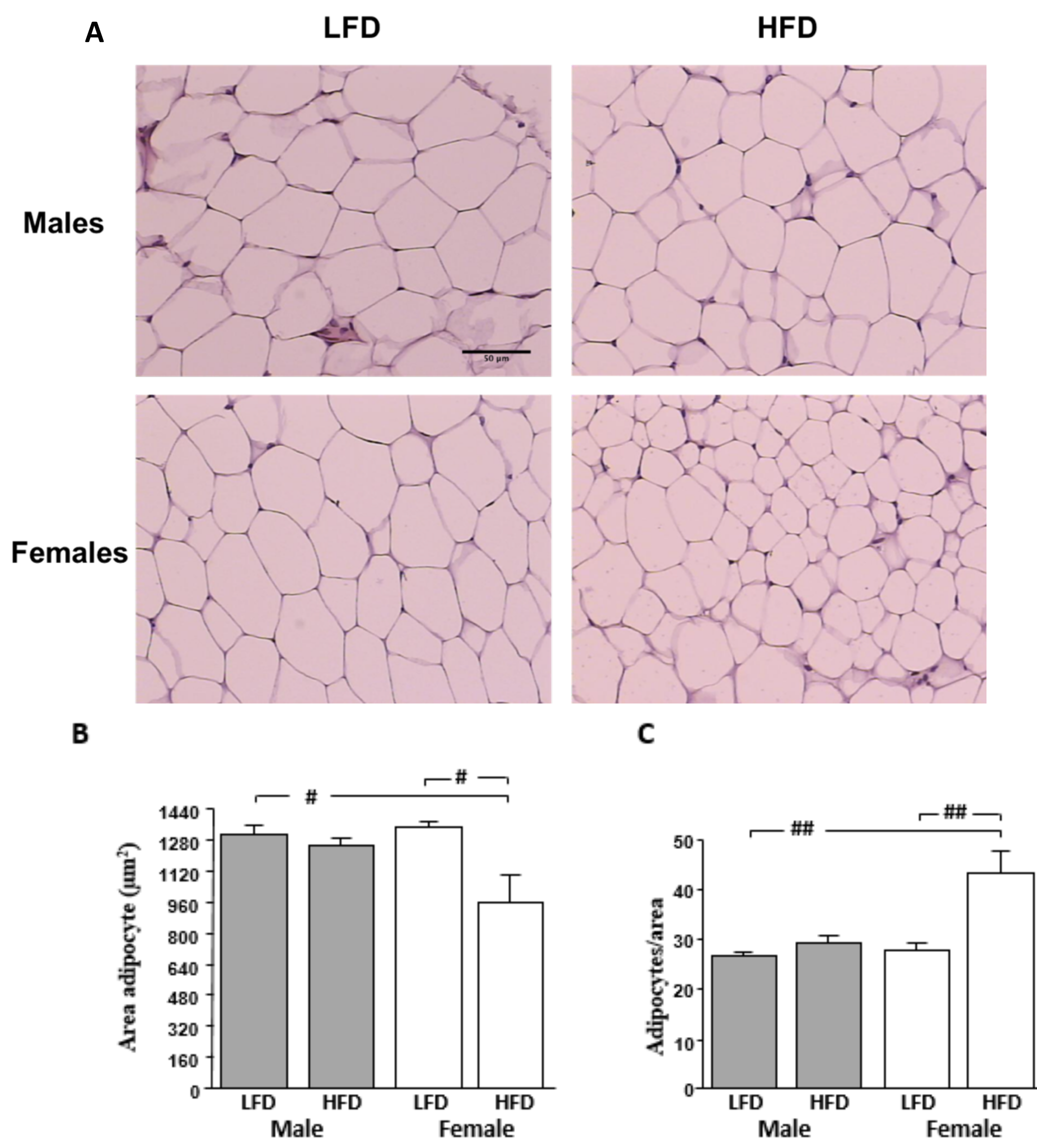

Figure 8. A: Photomicrographs of visceral adipose tissue. B: Mean adipocyte area. C: Number of adipocytes per area. # p < 0.02; ## p < 0.004.

1.5. Central inflammatory, glial and ER stress markers

In the hypothalamus, expression levels of IL-6 were influenced by sex ($F_{(1,24)}$ = 6.5, p < 0.02), with an interaction between sex and diet ($F_{(1,24)}$ = 10.7, p < 0.004). Females on a HFD had higher levels of IL-6 mRNA than females on a LFD and higher than males on a HFD ($F_{(3,24)}$ = 6.9, p < 0.002; Table 5).

Hypothalamic IL-1β, TNF-α, GFAP, DDIT-3 and IKBκB mRNA levels did not differ between groups (Table 5). Likewise, protein levels of GFAP did not differ between groups (Table 5). There was an effect of sex on Iba-1 protein levels ($F_{(1,24)}$ = 7.2, p < 0.02) with significantly higher levels in HFD males compared to HFD females ($F_{(1,11)}$ = 7.5, p < 0.03; Table 5).

	Male LFD	Male HFD	Female LFD	Female HFD	p-value
IL-1β mRNA	100.0 ± 3.5	73.0 ± 12.2	58.9 ± 4.5	70.1 ± 13.4	NS
TNF-α mRNA	100.0 ± 4.5	101.2 ± 20.6	93.5 ± 13.5	85.4 ± 10.8	NS
IL-6 mRNA	100.0 ± 9.2	84.0 ± 11.9	91.7 ± 9.9	151.6 ± 14.7**,##	a, b
GFAP mRNA	100.0 ± 6.4	92.2 ± 16.0	103.3 ± 15.7	90.5 ± 11.7	NS
DDIT-3 mRNA	100.0 ± 9.8	110.4 ± 25.5	135.1 ± 24.5	119.8 ± 24.3	NS
IKBκb mRNA	100.0 ± 15.4	110.3 ± 13.3	112.7 ± 17.6	136.5 ± 26.4	NS
GFAP protein	100.0 ± 7.6	105.8 ± 11.0	134.2 ± 39.0	100.2 ± 23.6	NS
IBA-1 protein	100.0 ± 16.6	110.3 ± 15.9	78.0 ± 12.5	54.0 ± 13.0 *	a
p-JNK protein	100.0 ± 6.2	108.5 ± 14.8	109.6 ± 18.2	79.2 ± 8.5	NS

Table 5. Inflammatory and gliosis markers in the hypothalamus of males and females on a low fat diet (LFD) or high fat diet (HFD). Values are expressed as a percentage from the male LFD group. N = 6. NS: non significant, a: overall effect of sex, b: interaction sex and diet, *p<0.03 compared to HFD males; **p<0.0002 compared to LFD females; ## p<0.0002 compared to HFD males.

To see if inflammatory/glial processes were affected in other brain areas, the hippocampus and cerebellum were analyzed. In the hippocampus, expression levels of IL-6 and IL-1β did not differ between groups (Table 6). However, there was a sex effect on TNF-α mRNA levels ($F_{(1,15)} = 5.7$, $p < 0.04$), with females having overall higher levels than males. There were no differences in GFAP mRNA or protein levels (Table 6). Protein levels of Iba-1 were also affected by sex in this brain area ($F_{(1,24)} = 7.15$, $p < 0.02$; Table 6), with females on a LFD tending to have lower levels than males on the same diet. In the cerebellum, mRNA levels of IL-6, IL-1β, TNF-α and GFAP and protein levels of GFAP and Iba-1 did not differ between groups (Table 7).

	Males LFD	**Males HFD**	**Females LFD**	**Females HFD**	**p**
IL-6 mRNA	100.0 ± 33.4	94.8 ± 27.3	112.0 ± 20.4	102.0 ± 10.2	NS
IL-1-β mRNA	100.0 ± 31.7	79.6 ± 11.6	79.2 ± 13.9	127.9 ± 35.3	NS
TNF-α mRNA	100.0 ± 11.0	127.9 ± 52.9	190.2 ± 27.2 *	362.4 ± 95.2	a
GFAP mRNA	100.0 ± 27.3	113.8 ± 37.8	115.3 ± 39.1	162.1 ± 37.4	NS
GFAP protein	100.0 ± 41.3	97.5 ± 38.6	86.0 ± 24.3	80.6 ± 17.3	NS
IBA-1 protein	100.0 ± 7.9	76.9 ± 6.8	87.0 ± 13.0	80.5 ± 15.5	a

Table 6**.** Inflammatory and gliosis markers in the hippocampus of male and female mice on a low fat diet (LFD) or high fat diet (HFD). Values are expressed as a percentage of the male LFD group. N = 6. NS: non significant, a: overall effect of sex, *p<0.04 compared to LFD males.

	Males LFD	Males HFD	Females LFD	Females HFD	*p*
IL-6 mRNA	100.0 ± 4.4	96.4 ± 8.6	134.7 ± 24.8	117.3 ± 16.8	NS
IL-1β mRNA	100.0 ± 5.4	95.7 ± 17.9	79.1 ± 10.8	66.9 ± 7.3	NS
TNF-α mRNA	100.0 ± 9.9	108.8 ± 12.1	106.2 ± 14.2	93.9 ± 6.8	NS
GFAP mRNA	100.0 ± 9.8	111.0 ± 18.4	106.7 ± 9.4	91.7 ± 15.1	NS
GFAP protein	100.0 ± 18.6	75.6 ± 24.7	86.0 ± 29.3	60.8 ± 17.4	NS
IBA-1 protein	100.0 ± 12.2	106.0 ± 20.9	94.1 ± 34.0	82.6 ± 10.4	NS

Table 7. Inflammatory and gliosis markers in the cerebellum of males and females on a low fat diet (LFD) or high fat diet (HFD). Values are expressed as a percentage of the male LFD group. N = 6. NS: non significant.

1.6. Sex differences in the expression levels of hypothalamic metabolic neuropeptides

There were no changes in AgRP or NPY mRNA levels in the hypothalamus in response to one week of HFD intake (Fig. 9 A & B). POMC mRNA levels were affected by sex ($F_{(1,24)} = 4.8$, $p < 0.05$), with the expression of this protein being lower in HFD males than in HFD females ($F_{(3,23)} = 3.5$, $p < 0.05$; Fig. 9C), but with no effect of diet.

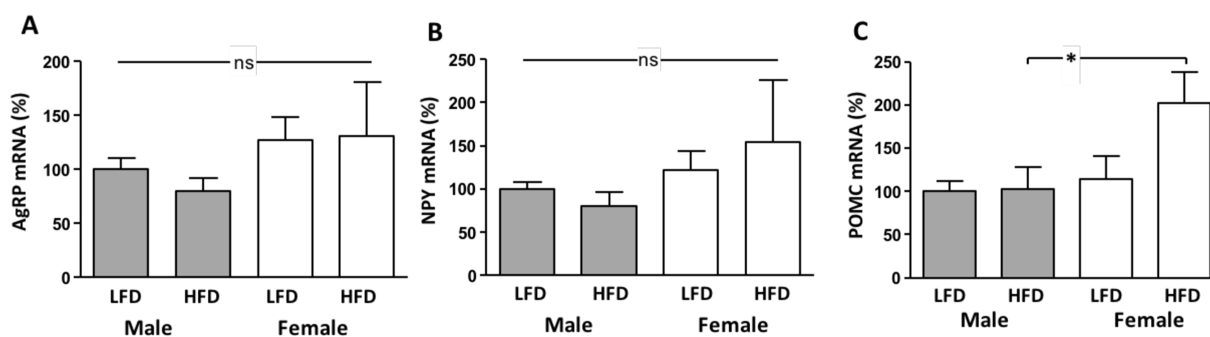

Figure 9. Hypothalamic mRNA levels of A) Agouti-related protein (AgRP), B) Neuropeptide Y (NPY) and C) Proopiomelanocortin (POMC) in peripubertal mice on a low fat diet (LFD) or high fat diet (HFD) for 1 week. ns: non significant; * p < 0.05.

2. Sex differences in the response to HFD intake in TgAPP and WT mice

2.1. Weight, weight gain and fat mass

At the onset of the study, males weighed more than females, but there was no effect of genotype in either sex.

Analyzing the total weight gain (Fig. 10A), there was a diet effect ($F_{(1,57)} = 54.7$; $p<0.0005$) and an interaction between sex and diet ($F_{(1,57)} = 4.8$; $p<0.04$) in the three-way ANOVA ($F_{(7,57)} = 10.4$; $p<0.0005$). Splitting data by sex and genotype, there were differences between LFD and HFD in APP males ($F_{(1,17)} = 28.5$; $p<0.0005$), WT females ($F_{(1,9)} = 34.2$; $p<0.0005$) and APP females ($F_{(1,17)} = 25.9$; $p<0.0005$), but not in WT males.

Final body weight (Fig.10B) was determined by diet ($F_{(1,48)} = 22.6$; $p<0.0001$), with an interaction between sex and diet ($F_{(1,48)} = 7.3$; $p<0.01$). On a LFD, males weighed more than females, with this reaching statistical significance only in WT mice. In males, HFD only increased final body weight in APP mice (split by genotype; $p<0.0002$). However, in females HFD increased body weight ($F_{(1,24)} = 22.8$; $p<0.001$) regardless of genotype ($F_{(7,48)} = 5.6$; $p<0.0001$).

The % VAT (Fig. 10C) was influenced by sex ($F_{(1,55)} = 71.6$; $p<0.0001$) and diet ($F_{(1,55)} = 9.6$; $p<0.004$), with an interaction between these two factors ($F_{(1,55)} = 19.4$; $p<0.0001$). Females had a higher % VAT than their male counterparts regardless of diet and genotype. HFD increased the percentage of VAT in both WT and APP females, while no effect was seen in males ($F_{(7,55)} = 16.3$; $p<0.0001$).

Sex ($F_{(1,55)} = 41.1$; $p<0.0001$) and diet ($F_{(1,55)} = 28.9$; $p<0.0001$) affected the % SCAT (Fig.10D). Females of all groups, except WT HFD, had a higher % SCAT than their corresponding male groups. HFD increased the % SCAT in all groups of both sexes ($F_{(7,55)} = 11.9$; $p<0.0001$), although this did not reach statistical significance in WT males.

There was an effect of sex on the % BAT with males having overall higher levels of BAT than females ($F_{(1,55)} = 16.2$; $p<0.001$) (Fig. 10E).

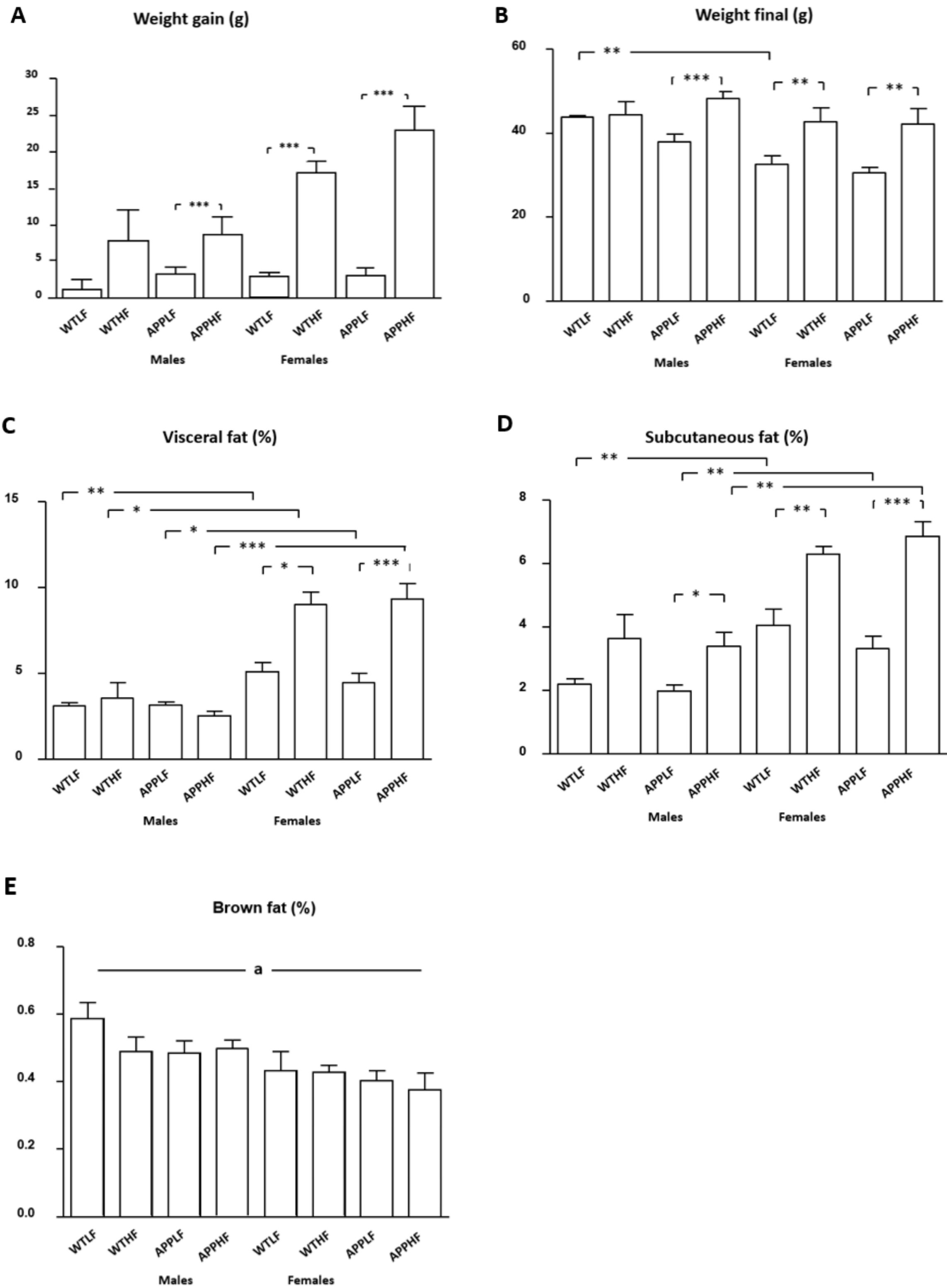

Figure 10. A: Total weight gain. B: Final weight. C: Percentage of visceral fat mass. D: Percentage of subcutaneous fat mass. E: Percentage of brown fat mass. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; a: overall effect of sex.

2.2. Circulating hormones and cytokines

There was an effect of diet on glycemia levels ($F_{(1,28)}$ = 20.0, p<0.0001; Fig. 11B). Glycemia was increased by HFD intake in all groups, but this only reached statistical significance in APP mice of both sexes ($F_{(7,28)}$ = 3.4, p<0.001).

Circulating insulin levels were determined by sex ($F_{(1,84)}$ = 8.4, p<0.006), with a significant ($F_{(1,84)}$ = 3.9; p = 0.05) effect of diet. Females had lower insulin levels than WT males, regardless of diet or genotype. HFD increased serum insulin levels in both genotypes and sexes ($F_{(7,84)}$ = 2.4, p<0.03) (Fig. 11C).

The homeostatic model assessment for insulin resistance (HOMA-IR) index was calculated from the circulating glucose and insulin levels using the following formula: $\frac{Insulin\left(\frac{IU}{L}\right)\cdot Glucose(mM)}{22.5}$. This index is used as a measure of insulin resistance and was introduced by Mathews and colleagues in 1985 (Matthews et al 1985). There was an interaction between sex and diet ($F_{(1,50)}$ = 5.0; p<0.004) on HOMA-IR. APP LFD males had higher values than APP LFD females and APP males on a HFD showed higher levels than their WT counterparts. HFD increased HOMA-IR significantly only in WT females ($F_{(7,50)}$ = 2.8; p<0.02), but showed a similar tendency in APP females and males (Fig. 11A).

Leptin levels were affected by sex ($F_{(1,87)}$ = 9.6, p<0.004) and genotype ($F_{(1,87)}$ = 6.1; p<0.02), with an interaction between sex and diet ($F_{(1,87)}$ = 6.2, p<0.02). Females had overall higher levels than males, and this was significant in mice on a HFD regardless of genotype (Fig. 11D). There was no significant effect of diet in males, while in females, APP mice on a HFD had higher levels of leptin than APP mice on a LFD and although this same tendency occurred in WT females, it did not reach significance ($F_{(7,87)}$ = 3,6; p<0.003).

Serum triglyceride levels (Fig. 11E) were dependent on genotype ($F_{(1,38)}$ = 6.5 p<0.02) and diet ($F_{(1,38)}$ = 4.8; p<0.04), with an interaction between sex and diet ($F_{(1,38)}$ = 6.3; p<0.02). In females, HFD significantly increased triglyceride levels in WT mice while this increase was not significant in APP females. APP males had higher triglyceride levels on a HFD than WT males. Furthermore, WT females had higher triglyceride levels on a HFD than WT males ($F_{(7,58)}$ = 3.0; p<0.02). There was no effect of any parameter on circulating NEFA levels (Fig. 11F).

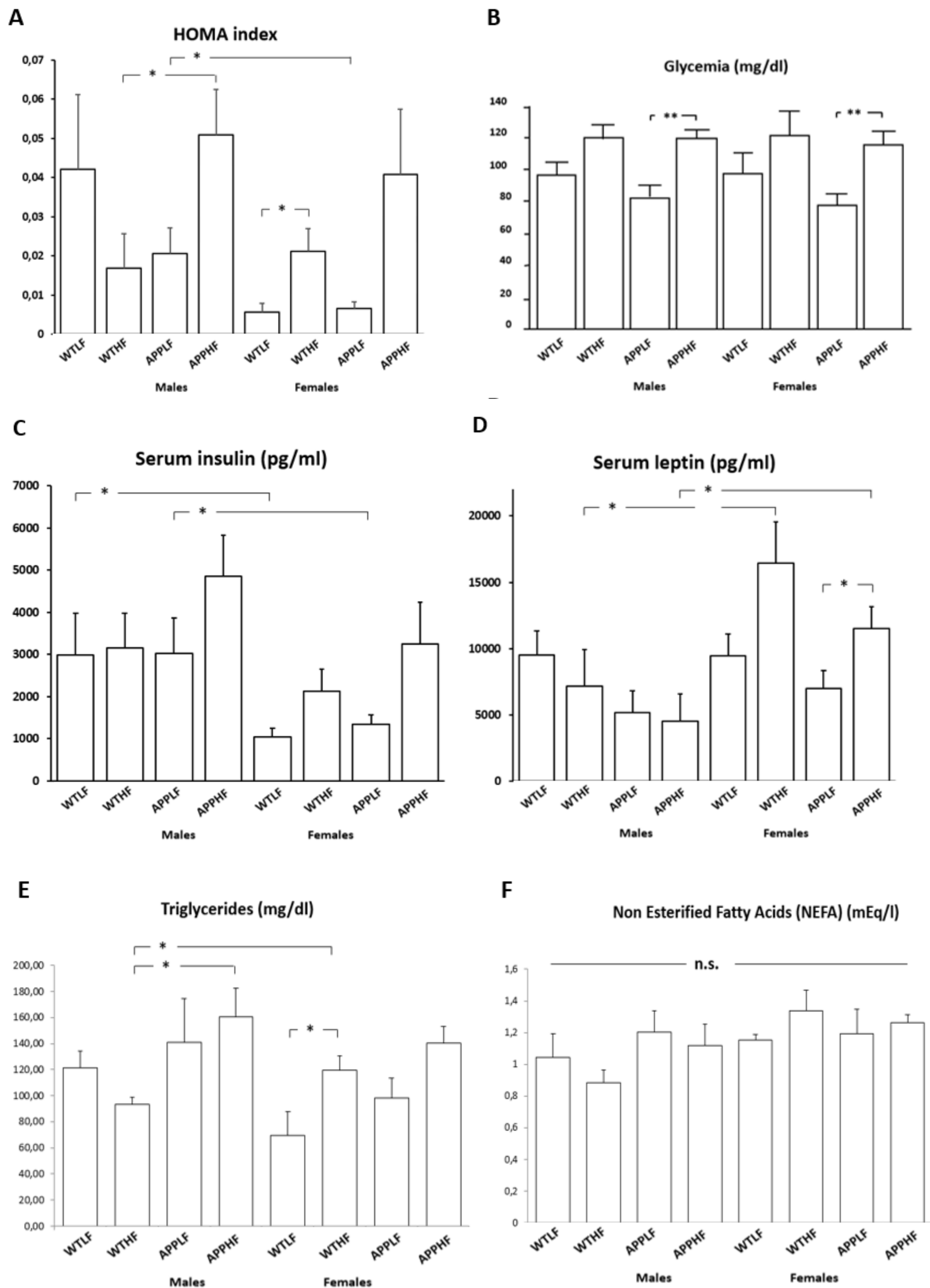

Figure 11. A: Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) index. B: Glycemia. C: Circulating insulin levels. D: Circulating leptin levels. E: Circulating triglycerides levels. F: Non Esterified Fatty Acids (NEFA) levels. n.s.: non-significant; * $p < 0.05$; ** $p < 0.01$.

There were no significant differences in MCP1 or PAI1 levels (Table 8). Circulating IL6 and TNFα levels were below the level of detection of the assay employed in samples of all experimental groups. Hence, these results are not reported.

	MCP-1	PAI-1
Males WT LFD	28.4 ± 6.8	5713.0 ± 2350.0
Males WT HFD	17.2 ± 4.2	9940.7 ± 4717.9
Males APP LFD	27.1 ± 6.1	9223.3 ± 3681.6
Males APP HFD	16.2 ± 3.4	9152.3 ± 2917.1
Females WT LFD	14.0 ± 6.0	1830.2 ± 371.7
Females WT HFD	24.2 ± 3.2	3972.4 ± 1051.5
Females APP LFD	21.8 ± 4.8	2864.1 ± 516.1
Females APP HFD	14.5 ± 2.6	5988.7 ± 2790.9

Table 8. Circulating levels of monocyte chemoattractant protein (MCP)-1 (pg/mL) and plasminogen activator inhibitor (PAI)-1 (pg/mL).

2.3. Hormone receptors and cytokine expression in adipose tissue

2.3.1. Visceral adipose tissue

There was an effect of sex on LepR expression in VAT with levels being higher in females (sex: $F_{(1,30)} = 7.6$, $p<0.01$) compared to males. There was also an interaction between sex and genotype ($F_{(1,30)} = 11.8$, $p<0.002$) and when split by sex, there was an effect of diet ($F_{(1,15)} = 5.7$, $p<0.03$). HFD induced the expression of this receptor reaching statistical significance in APP males ($F_{(3,15)} = 3.7$, $p<0.04$; Table 9). There was

also a sex effect on leptin expression levels, with females having overall higher levels than males.

Diet also affected IL-6 ($F_{(1,31)}$ = 16.6, p<0.0003) and TNF-α ($F_{(1,27)}$ = 7.2, p<0.02) mRNA levels, with HFD increasing the expression of both cytokines in VAT. The dietary effect on both cytokines reached statistical significance in APP females ($F_{(7,31)}$ = 3.0, p<0.02). However, the levels of IL-1β mRNA did not differ significantly between groups (Table 9).

	Lep	**LepR**	**IL6**	**TNFα**	**IL1β**
Males WT LFD	100.0 ± 14.7	100.0 ± 30.5	100.0 ± 17.3	100.0 ± 19.9	100.0 ± 8.6
Males WT HFD	119.8 ± 53.8	140.4 ± 37.4	663.3 ± 389.5	115.5 ± 38.0	154.0 ± 54.7
Males APP LFD	137.8 ± 61.1	153.7 ± 44.6	207.3 ± 80.9	63.1 ± 27.4	124.0 ± 48.0
Males APP HFD	116.1 ± 27.0	250.4 ± 58.1	591.2 ± 259.1	134.5 ± 33.6	501.2 ± 213.8
Females WT LFD	23.6 ± 12.8	305.9 ± 115.0	44.7 ± 9.7	54.8 ± 34.3	50.1 ± 7.6
Females WT HFD	180.2 ± 48.8	412.0 ± 93.7	808.3 ± 282.3	127.1 ± 39.6	284.0 ± 83.9
Females APP LFD	76.2 ± 16.8	187.5 ± 29.3	117.1 ± 28.6	37.5 ± 12.1	88.6 ± 32.3
Females APP HFD	272.8 ± 119.2	183.5 ± 62.3	476.1 ± 284.0	169.1 ± 66.9	323.4 ± 132.3

Table 9. Relative levels of mRNA in visceral adipose tissue in male and female wild-type (WT) and transgenic APP mice on a low fat diet (LFD) or a high fat diet (HFD) for 18 weeks. Data are expressed as a percentage of the male WT LFD group.

2.3.2. Subcutaneous adipose tissue

LepR mRNA levels on SCAT were sex dependent ($F_{(1,33)}$ = 7.6, p<0.01), but with females having overall higher levels than males (Table 10). There was also a sex

effect on leptin expression levels, with males having overall higher levels than females, this being significant in WT mice on a LFD and APP mice on a HFD.

In SCAT, IL-6 mRNA levels were sex ($F_{(1,36)}$ = 5.7, $p<0.03$) and diet ($F_{(1,36)}$ = 5.4, $p<0.03$) dependent. Males had higher IL-6 mRNA levels in SCAT when on a HFD ($F_{(7,36)}$ = 2.6, $p<0.03$) than females. When split by sex, males were found to be affected by diet ($F_{(1,18)}$ = 5.9, $p<0.03$), with HFD increasing IL-6 mRNA levels (Table 10).

There was an interaction between sex and diet ($F_{(1,34)}$ = 6.2, $p<0.02$) on the mRNA levels of TNF-α. When split by sex, the HFD significantly increased expression of this cytokine in males ($F_{(1,16)}$ = 34.9, $p<0.0001$), but had no significant effect in females (Table 10). There were no differences between experimental groups in the expression of IL-1β.

	Lep	**LepR**	**IL6**	**TNFα**	**IL1β**
Males WT LFD	100.0 ± 41.4	100.0 ± 31.4	100.0 ± 30.9	100.0 ± 14.6	100.0 ± 12.2
Males WT HFD	99.0 ± 32.8	142.0 ± 64.3	243.1 ± 41.7	246.1 ± 28.3	185.2 ± 69.9
Males APP LFD	61.0 ± 23.5	184.8 ± 126.4	189.2 ± 80.1	103.7 ± 28.6	223.2 ± 80.7
Males APP HFD	84.6 ± 15.4	82.2 ± 20.4	323.7 ± 49.7	240.6 ± 21.6	203.6 ± 32.7
Females WT LFD	9.4 ± 3.1 (a)	536.6 ± 209.7	120.1 ± 34.7	266.1 ± 116.3	426.4 ± 186.1
Females WT HFD	51.2 ± 17.2	245.2 ± 71.4	95.9 ± 18.5	203.6 ± 41.6	111.0 ± 23.6
Females APP LFD	27.4 ± 9.2	328.9 ± 124.7	105.6 ± 37.3	237.4 ± 74.5	217.6 ± 110.3
Females APP HFD	40.0 ± 15.6 (a)	239.4 ± 50.0	156.0 ± 65.7	165.3 ± 39.9	170.9 ± 78.5

Table 10. Levels of mRNA in subcutaneous adipose tissue in male and female wild-type (WT) and transgenic APP mice on a low fat diet (LFD) or a high fat diet (HFD) for 18 weeks. Data are expressed as a percentage of the male WT LFD group. a: sex effect.

2.4. Hypothalamic Amyloid-β levels

There was an interaction between the factors of sex and diet ($F_{(1,34)} = 9.5$, $p<0.006$) on the levels of amyloid-β peptide in the hypothalamus ($F_{(7,34)} = 2.5$, $p<0.05$; Fig. 12). When split by sex, there were differences within the male groups ($F_{(3,18)} = 7.0$, $p<0.005$), with an effect of diet ($F_{(1,18)} = 19.4$, $p<0.002$). Moreover, splitting data by genotype, there were significant differences in APP mice ($F_{(3,17)} = 4.6$, $p<0.03$), with an effect of diet ($F_{(1,17)} = 5.0$, $p<0.05$) and an interaction between sex and diet ($F_{(1,17)} = 8.2$, $p<0.02$). This effect was especially significant in APP males, which responded to a HFD with higher levels of the peptide (splitting by sex and genotype: $F_{(1,7)} = 30.8$, $p<0.002$). Furthermore, splitting the data by diet, there were also differences in LFD mice ($F_{(3,18)} = 3.6$, $p<0.05$), with an effect of sex ($F_{(1,18)} = 9.7$, $p<0.009$). These differences were mainly due to APP LFD females having higher peptide levels than APP LFD males (splitting by diet and genotype: $F_{(1,8)} = 9.7$, $p<0.05$).

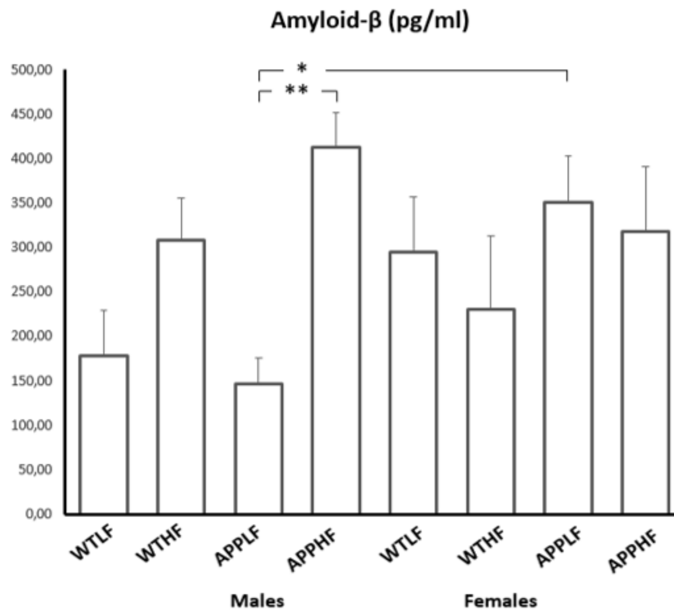

Figure 12. Peptide amyloid-β 42 levels (pg/mL) in the hypothalamus of male and female wild-type (WT) and transgenic APP mice on a low fat diet (LFD) or a high fat diet (HFD) for 18 weeks. * $p < 0.05$; ** $p < 0.01$.

2.5. Hypothalamic neuropeptide expression

Expression of the orexigenic neuropeptide AgRP was affected by sex ($F_{(1,31)} = 12.2$, $p<0.002$) and diet ($F_{(1,31)} = 12.9$, $p<0.001$) with an interaction between these two

factors ($F_{(1,31)} = 7.2$, $p<0.02$). When on a LFD, males had higher AgRP mRNA levels than females regardless of genotype ($F_{(7,31)} = 7.1$, $p<0.0001$; Fig. 13A). In males, there was a significant effect of diet ($F_{(1,16)} = 17.9$, $p<0.0005$), with mice on a HFD having lower AgRP mRNA levels than those on a LFD regardless of genotype ($F_{(3,16)} = 6.4$, $p<0.005$). In females there was no effect of either genotype or diet.

The orexigenic neuropeptide NPY was also affected by genotype ($F_{(1,33)} = 4.3$, $p<0.05$) and sex ($F_{(1,33)} = 4.7$, $p<0.04$), with an interaction between sex and diet ($F_{(1,33)} = 4.4$, $p<0.05$). When on a LFD, WT males had higher NPY mRNA levels than WT females and APP males higher than APP females ($F_{(7,32)} = 3.8$, $p<0.005$). These differences were not observed on a HFD. When split by sex, there was an effect of diet ($F_{(1,16)} = 10.1$, $p<0.005$), with both WT and APP males on a HFD having lower NPY mRNA levels than those on a LFD (Fig. 13B). There was no effect of genotype or diet in females.

The mRNA levels of the anorexigenic neuropeptide POMC were dependent on genotype ($F_{(1,30)} = 5.9$, $p<0.02$), with an interaction between genotype and sex ($F_{(1,30)} = 4.3$, $p<0.05$). In males, APP mice had lower POMC levels than WT mice ($F_{(1,14)} = 9.5$, $p<0.01$), regardless of diet ($F_{(7,30)} = 2.6$, $p<0.05$; Fig. 13C), while in females there was an effect of diet ($F_{(1,16)} = 5.5$, $p<0.05$) with HFD increasing POMC expression regardless of genotype.

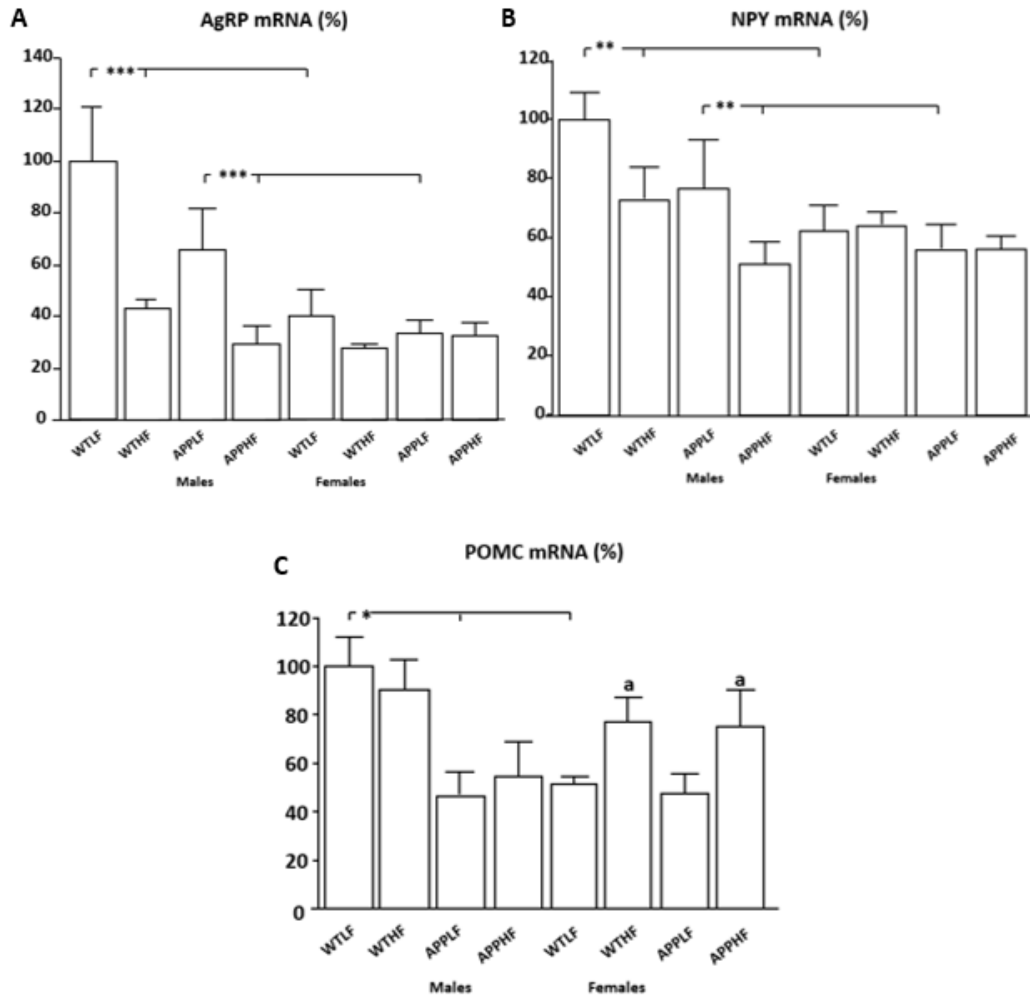

Figure 13. Hypothalamic mRNA levels of neuropeptides A) Agouti related protein (AgRP), B) Neuropeptide Y (NPY) and C) Pro-opiomelanocortin (POMC) on male and female wild-type (WT) and transgenic APP mice on a low fat diet (LFD) or a high fat diet (HFD) for 18 weeks. Data are expressed as a percentage of the male WT LFD group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

There was an overall effect of genotype ($F_{(1,33)} = 4.1$, $p = 0.05$) and diet ($F_{(1,33)} = 3.9$, $p = 0.05$) on hypothalamic IL-1β mRNA levels with APP mice tending to have lower levels than WT mice and with HFD tending to increase the levels of this cytokine (Table 11).

There was an interaction between genotype and sex on the expression of IL-6 in the hypothalamus ($F_{(1,33)} = 5.2$, $p<0.03$). When split by genotype, there was an effect of sex in WT mice ($F_{(1,13)} = 7.0$, $p<0.2$), with females having overall higher levels than males (Table 11). Hypothalamic TNF$-\alpha$ mRNA levels did not differ significantly between groups (Table 11). Expression of DDIT3 did not change.

	IL6	TNFα	IL1β	DDIT-3
Males WT LFD	100.0 ± 25.4	100.0 ± 28.3	100.0 ± 13.7	100.0 ± 7.6
Males WT HFD	162.1 ± 65.6	85.7 ± 35.7	60.7 ± 12.9	95.4 ± 10.3
Males APP LFD	122.0 ± 27.4	88.7 ± 32.3	66.2 ± 13.9	81.2 ± 10.2
Males APP HFD	190.7 ± 29.3	65.4 ± 13.2	61.3 ± 8.9	95.9 ± 2.3
Females WT LFD	185.8 ± 29.6	80.6 ± 23.8	76.2 ± 17.1	88.6 ± 5.8
Females WT HFD	149.4 ± 11.4	80.2 ± 13.8	107.4 ± 13.0	88.5 ± 8.4
Females APP LFD	288.0 ± 86.7	65.4 ± 13.2	66.8 ± 13.4	89.4 ± 10.1
Females APP HFD	181.0 ± 20.1	83.7 ± 22.0	112.3 ± 18.2	91.1 ± 3.4

Table 11. Relative mRNA levels of the cytokines interleukin (IL)-6, interleukin (IL)-1β and tumor necrosis factor (TNF)-α and transcription factor DNA damage inducible transcript (DDIT)-3 in the hypothalamus in male and female wild-type (WT) and transgenic APP mice on a low fat diet (LFD) or a high fat diet (HFD) for 18 weeks. Data are expressed as a percentage of the male WT LFD group.

2.6. Glial cell markers and inflammatory signaling in the hypothalamus

The glial markers GFAP and vimentin were not different between groups in either males or females. In contrast, although the levels of Iba1 were unaffected in females, in males HFD significantly increased this microglial marker ($F_{(1,22)} = 5.3$, $p<0.04$), with this effect being significant in APP males ($F_{(1,11)} = 6.2$, $p<0.04$; Fig.14).

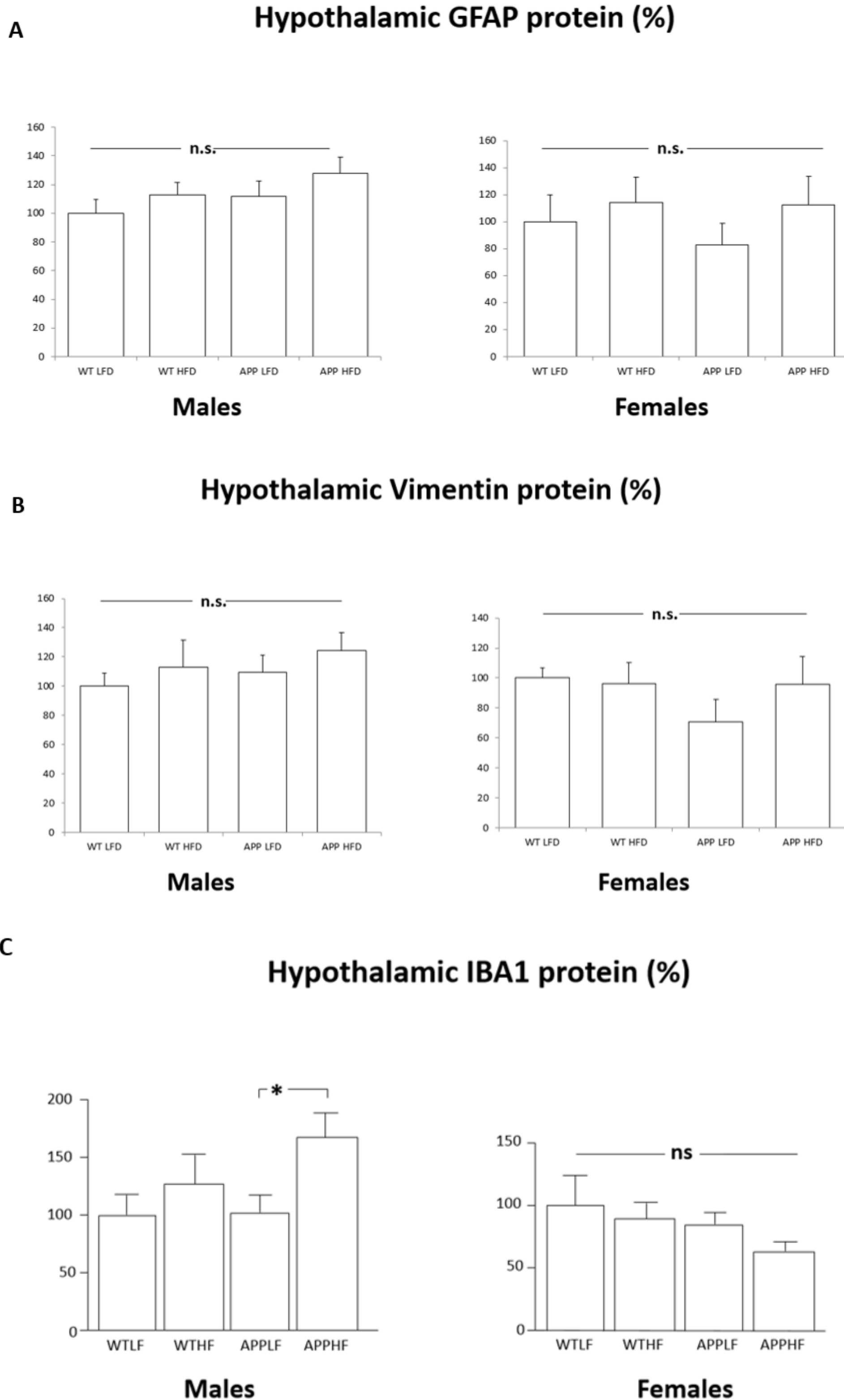

Figure 14. Relative protein quantities of A) glial fibrillary acidic protein (GFAP), B) Vimentin and C) ionized calcium binder adapter (IBA)-1 in male and female wild-type (WT) and transgenic APP mice on a low fat diet (LFD) or a high fat diet (HFD) for 18 weeks. Data are expressed as a percentage of the male WT LFD group. ns: non-significant; * $p < 0.05$.

No effect of genotype or diet was found on hypothalamic pIRS1, pIκBα, pNFκB (Fig. 15) or pJNK2 (p54) (Fig. 16C) levels in either sex. However, pJNK1 (p46) showed a tendency to increase with HFD in WT males, but this did not reach statistical significance (Fig. 16B).

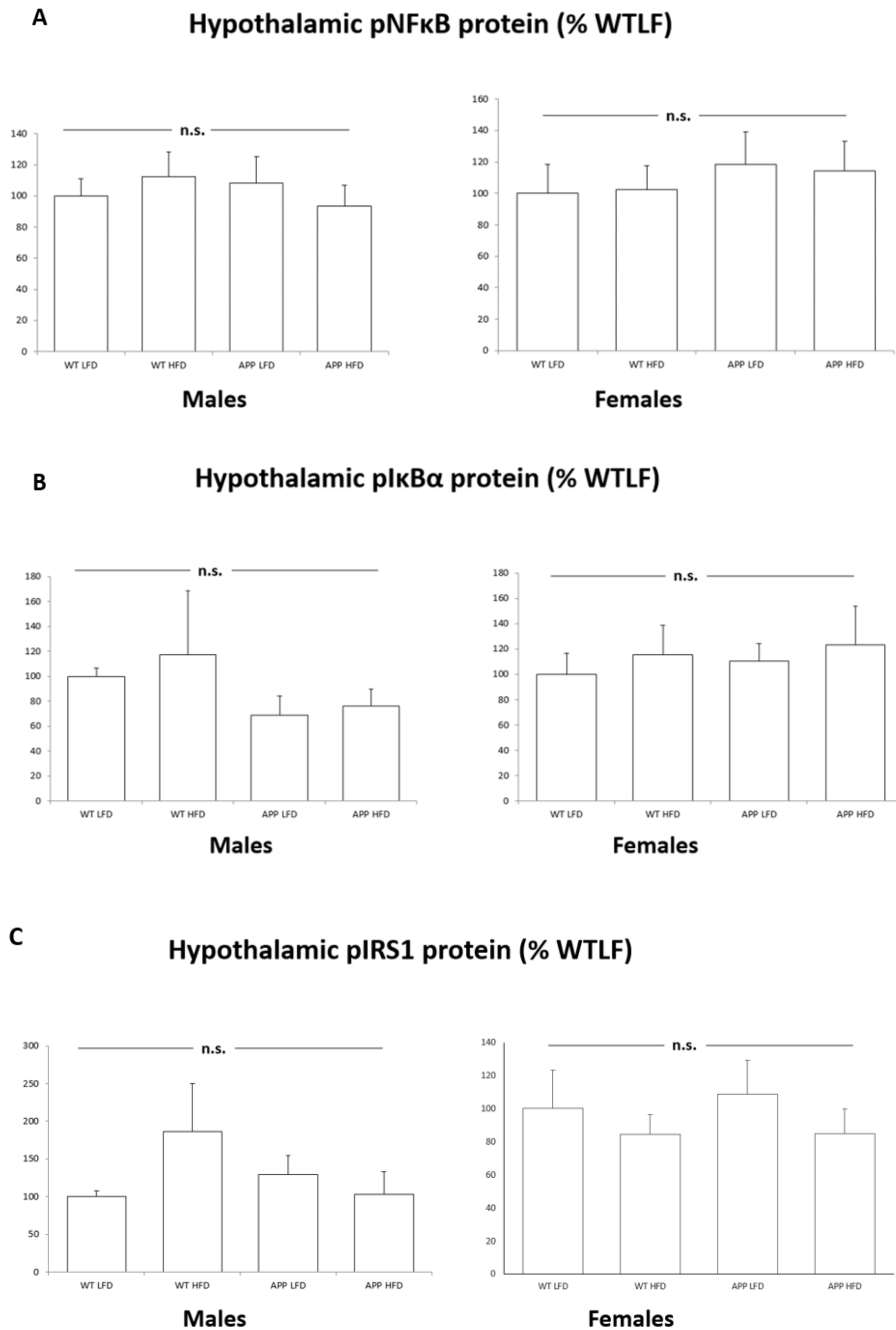

Figure 15. Hypothalamic protein levels of A) nuclear factor kappa B (NFκB), B) IκBα and C) phosphor-IRS-1 in male and female wild-type (WT) and transgenic APP mice on a low fat diet (LFD) or a high fat diet (HFD) for 18 weeks. Data are expressed as a percentage of the male WT LFD group. ns: non-significant.

In the hypothalamus there was an interaction between diet and genotype in males ($F_{(1,22)} = 4.8$, $p<0.05$; Fig. 16A) on pAKT levels and when separated by genotype, HFD induced a significant decrease in pAKT levels in the hypothalamus of APP males ($F_{(1,11)} = 7.2$, $p<0.03$), but not WT males. There was no effect on pAKT levels in females.

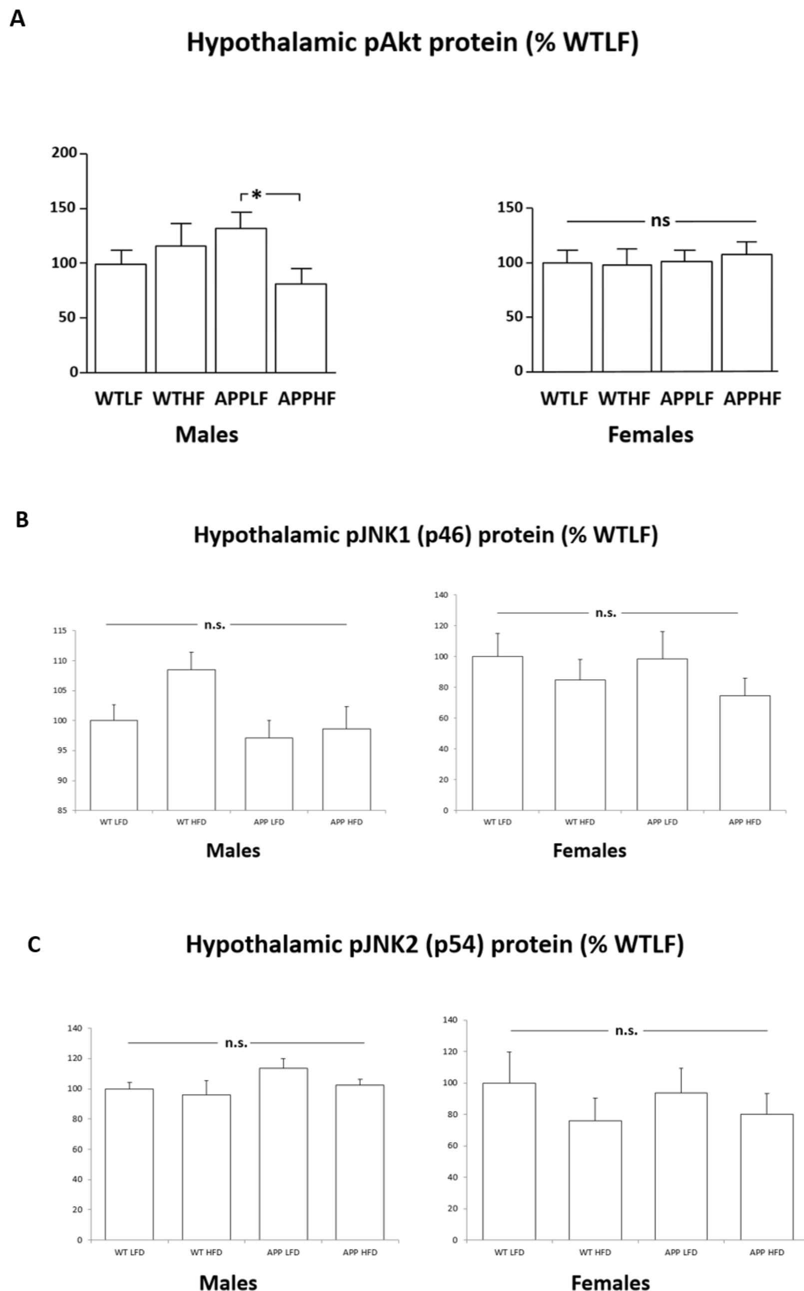

Figure 16. Hypothalamic protein levels of activated kinases A) p-Akt, B) pJNK1 and C) pJNK2 in male and female wild-type (WT) and transgenic APP mice on a low fat diet (LFD) or a high fat diet (HFD) for 18 weeks. Data are expressed as a percentage of the male WT LFD group. ns: non-significant; * $p < 0.05$.

3. Primary hypothalamic astrocyte cultures

3.1. Effects of palmitic acid and interaction with sex steroids

Based on previous results from our team (Frago et al 2017a) and others (Gupta et al 2012), which have found deleterious effects of PA on cultured astrocytes, we tested the protective effects of estradiol and testosterone on primary hypothalamic astrocyte cultures. An exploratory study on the effect of PA on cell stress and inflammation in male astrocytes showed that PA 0.1 M increased DDIT-3 and IL-6 expression (N=2) (data not shown), as previously reported in our laboratory (Argente-Arizón et al 2018).

3.1.1. Palmitic acid and 17β-estradiol

There were no significant differences between groups in the relative quantities of the protein levels of GFAP, SOD or Hsp-70 (Table 12). Nevertheless, Student's t tests showed that PA lowered Hsp-70 levels in male astrocytes ($t = 4.5$; $p<0.02$) and that the combined treatment of estradiol and PA compared to controls lowered protein levels of Hsp-70 in female astrocytes ($t=3.2$; $p<0.04$).

	GFAP	SOD	HSP-70
Males Control	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Males PA	118.0 ± 21.2	118.4 ± 18.4	61.3 ± 8.6
Males Estradiol	95.5 ± 7.8	105.4 ± 20.3	83.1 ± 13.3
Males Estradiol + PA	87.8 ± 9.3	105.0 ± 19.0	66.3 ± 24.7
Females Control	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Females PA	166.9 ± 73.3	105.6 ± 11.3	123.6 ± 24.0
Females Estradiol	95.7 ± 12.0	103.4 ± 23.6	73.1 ± 22.8
Females Estradiol + PA	504.4 ± 407.1	107.6 ± 19.3	63.3 ± 11.5

Table 12. Relative protein quantities of glial fibrillary acidic protein (GFAP), super-oxide dismutase (SOD) and heat-shock protein (Hsp)-70 in primary hypothalamic cultures of astrocytes treated with palmitic acid, 17β-estradiol or the combination of both. Data are expressed as a percentage of the male WT LFD group.

3.1.2. Palmitic acid and testosterone

We tested the effects of PA and testosterone on different key proteins from the steroidogenic pathway: aromatase, steroidogenic acute regulatory protein (StAR) and translocator protein TSPO (Fig. 17).

Aromatase expression was increased by PA and by T (10^{-10} M) in male astrocytes, but did not change in female astrocytes. There was an effect of PA (F; $p<0.01$) on StAR expression levels in male astrocytes, with groups treated with PA showing higher levels of StAR mRNA (F; $p<0.05$). In female astrocytes, there was also an effect of PA increasing StAR expression. In TSPO expression levels in male astrocytes, there was an effect of testosterone and an interaction between PA and testosterone. Testosterone produced a relative decrease at higher concentrations and the effect in combination with PA was dose-dependent. Treatment with testosterone (10^{-10} M) and PA decreased the expression of TSPO compared to testosterone alone and PA in any combination with testosterone (10^{-8} M or 10^{-10} M) also reduced TSPO mRNA levels in comparison with PA alone. In contrast, no effect of any of the treatments was found in TSPO expression levels in female astrocytes.

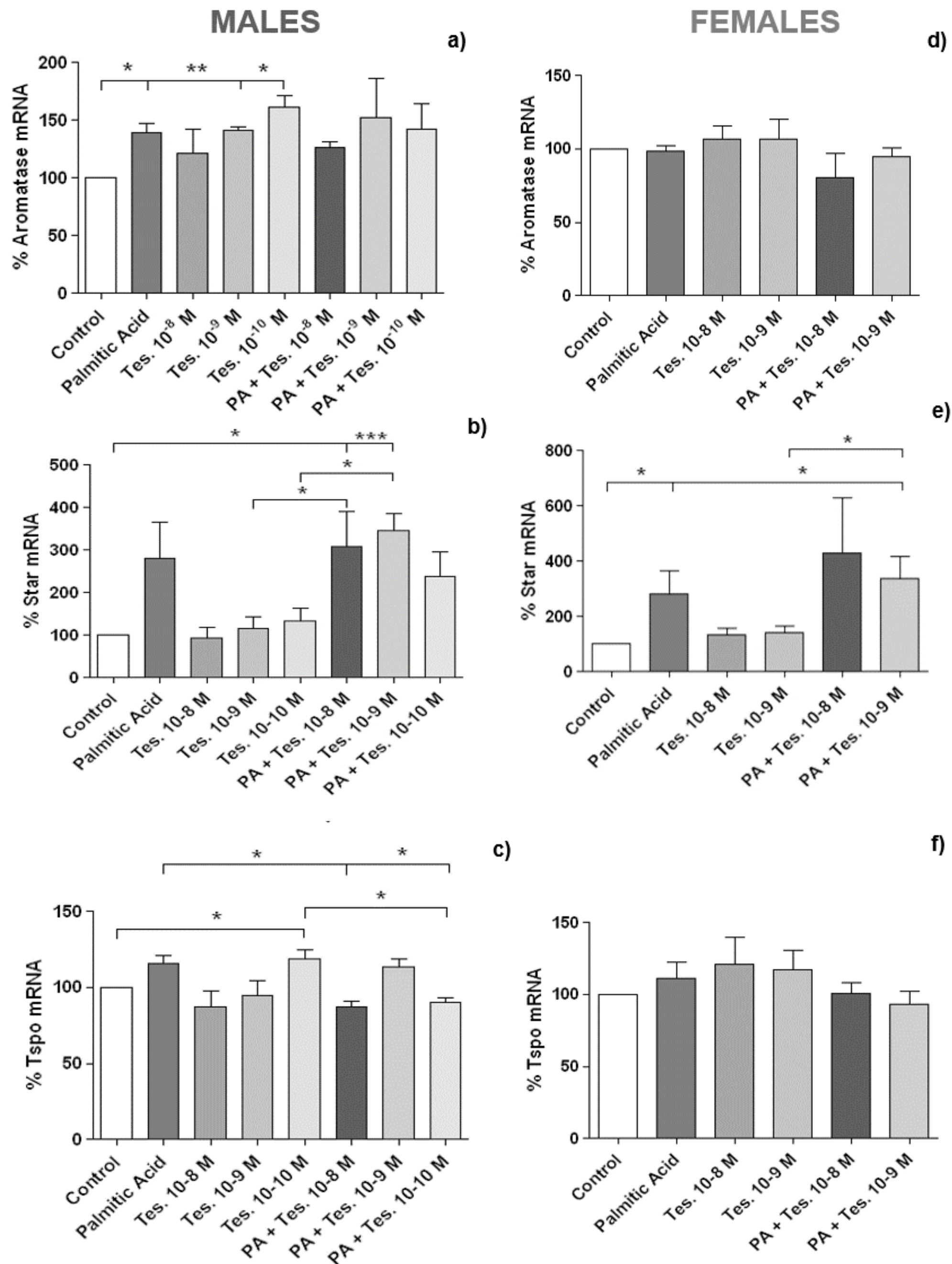

Figure 17. Levels of mRNA of proteins related to the steroidogenic pathway in primary hypothalamic cultures of astrocytes treated with palmitic acid, testosterone or the combination of both. Data are expressed as a percentage of the male WT LFD group. * $p < 0.05$; ** $p < 0.01$; $p < 0.001$.

3.2. Amyloid-β

To determine whether amyloid-β (1-40) modifies the number of hypothalamic astrocytes *in vitro,* crystal violet assays were performed. There were no significant

differences in the number of cells after treatment with any of the amyloid-β concentrations employed (Fig. 18A).

However, in male astrocytes, GFAP expression was significantly decreased by amyloid-β at both 5 and 20 μg/mL; ($F_{(5,18)}$=10.5; p<0.0005; Fig. 18B).

There were no differences between groups in DDIT-3 expression or IL-6 expression (data not shown).

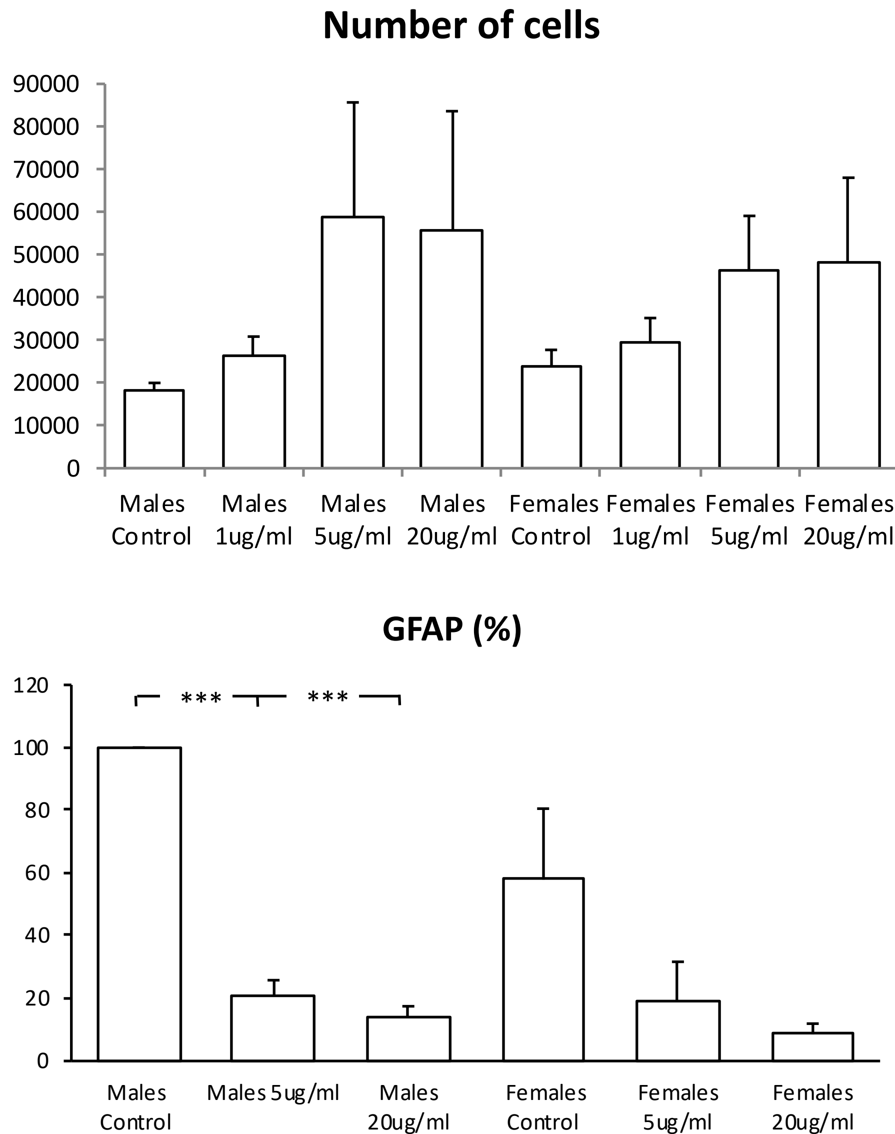

Figure 18. A: Number of cells after 24 h treatment with different concentrations of amyloid-β. B: Glial fibrillary acidic protein (GFAP) expression in hypothalamic cells after 24 h treatment with different concentration of amyloid-β. *** p < 0.0005.

DISCUSSION

It is well known that excess weight gain is influenced by various factors, including genetics, epigenetics, age, sex, dietary habits and lifestyle, with a clear interaction between these factors. Moreover, these factors, in addition to obesity itself, can also directly influence disease processes. Thus, our health and wellbeing depend on numerous factors and understanding the interaction between these factors could lead to improvement of overall health and help to implement individualized medicine protocols. The studies performed in this thesis were designed to shed some light on the differences between males and females in their response to a high-fat diet and to explore these differences at critical age points.

1. Sex differences in the effect of a short-term HFD in peripubertal mice

The results presented here suggest that prepubertal mice respond differently to HFD intake during this period when compared to the introduction of a HFD at other developmental phases. Indeed, as discussed below, the metabolic changes in the peripubertal/pubertal period might be tempered by the multiple hormonal changes that are occurring at this time.

1.1. Weight gain and food intake

There is a known sex difference in the weight gain and metabolic changes in response to poor dietary habits (Argente-Arizón et al 2018, Argente-Arizón et al 2016, Lenglos et al 2013, Morselli et al 2014, Palmer & Clegg 2015, Pradhan 2014). Differential responses to nutritional challenges according to age have also been observed (Argente-Arizón et al 2018, Argente-Arizón et al 2016, Aurich et al 2013). Indeed, neonatal overnutrition induced by reducing the litter size, and thus increasing the availability of maternal milk, results in increased weight gain of both males and females during the neonatal period; however, the long-term effects of this early nutritional manipulation were only seen in males (Argente-Arizón et al 2018). An interesting observation in the study by Argente-Arizon et al., (Argente-Arizón et al 2018) is that the effect of overnutrition is blunted during the peripubertal/pubertal period. Likewise, in other studies analyzing long-term effects of early hormonal or nutritional manipulation (Fuente-Martin et al 2012, Fuente-Martín et al 2012, Granado et al 2011, Mela et al 2012b) it is of note that the metabolic effects begin to appear after the peripubertal/pubertal period. As this is a period of rapid growth and changes in

hormonal status, it follows that the response to nutritional manipulations may also vary compared to other periods of life.

In our study of peripubertal mice, body weight, weight gain, energy intake and energy efficiency (grams gained per each calorie eaten) were significantly higher in males than in females. This is similar to that observed in adult mice (Palmer & Clegg 2015, Shi et al 2009). However, the HFD-induced body weight gain described in other studies (Lai et al 2014, Morselli et al 2014) was not observed here. Although our study employs a relatively acute exposure to HFD intake, other studies have reported significant weight gain in adult animals on one week or less of the HFD (Balland & Cowley 2017, Lai et al 2014, Snook et al 2017, Voigt et al 2013, Williams et al 2014b). Thus, it is possible that the lack of significant weight gain in the study reported here could be due to the developmental stage of these animals, with animals of this age being more resistant to the weight-increasing effect of a HFD.

The higher food intake in males is a common trait of mammals (Wade 1972), when the sex differences in body weight are not taken into consideration (Mauvais-Jarvis 2015). Indeed, if food intake is normalized to body weight this difference diminishes. Many studies have reported that, post-pubertally, male mice are heavier and gain more weight when on a HFD than females, which is determined both by sex hormones and genetic make-up/chromosomes (Chen et al 2012, Grove et al 2010, Palmer & Clegg 2015, Shi et al 2009). However, in our pre-pubertal study we found an increase in energy intake, but not in body weight, in both males and females on a HFD. In rodents, it has been reported that energy balance is regulated differently in each sex: females change their energy expenditure, whereas males modify their energy intake (Shi et al 2009). Interestingly, the increased expression of the anorexigenic neuropeptide POMC in HFD-fed females could be related to this strategy, raising their energy expenditure and, thus, protecting from weight gain on a longer-term HFD. It would indeed be of interest to determine energy expenditure in future studies to determine if pubertal animals are more capable of modulating energy expenditure in response to hypercaloric diets than adult animals.

Animals exposed to a palatable HFD normally have an initial period of hyperphagia before regulating their food intake to prevent an exaggerated increase in caloric intake (Benani et al 2012, Branch et al 2015, Buckman et al 2014, Priego et al 2009, Williams et al 2014b). In our study, all groups showed interest in the new diets, having a higher food intake during the first period (days 1-3) than during the second

period (days 4-8). Although basal food intake on the chow diet was not measured in these animals before changing their diet, it appears that mice increased their food intake regardless of the type of novel diet to which they were exposed. Indeed, most studies do not take into consideration that the introduction of a LFD can also influence study variables such as novelty. Thus, in accordance with short-term hyperphagia being induced when exposed to a novel and appetizing diet, during the second 3-day period energy intake was reduced in the four groups. However, there was a significant difference between HFD- and LFD- fed mice, the former ingesting more calories than the latter, especially in the females. The increase in body weight induced by a HFD has been shown to appear as soon as 3 days to 1 week of HFD in adult male mice (Balland & Cowley 2017, Snook et al 2017, Voigt et al 2013, Williams et al 2014b). Venancio and colleagues found that, in prepubertal female mice, one week of HFD increased body weight (Venancio et al 2017). On the other hand, it has been reported that feeding rats from weaning onward with a HFD does not cause weight gain during the prepubertal or pubertal period, but at around PND50 weight gain became significantly increased in males (Sánchez-Garrido et al 2013). In the study by Sanchez-Garrido, females were only studied until PND40, but with no weight increase at this time. Likewise, our group previously reported similar results in a study with rats, where males started to gain more weight on a HFD at PND65 (Mela et al 2012b). In this study by Mela et al, HFD-fed females gained more weight than controls during puberty, but this increase disappeared in young adults, reappearing later around PND100. Although rats and mice are not necessarily similar, it seems that age, as well as sex, is an important factor in rodents when they are exposed to a dietary challenge. This is important to consider when comparing results from various studies, as well as when designing experimental interventions. But more importantly, this suggests that more studies are needed to understand the susceptibilities and long-term effects of poor dietary habits not only in experimental animals, but also in humans.

1.2. Circulating levels of hormones, glucose and lipids

Serum leptin levels have been reported to increase in response to HFD intake (De Souza et al 2005, Mela et al 2012a, Priego et al 2009, Sánchez-Garrido et al 2013, Williams et al 2014b), as observed here. This is a rapid effect of diet, probably due to increased expression in adipose tissue, as fat mass does not change as rapidly. Williams

and colleagues previously reported that leptin levels in blood increase shortly after HFD intake, then decrease and rise again when adipose tissue is increased (Williams et al 2014b). This rapid rise in leptin levels is considered to be an acute anorexic metabolic signal, whereas the chronic rise in levels of this hormone is associated with the more long-term effects of leptin on body mass regulation. Moreover, as seen in adult mice and rats (Argente-Arizón et al 2016, Grove et al 2010, Mela et al 2012b, Mulet et al 2003, Priego et al 2009, Wang et al 2017), leptin levels in our study were higher in males than in females, even at this peripubertal age.

Circulating glucose levels were higher in HFD-fed male mice. There are sex differences in fasting glycemia in rodents and humans (Færch et al 2010, Garg et al 2011), with males having higher levels than females. Also, HFD-induced changes in glucose metabolism are more frequent in male than in female rodents (Amengual-Cladera et al 2012, Argente-Arizón et al 2016, Cecconello et al 2015, Mela et al 2012b, Morford & Mauvais-Jarvis 2016). In our study, the increase in glycemia is not accompanied by an increase in body weight, which suggests a direct effect of diet on this parameter. Circulating insulin levels were also higher in males than in females, being significantly different when on a HFD. Glucose tolerance in male C57BL/6 mice is lower than in females (Toye et al 2005) and, accordingly, we found that the HOMA-IR index is higher in males than females in our study. A low glucose tolerance correlates with a high HOMA-IR index. Other dietary challenges, like intake of a high-fructose diet (Sharma et al 2015) and neonatal overnutrition (Argente-Arizón et al 2016) also reflect this sex difference in glucose tolerance.

Circulating NEFA and triglycerides have been associated with HFD and obesity (Björntorp et al 1969, Brunzell 2007, Opie & Walfish 1963, Yuan et al 2007), but our results reflect no changes in either of these factors. As for NEFA levels, a study by Williams and colleagues found similar results in adult mice fed HFD for one week (Williams et al 2014b). However, in their study they found a decrease of triglyceride levels in animals fed a HFD, which we did not see in our mice, possibly because of their young age. Adipose tissue has a higher proliferative capacity in children than in adults (Martos-Moreno et al 2013), which could account for some of these differences. Our data support this, as we have found an increased number of adipocytes per area in VAT of HFD-fed animals, especially in females, which could be a signal of proliferation. These cells did not show, however, signs of hypertrophy, and this could be due to the short term of the HFD. It is important to note that it is prolonged hypertrophy of adipose

tissue that is associated to metabolic complications; hence, increased proliferative capacity of these cells in younger individuals, thus requiring less lipid storage/adipocyte, may be involved in increased protection against secondary complications of obesity.

1.3.Hypothalamic inflammation and gliosis

Central inflammation is commonly reported after long-term HFD intake. This involves an increase of hypothalamic cytokine and IκBKβ expression, JNK phosphorylation (De Souza et al 2005) and ER stress, which is linked to the pro-inflammatory action of IκBKβ (Özcan et al 2004, Zhang et al 2008). The outcome of HFD-induced leptin and insulin resistance is also linked to central inflammation (Milanski et al 2009, Özcan et al 2004, Zhang et al 2008). Thaler and colleagues studied hypothalamic inflammation at different time points after introduction of HFD in adult mice. They found increased inflammatory signals as early as 1 day after the begining of the diet, but levels of inflammatory markers descended after 1 week. At this point, GFAP levels increased, but normalized after 2 weeks of diet. On the other hand, the number and size of microglial cells were increased from 3 days of diet (Thaler et al 2012b). Others have found an increase in hypothalamic astrogliosis after 10 days of HFD in adult male mice (Balland & Cowley 2017). This inflammatory/astroglial response has been interpreted as a biphasic reaction, with an early protective step that can be followed by a chronic and possibly damaging response.

Our peripubertal mice showed a sex difference in hypothalamic glial and inflammatory markers, even at this early age. However, in response to HFD, only hypothalamic IL-6 expression in females was significantly increased, which could imply a protective effect (Astiz et al 2017). IL-6 is also linked to control of metabolism (Wallenius et al 2002), that is also reported to be different between males and females (Señarís et al 2011). Thus, at this early age one week of HFD did not induce apparent signs of hypothalamic inflammation or gliosis. This could be due to the fact that these animals did not gain sufficent weight, but the diet itself has also been shown to induce glial changes and increase inflammatory markers (Clegg et al 2011, Doerner et al 2016, Gao et al 2014, Thaler et al 2012a). Thus, again this lack of response could indicate increased resistance to the effects of HFD at this early age. Likewise, we did not detect

any differences in the hippocampus or the cerebellum in the expression of inflammatory/gliosis markers.

Further studies are necessary to determine whether sex steroid levels could account for the differences between the sexes observed here. Moreover, it would be interesting to study the long-term metabolic effects of the dietary modifications employed in this experiment. Indeed, the period of pubertal transition includes multiple hormonal changes (Plant 2015), as well as structural modifications in the hypothalamus (Clasadonte & Prevot 2017), suggesting that it may be more suceptible to the impact of dietary challenges. If so, this could have important implications in the general population and the fight against obesity.

2. Interaction of sex and diet in a mouse model of Alzheimer's Disease

Alzheimer's Disease is one of the most prevalent neurodegenerative diseases in humans. The increasing life expectancy is, at least, one of the causes behind the high number of diagnoses. The incidence of AD is higher in women than in men (Li & Singh 2014) and it has been suggested that the levels of sex steroids could be one of the underlying causes (Manly et al 2000, Pike et al 2009). As women have a longer lifespan and AD frequently manifests at older ages, a logical outcome would be an increased number of women suffering this disease. However, taking this into account, there is still a higher prevalence of AD in women than in men. Thus, age-related loss of estrogens is the main suspect for this sex difference, a hypothesis that has been supported by animal models (Carroll et al 2007). Interestingly, it has also been suggested that the role of sex steroids in early brain development and sexual differentiation could underlie the greater risk of females to suffer AD (Carroll et al 2010). Another factor to take into account when talking about AD is that the aged brain does not respond to damage as effectively as the young brain (Patterson et al 2015, Rodríguez-Arellano et al 2016).

In the last decade, obesity has been suggested as a risk factor for the development of AD and other neurodegenerative diseases (Emmerzaal et al 2015, Hayden et al 2006, Moser & Pike 2016). One of the probable causes of this incresed risk is the central inflammation associated with obesity, which could be involved in cognitive decline (Freeman et al 2014, Moser & Pike 2016, Puig et al 2015).

Transgenic mice over-expressing amyloid precursor protein (APP) have been widely used to study the pathogenicity of Alzheimer's Disease; however, few studies

have addressed how metabolism is affected as a result of increased central APP expression. Here we not only analyzed how metabolism is affected in this mouse model in response to excess fat intake, but also how males and females are differently affected.

2.1. Sex differences in weight gain and fat distribution

As expected, WT male mice were heavier than WT females at the beginning of the study when all were on a normal chow diet. All groups, except WT males, increased their weight gain when fed a HFD. In fact, female mice gained significantly more weight when subjected to the HFD than males, reaching a similar weight to the males by the end of the study. This is in contrast to what has been reported in many rodent studies, where males are generally more susceptible to the adverse effects of a HFD (Argente-Arizón et al 2016, Dorfman et al 2017, Mela et al 2012b). However, it is possible that the age at which the mice were subjected to the dietary challenge is of importance here. Indeed, the mice used in this study were older than those normally subjected to dietary challenges. While most studies use young adult mice, our mice were already middle aged at the onset of the experiment. Dr. Kristen L. Zuloaga and her team found that when they used middle aged mice sex differences in diet-induced weight gain and glucose impairment were opposite to that found when they used juvenile mice (Salinero et al 2018). This result is also supported by previous studies (Nishikawa et al 2007). Thus, once again, studies analyzing the sex differences in metabolic responses to poor dietary habits must take into consideration the age of the animals subjected to the diet.

Here we found that females had a higher percentage of both SCAT and VAT than males. Moreover, HFD-fed females had significantly larger fat depots than their LFD-fed counterparts. In contrast, males on a HFD showed a higher percentage of subcutaneous fat, but not visceral fat, and this was only significant in transgenic mice. Sex differences in fat distribution are well studied in humans: women tend to accumulate fat in subcutaneous depots, while men store fat in visceral depots (White & Tchoukalova 2014). However, this distribution becomes less sex-dependant as women become postmenopausal (Brown & Clegg 2010, Meyer et al 2011, Shi & Clegg 2009), suggesting the implication of sex steroids, or the loss of sex steroids. In mice, this sex difference is not so clear, but there are studies that have found a tendency in male mice to accumulate a higher percentage of intraabdominal or visceral fat than females (Grove

et al 2010, Maejima et al 2017). However, these studies were performed in young mice, in which sex differences in body weight, as stated above, are opposite to that in older mice. Moreover, some studies have found that middle age to old female mice present a higher adiposity than males (Nishikawa et al 2007, Salinero et al 2018), and also with an increase in their visceral fat (Grove et al 2010, Krishna et al 2016). Here, the differential gain in fat mass is consistent with weight gain in females. Brown adipose tissue was more abundant in males than in females, which is consistent with previous studies that show BAT increasing at a higher rate than body weight in males, but not in females (Valle et al 2008).

2.2. Sex differences in circulating levels of hormones, nutrients and peripheral inflammation.

Circulating triglyceride levels in female WT mice are more elevated on a HFD, while in males on a HFD triglyceride concentrations tend to be lower than in males on a LFD. This is consistent with the female strategy of fat storage, thus converting free fatty acids into triglycerides. On the other hand, APP males on a HFD have higher levels of triglycerides than WT males. Serum NEFA levels in WT mice follow a similar tendency between males and females, although in this case both genotypes respond similarly.

There were few changes in circulating inflammatory markers in response to HFD in either sex or genotype. Obesity is often reported to be a state of chronic inflammation (Gregor & Hotamisligil 2011, Medzhitov 2008), however, circulating cytokines are not always found to be increased. Once again this lack of change could be due to the age of the animals used in this study, as age is also associated with increased inflammatory processes. Surprisingly, circulating MCP-1 levels were lowered with HFD in males, but increased with HFD in females. The increase observed in females is a normal inflammatory response to a HFD, but the decrease seen in males cannot be easily explained. A possible explanation is that males are aging faster and the route involving MCP-1 has been impaired in males but not yet in females.

Pro-inflammatory cytokine expression in the subcutaneous fat pad was increased in HFD males, but did not change in females. On the other hand, there was a strong diet effect in cytokine expression in the visceral fat pad in both sexes. A similar result has been also found by Vasconcelos and colleagues in middle-aged rats (Vasconcelos et al 2018).

Circulating levels of leptin were only increased with HFD in female mice, but not in males. Likewise, leptin expression in VAT was increased with HFD only in females, with these changes being normally seen in response to chronic HFD intake. Leptin signaling has been found to be impaired in middle-aged male mice (Jacobson 2002) and here it appears that the normal increase in leptin in response to a HFD is also impaired. The absence of an increase in circulating leptin with HFD in males could be partially explained by the fact that they did not gain VAT. The fact that males did not have an increase in VAT, leptin expression or circulating leptin levels could indicate that during aging males lose the normal (or that seen in younger animals) metabolic response to this dietary challenge earlier than females.

Circulating levels of insulin were also increased with HFD only in females, but not in males. On the other hand, HFD increased glycemia in both sexes. This indicates that females are still capable of producing higher levels of insulin in response to the HFD-induced rise in glycemia. However, as their glycemia levels rose, this might indicate that they are becoming insulin resistant on the HFD. This was not observed in WT males, where glycemia rose, but not insulin levels. This could indicate that the male mice are no longer capable of up-regulating insulin secretion to reduce glycemia levels. These observations again point to differential metabolic aging between the sexes.

2.3. Sex differences in hypothalamic neuropeptides, central inflammation and gliosis.

Hypothalamic expression of the orexigenic neuropeptides NPY and AgRP is decreased in HFD-fed males, while HFD-fed females presented an increase in the expression of the orexigenic peptide POMC. This sex difference was also found in our previous study in peripubertal mice and may be related with the different strategies that males and females follow in response to a dietary challenge. Thus, some of the responses to HFD intake seen in young mice are not found in older mice, while others are completely different. Indeed, age-related disruption of metabolic homeostasis could underlie some of the age-related differences reported here and in the literature; moreover, this effect is most likely a combination of the aging effects on metabolic hormones with age-associated increases in inflammatory processes, including in the CNS, and increased cell stress. These processes use similar intracellular signaling mechanisms (Cai & Liu 2012) and with continued activation of these signaling

mechanisms, protective processes may become less effective, leading to development of secondary complications.

Hypothalamic inflammation and gliosis have been reported in response to chronic HFD intake and weight gain (Thaler et al 2012a). Here we found no indication of these processes in the WT mice of either sex, despite significant weight gain. There are some possible explanations that should be taken into consideration. First, as neuroinflammation has been associated with aging (Franceschi & Campisi 2014), the baseline central inflammation may already be elevated and not significantly changed by the dietary challenge. Leptin has also been reported to participate in the activation of glial cells (Gao et al 2014, Garcia-Caceres et al 2011), and the reported leptinemia in conjunction with HFD and weight gain was observed here only in females. Also, the levels of active JNK1 in WT males were higher in the HFD group. However, as other inflammatory markers are not increased, we cannot conclude that this elevation indicates hypothalamic inflammation. Moreover, morphological analysis of astrocytes and microglia could shed more light on affectation of these glial cells and their activational state.

2.4. Differences between WT and APP mice in response to a HFD.

Although TgAPP mice cannot be directly compared to what occurs in AD, they are considered useful for the study of preclinical AD. Indeed, middle-age mice, as used here, are too young to develop all signs of AD, but they already show some cognitive decline (unpublished data). In our study, WT and APP mice present similar responses to the HFD. However, in males, amyloid β hypothalamic levels are higher in the HFD than in the LFD groups, especially in APP males. Such increase is possibly related to the metabolization of circulating fats, as this peptide is involved in lipid processing (Czeczor & McGee 2017). As it has been found in other studies (Puig et al 2017), our results suggest that there is an interaction between APP over-expression and energy homeostasis. This is more apparent in APP males on a HFD, which gain more weight and more % SCAT, have less POMC expression, increase their HOMA index and Iba-1 protein levels in the hypothalamus, decrease p-Akt protein levels in the hypothalamus and do not change hypothalamic levels of JNK1, in comparison with WT males. In contrast, APP females on the HFD show lower circulating MCP-1 levels and less LepR expression in VAT than WT females. The mechanisms underlying these differences

between WT and APP genotypes cannot be deduced from the studies reported here, but these results indicate that alterations in APP expression do affect metabolism and that males appear to be more metabolically affected than females by APP over-expression. Indeed, the increase in HOMA index with HFD in APP males and not in WT males could be related to the interaction between APP and insulin signaling pathways (Chan et al 2015, Kulas et al 2018, Yamamoto et al 2018). On the other hand, the higher levels of Iba-1 in the hypothalamus could be a sign of microgliosis triggered by HFD in an aged, and possibly already inflamed, hypothalamus. The changes in metabolism, as well as the signs of cognitive decline, may become more apparent with further aging, as occurs in humans.

Interaction between diet, aging and the development of neurodegenerative diseases has only begun to be exposed (Moser & Pike 2016). The number of concurring factors at the onset of a disease makes it difficult to find the critical ones that could be targeted for its prevention. Either way, the avoidance of a diet high in saturated fats is a good choice, as there is little we can do about our aging and sex, which is a very complex subject.

3. Effects of palmitic acid and steroid hormones on hypothalamic astrocytes

Palmitic acid is a saturated fatty acid frequently present in food. It affects energy homeostasis at the central level by inducing the production of pro-inflammatory cytokines, causing ER stress and leptin resistance (Gregor & Hotamisligil 2011, Gupta et al 2012, Karaskov et al 2006, Milanski et al 2009, Morselli et al 2014). In astrocytes, palmitic acid has been reported to increase GFAP expression and switch them to their reactive form (Horvath et al 2010, Hsuchou et al 2009). Estradiol has been shown to have anti-inflammatory actions in the brain (Arevalo et al 2010, Cerciat et al 2010, Guo et al 2012). In a similar way, testosterone has also been suggested as a neuroprotective factor (Cai et al 2017, Gürer et al 2015, Kurth et al 2014). Thus, we tested the protective actions of these sex steroids in hypothalamic astrocytes exposed to a moderate concentration of palmitic acid.

3.1. Palmitic acid and 17β- estradiol

The lack of effect of PA on the protein levels of the ER stress markers Hsp-70 and SOD and the gliosis marker GFAP made it impossible to test the neuroprotective effect of estradiol on these factors. However, a result similar to what we observed in Hsp-70 was also found by Frago and colleagues (Frago et al 2017b) in primary hippocampal astrocyte cultures. It is possible that hypothalamic astrocytes do not respond in the same way as astrocytes from the hippocampus, but further studies are necessary to determine if estradiol has effects on these factors.

3.2. Palmitic acid and testosterone

Our results suggest that palmitic acid interacts with the steroidogenic pathway in male astrocytes. Aromatase, the enzyme that converts testosterone to estradiol, is expressed in astrocytes in response to an injury (Azcoitia et al 2003, García-Segura et al 1999). TSPO and StAR are widely expressed proteins involved in the transport of cholesterol to the mitochondrial inner membrane, one of the first steps of steroidogenesis. Their expression is also enhanced after a brain injury (Chen & Guilarte 2008, Gehlert et al 1997). Considering that steroids can exert neuroprotective effects, the increase of these factors could be an attempt to protect the brain against injuries.

These results in hypothalamic astrocytes are in contrast with those found by Frago and colleagues in hippocampal astrocytes, with PA reducing the expression of TSPO and aromatase in male astrocytes and increasing StAR in astrocytes of both sexes (Frago et al 2017b). The reason for these contradictory results may be the different brain regions from where the astrocytes have been collected and/or the fact that we used a concentration of PA five times lower than that in the previous study. In either way, our astrocytes appear to increase steroidogenesis as a neuroprotective measure in response to PA. The fact that testosterone did not modify this response suggests that the central production of steroids may be independent from circulating steroid levels

4. Effects of amyloid-β on hypothalamic astrocytes

The aggregation of peptide amyloid-β and the consequent formation of plaques is characteristic of Alzheimer's disease. Amyloid plaques are found in the hypothalamus, among other regions, (Braak & Braak 1991) and this brain area has been

suggested to be affected at early stages of the disease (Hiller & Ishii 2018, Ishii & Iadecola 2015). The implication of astrocytes in the progression of AD has been, and continues to be studied, with these cells being involved in the clearance of amyloid-β (Wyss-Coray et al 2003) and with increased astrogliosis in the areas surrounding amyloid plaques (Olabarria et al 2010). However, we found no increase in the expression of inflammatory or gliosis factors, as well as no decrease in the number of cells after 24 hours of treatment with different concentrations of the peptide, which had been previously treated to promote its aggregation. It is possible that amyloid-β may require more than 24 hours in contact with hypothalamic astrocytes to trigger a protective response. Results from our laboratory show that, when primary astrocytes from the hippocampus are treated, instead of those from the hypothalamus, amyloid-β produces a reduction in the number of cells and an increased expression of inflammatory markers. Indeed, there is growing evidence that points to the differences between astrocytes from distinct brain regions (Bayraktar et al 2014, Morel et al 2017).

Taken together, the results reported here indicate that both age and sex are of great importance in determining the response to different dietary challenges. Lifestyle and dietary intake influence the aging process, but the aging process also influences our response to diet. Genetics must also be taken into this equation as it is clear that our genetic background, including our sex, has an important impact on our metabolism, as well as on our aging process. Hence, it is clear that individuals will respond differently to similar diets and treatment protocols, emphasizing that the trend towards implementation of personalized medicine may help to slow the obesity pandemic.

CONCLUSIONS

1. **Age is an important determinant in the response to dietary challenges, with the peripubertal/pubertal period being a specific developmental time period that deserves more attention regarding analysis of the detrimental responses to poor dietary habits that may be specific to this special period.**

2. **Males and females respond differently to dietary challenges and these differential responses are age dependant. Thus, these factors must be taken into consideration in experimental design and interpretation, and possibly as well clinical assessment and treatment.**

3. **The development of secondary complications due to poor nutrition/excess weight gain is sex and age dependant.**

4. **Astrocytes participate in neuroprotection and this process can differ in males and females. The sex differences in production of sex steroids by these glial cells may participate in this process.**

CONCLUSIONES

1. La edad es un factor determinante en la respuesta a una dieta alta en grasas, siendo la pubertad un período del desarrollo que merece especial atención en cuanto al análisis de las respuestas a los malos hábitos alimentarios que pueden tener lugar en este estadio.

2. Machos y hembras responden de manera diferente a las alteraciones nutricionales y estas respuestas también dependen de la edad. Por tanto, estos factores han de ser tenidos en cuenta en la interpretación y diseño de los experimentos, así como, posiblemente, en la evaluación y tratamiento clínicos.

3. El desarrollo de complicaciones secundarias derivadas de una mala alimentación o un exceso de peso depende del sexo y de la edad.

4. Los astrocitos participan en la neuroprotección y este proceso puede ser distinto en función del sexo. Las diferencias entre machos y hembras en la producción de esteroides sexuales por parte de estas células pueden estar participando en este proceso.

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