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SEX AND AGE AS DETERMINING FACTORS IN THE PERIPHERAL AND CENTRAL RESPONSES TO NEONATAL OVERNUTRITION. POSSIBLE IMPLICATIONS OF HYPOTHALAMIC ASTROCYTES IN THE SEXUAL DIMORPHIC RESPONSE TO METABOLIC CHALLENGES

Memoria para optar al grado de Doctor con Mención Internacional de la licenciada

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RESUMEN

Resumen

Diversos estudios sugieren que la respuesta a los desafíos metabólicos es diferente entre machos y hembras. Como consecuencia de un aumento en el consumo de calorías, se producen diferencias en la ganancia de peso, así como en el aumento y distribución de tejido adiposo, pero, además, la respuesta al sobrepeso y obesidad es diferente entre los sexos. Sin embargo, aunque se sabe que las alteraciones nutricionales tempranas pueden tener efectos a largo plazo en el metabolismo, las diferentes respuestas entre los sexos son insuficientemente conocidas hasta la fecha. Junto a ello, estudios recientes indican que los astrocitos hipotalámicos están implicados en el control neuroendocrino del metabolismo, así como en el desarrollo de las complicaciones secundarias asociadas a la obesidad. No obstante, se desconoce en gran medida si los astrocitos hipotalámicos de machos y hembras responden de manera diferente a los desafíos metabólicos.

El objetivo de esta Tesis fue determinar si la sobrenutrición neonatal como resultado de una reducción en el número de crías por madre durante el período de lactancia, produce efectos similares en el crecimiento y metabolismo de ratas macho y hembra a lo largo del desarrollo. Es bien sabido que los esteroides sexuales desempeñan una función crítica en las diferencias post-puberales entre machos y hembras en su metabolismo y distribución y función del tejido adiposo. No obstante, los efectos metabólicos de las variaciones en los esteroides sexuales durante la etapa neonatal deben ser estudiados en mayor profundidad. Por esta razón, empleamos también un modelo de androgenización neonatal en hembras para determinar sus efectos a corto y largo plazo en el metabolismo. Igualmente, la respuesta hipotalámica y, concretamente de los astrocitos hipotalámicos a la sobrenutrición neonatal, fue estudiada en ambos sexos.

Por tanto, en el estudio de sobrenutrición neonatal en machos y hembras, se analizó el perfil metabólico, las respuestas inflamatorias y distribución y función del tejido adiposo, así como posibles alteraciones en los astrocitos

hipotalámicos. Para ahondar en el estudio de las posibles diferencias entre los sexos específicamente en los astrocitos hipotalámicos, se estudió la respuesta *in vitro* de estas células gliales a determinados ácidos grasos, así como el posible efecto protector de los estrógenos a la inflamación producida por los ácidos grasos.

Los resultados de estos estudios indican la diferente respuesta entre los sexos a alteraciones nutricionales en el periodo neonatal, siendo esta respuesta diferente también, en función de la edad o etapa del desarrollo en la que se encuentran. Asimismo, las modificaciones en los esteroides sexuales en el neonato, podrían contribuir a las diferencias metabólicas observadas a largo plazo a consecuencia de la sobrenutrición neonatal, así como en los diferentes efectos vistos en machos y hembras. Aún más, los astrocitos hipotalámicos procedentes de ratas macho y hembra, responden de manera diferente a la sobrenutrición neonatal, así como a los tratamientos *in vitro* con ácidos grasos y estrógenos.

Por consiguiente, los astrocitos hipotalámicos no solamente estarían implicados en la fisiología y fisiopatología del control neuroendocrino del metabolismo, sino que también podrían estar participando en las respuestas a los cambios nutricionales tempranos y en las respuestas sexualmente dimórficas del sistema metabólico.

PALABRAS CLAVE: dimorfismo sexual, sobrenutrición neonatal, desarrollo, hipotálamo, astrocitos.

SUMMARY

Summary

The responses to metabolic challenges are suggested to be different in males and females. Not only is there a difference in weight gain and adipose tissue accumulation and distribution in response to increased energy intake, but the secondary complications in response to being overweight or obese also differ between the sexes. However, less is known about the differential sex response to early nutritional changes that can affect long-term metabolism. In addition, recent studies have indicated that hypothalamic astrocytes are involved in the neuroendocrine control of metabolism, as well as the development of secondary complications in response to obesity. However, whether hypothalamic astrocytes from male and female rats respond differently to metabolic challenges remains to be thoroughly analyzed.

The aim of this thesis was to determine if neonatal overnutrition as a consequence of the reduction in the number of pups per dam during nursing produces similar effects on growth and metabolism in male and female rats throughout development. Moreover, although sex steroids are clearly involved in the post-pubertal differences between males and females in metabolism and adipose tissue distribution and function, less is known regarding the effect of changes in the neonatal sex steroid environment on metabolism. Hence, we employed a model of neonatal androgenization of females to determine how this affected both short-term and long-term metabolism.

Metabolic profile, inflammatory responses and adipose tissue distribution and function, as well as possible alterations in hypothalamic astrocytes were analyzed in these studies. To further explore the sex differences in hypothalamic astrocytes, the response of these glial cells to fatty acids and the possible protective effect of estrogens against fatty acid-induced inflammation were analyzed *in vitro* employing primary hypothalamic astrocyte cultures.

The results of these studies indicate that the response to nutritional disturbances during the neonatal period differ between the sexes and are age

dependent. In addition to changes in insulin and leptin levels, differences in the sex steroid environment during the neonatal stage could participate in the distinct long-term metabolic responses to neonatal overnutrition and the differential effects in males and females. We also demonstrate that hypothalamic astrocytes of male and female rats respond differently to neonatal overnutrition, as well as to fatty acid and hormonal treatments *in vitro*.

Thus, hypothalamic astrocytes are not only involved in the physiological and physiopathological neuroendocrine control of metabolism, but they may also participate in developmental responses to early nutritional changes and sexually dimorphic metabolic responses.

KEY WORDS: sexual dimorphism, neonatal overnutrition, development, hypothalamus, astrocytes.

ABREVIATIONS

Ab/Am: Antibiotic/Antimitotic.

ACS: acetyl-coA synthase

AgRP: Agouti related protein.

Akt: Protein kinase B.

AMPK: AMP-protein kinase

ANOVA: Analysis of variance.

ApoE: Apolipoprotein E.

ARC: Arcuate nucleus of the hypothalamus.

ABC: Avidin-biotin complex.

BDNF: Brain derived neurotrophic factor.

B-EP: Beta-endorphin.

BMI: Body mass index.

BSA: Bovine serum albumin.

BL: Body length.

BW: Body weight.

CART: Cocaine and amphetamine-related transcript.

CCK: Cholescystokinin

cDNA: Complementary DNA.

cm: centimeters.

CNS: Central nervous system.

CPT-1: Carnitine palmitoyl transferase 1.

CREB: cAMP response element-binding protein.

CVDE: Cristal violet dye elution.

DMEM/F12: Dulbecco's modified Eagle's medium: Nutrient mixture F-12.

DMH: Dorsomedial nucleus of the hypothalamus.

ELISA: Enzyme-linked immunosorbent assay.

ER: Endoplasmic reticulum stress.

ER: Estrogen receptors.

ER-α: Estrogen receptor alpha.

ER-β: Estrogen receptor beta.

ERK: Extracellular signaling-regulated kinase.

FA: Fatty acid.

FBS: Fetal bovine serum.

FFAs: Free fatty acids.

FI: Food intake.

FOXO: forkhead-O transcription factor.

g: Grams.

h: Hours.

GA: glutaraldehyde

GABA: Gamma-aminobutyric acid.

Galc: Galactocerebroside.

GFAP: Glial fibrillary acidic protein.

GLUT: Glucose transporter.

GLP-1: Glucagon-like peptide 1.

HFD: High fat diet.

Iba1: Ionized calcium-binding adapter molecule 1.

ICV: Intra-cerebral ventricular.

IKBKB: Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta.

JNK: c-Jun N-terminal kinase.

IL-1β: Interleukin 1 beta.

IL-10: Interleukin 10.

IL-6: Interleukin 6.

IP: Intraperitoneal.

IR: Insulin receptor.

JNKs: c-Jun N-terminal kinases

kDa: Kilodalton.

KO: Knock-out.

LCFAs: Long chain fatty acids.

LCFA-CoA: Long chain fatty acyl-CoA.

LHA: Lateral hypothalamic area.

MAPK: Mitogen-activated protein kinase.

MC3-R: Melanocortin receptor 3.

MC4-R: Melanocortin receptor 4.

mM: Mili molar.

mRNA: Messenger ribonucleic acid.

MSH: Melanocyte-stimulating hormone.

$NaNO_2$: Sodium nitrate.

NEDD: Ethylenediamine dihydrochloride.

NF-κB: Nuclear factor of kappa-light-chain-enhancer of activated B cells.

NFKBIA: Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha.

NIH: National Institute of Health.

NO_2: Nitrites.

NO_3: Nitrates.

NON: Neonatal overnutrition.

NPY: Neuropeptide Y.

NS: Not significant.

OA: Oleic acid.

Ob-R: Leptin receptor.

O/N: Overnight.

OGTT: Oral glucose tolerance test.

OVN: Over-nutrition

P38: P38 mitogen activated protein kinase.

$P70^{s6k}$: P70 S6 kinase.

PA: Palmitic acid

PB: Phosphate buffer.

PBS: Phosphate buffered saline.

PFA: Paraformaldehyde.

PI3/Akt: Phosphoinositide-3 kinase pathway

PND: Postnatal day.

POMC: Proopiomelanocortin.

PPAR-α: Proliferator activated receptor α.

PPAR-γ: Proliferator activated receptor γ.

PVN: Paraventricular nucleus of the hypothalamus.

RPM: Revolutions per minute.

RNA: Ribonucleic acid

RT: Room temperature.

RT-PCR: Real time-polymerase chain reaction**.**

S100A4: S100 calcium binding protein A4.

SCAT: Subcutaneous adipose tissue.

SCG: Superior cervical ganglion.

Ser727: Serine-727.

STAT3: Signal transducer and activator of transcription 3.

STAT5: Signal transducer and activator of transcription 5.

T2D: Type 2 diabetes.

TG: Triglycerides.

TNF-α: Tumor necrosis factor alpha.

Tyr705: Tyrosine-705

µg: Micrograms.

µl: Microliters.

µm: Micra

VAT: Visceral adipose tissue.

VCl3: Vanadium (III) chloride.

WAT: White adipose tissue.

WB: Western blotting.

WHO: World Health Organization.

WT: Wild type

Experimental groups:

AF: Androgenized females.

Ct: Control group.

Ct 0 h: Control group after 0 hours.

Ct 24 h: Control group after 24 hours.

F: Females.

FL12: Females from control litters.

FL4: Females from small litters.

L12: Litters of 12 pups per dam (control litter).

L4: Litters of 4 pups per dam (small litter).

M: Males.

ML12: Males from control litters.

ML4: Males from small litters.

OA: Oleic acid treatment.

PA: Palmitic acid treatment.

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I. INTRODUCTION

1. Obesity and energy balance

Obesity and overweight are major public health concerns throughout the industrialized world as both conditions are risk factors for the development of additional diseases such as type 2 diabetes (T2D), cardiovascular diseases or cancer **(Lustig and Weiss, 2008; Daniels, 2009; Anteneh et al., 2015)**. Obesity can be defined as an abnormal/excessive fat accumulation **(Garrow, 1988; Ofei, 2005)** that usually leads to an increase in total body weight. Body mass index (BMI) is used as a simple indirect measurement of obesity. A person's BMI is calculated as their weight (in kilograms) divided by the square of his or her height (in meters). When the BMI is 30 or higher, a person is considered to be obese **(WHO, 1995)**.

It is well accepted that the accumulation of excessive adiposity is due to an imbalance between energy intake and energy expenditure over time **(Garrow, 1988)**, although the specific causes of the dramatic rise in this epidemic are still under debate. The genetic make-up of each individual, as well as the hormonal and nutritional environment and psychosocial factors are all involved in the etiology of this metabolic imbalance **(Dong et al., 2003; Mendieta-Zeron et al., 2008; Argente, 2011; Stein et al., 2011)**. Therefore, development of obesity in an individual depends on their genetic predisposition, environmental factors such as lack of exercise and/or poor dietary habits, and the interaction between these factors **(Figure 1)**.

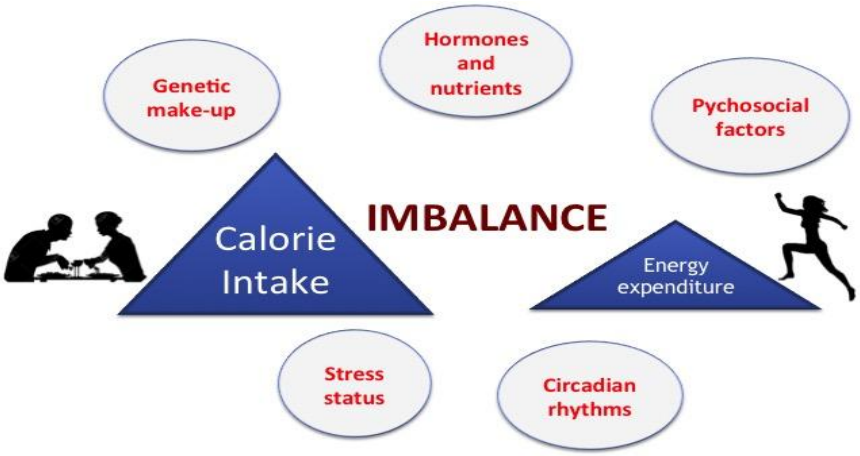


Figure 1. Overweight and obesity occur due to an imbalance between calorie intake and energy expenditure over time, with the genetic make-up of each individual, hormonal and nutritional environment, psychosocial factors, stress and circadian rhythms influencing this imbalance.

The prevalence of obesity began to increase at an alarming rate at the end of the 20th century and continues to rise, with no signs of abating. Indeed, obesity is considered to be the most important epidemic of the 21st century, with at least 2.8 million people dying each year as a result of being overweight or obese, according to the World Health Organization (WHO) **(WHO, 2015)**. Obesity affects all ages, including children and adolescents, particularly in developing countries **(Ogden et al., 2007; Prevention, 2013)**. Obesity during infancy is currently one of the most serious public health concerns and its rates have doubled over the past 20 years, while in adolescents the rate has tripled **(Ford et al., 2014)**. Obese children will likely continue to be obese as adults and thus, will have an increased probability of developing obesity-associated comorbidities in the future including T2D, cardiovascular diseases, insulin resistance syndrome, arthritis, dyslipidemias, infertility or psychological disorders among others **(Horvath, 2005; Despres, 2007; Gundogan et al., 2009; Martos-Moreno and Argente, 2011; Gungor, 2014)**. The dramatic rise in the incidence of obesity, in concert with the huge economic impact attributed to its associated disorders, makes obesity a primary health concern and emphasizes the need for a deeper understanding of its etiology and the identification of new targets to fight against this disease.

2. Early life nutritional influences on metabolism

Poor nutrition, the lack of physical activity and the interaction of these two factors with an individual's genetic background, predispose a person to weight gain, due to excessive fat accumulation, that finally leads to them being overweight and/or obesity. In addition, animal models and human studies have demonstrated that early environmental influences, including nutritional factors, can have effects on adult metabolic homeostasis **(Roseboom et al., 2006; Barker, 2007; Levin, 2008; Fuente-Martin et al., 2012b; Spencer, 2012; Liu et al., 2013; Collden et al., 2015; Long et al., 2015)**. Thus, not only does a person's current lifestyle and genetic background make them susceptible to becoming obese, but an increased propensity to develop obesity can begin *in utero* or during the early postnatal stages, such as during lactation. Indeed, an excessive nutrient supply and rapid weight gain in early life

can have long-term effects, even when the individual or animal is exposed to a normal diet during postnatal life **(Barker, 2007; Levin, 2008; Fuente-Martin et al., 2012b; Spencer, 2012; Liu et al., 2013; Collden et al., 2015; Long et al., 2015)**.

Maternal health and nutrition during gestation, as well as fetal exposure to stress, abnormal levels of hormones and environmental toxins, have all been shown to disrupt metabolic homeostasis in later life **(Roseboom et al., 2006; Barker, 2007; Shin et al., 2012; Regnier et al., 2015)**. For example, maternal obesity and diabetes frequently complicate pregnancy **(Anna et al., 2008)**, altering maternal metabolism and thus potentially disrupting metabolic homeostasis in the offspring **(Yu et al., 2013)**. In rodents, the initial postnatal weeks are critical for the formation of hypothalamic neurocircuits, with approximately postnatal day (PND) 20 being the end of this critical period for hypothalamic differentiation and development **(Bouret et al., 2004a; Grayson et al., 2006)**. In contrast, this sensitive period in humans predominantly occurs *in utero* during the third trimester of gestation **(Clancy et al., 2007)**.

Although the majority of studies analyzing the long-term effects of early environmental influences on metabolism have been performed in males, here is evidence that the metabolic responses to these early disturbances may be different in females **(Garcia-Caceres et al., 2010; Fuente-Martin et al., 2012c; Mela et al., 2012; Reynolds et al., 2015b)**. In addition, these responses may also be age dependent, for example appearing in some occasions only in later adulthood **(Habbout et al., 2013; Granado et al., 2014)**.

Animal studies have illustrated that modification of the litter size in which animals are reared can have long term-effects on their metabolic homeostasis. Diverse studies show that when the number of pups per dam is reduced, weight gain is promoted, while the opposite occurs when the number of pups per dam is greatly enlarged during nursing **(Frolkis et al., 1993; Plagemann et al., 1999c; Cunha et al., 2009; Fuente-Martin et al., 2012b; Fuente-Martin et al., 2012c; Kayser et al., 2015)**. Some studies have attributed this weight gain to overfeeding as a result of increased food

availability during suckling that engenders increased energy intake **(Fiorotto et al., 1991; Fink et al., 2001; Cunha et al., 2009).** The consequences of this increased food intake and weight gain are involved in the observed long-term metabolic effects. Other factors, such as maternal nurturing, can also participate in the long-term effects due to litter size modification **(Connor et al., 2012; Reynolds et al., 2014; Segovia et al., 2014)**. In rodents, these early nutritional modifications during nursing are taking place precisely when neuronal circuits are developing and the central nervous system (CNS) is still maturating. Hence, the perinatal nutritional environment could have a decisive influence on the development of neuronal circuits involved in energy balance and metabolic homeostasis.

Under normal physiological conditions, at the same time that hypothalamic neurocircuits are maturing, there is a surge in circulating leptin levels in neonatal rodents. Leptin levels increase progressively by PND 5, and peak between PND 9 and PND 10 remaining elevated until approximately day 13 **(Ahima et al., 1998; Delahaye et al., 2008)**. This increase in leptin is crucial for the outgrowth of key neuronal projections in the hypothalamus. Indeed, either a deficiency or an excess in leptin levels during the perinatal period can cause important changes in hypothalamic circuits involved in metabolic homeostasis, appetite and food intake behavior, possibly predisposing an individual to obesity and metabolic disorders later in life **(Bouret and Simerly, 2007)**. Importantly, not only are neurons affected during this critical period but also, astrocytes proliferate and expand within the brain, with leptin suggested to promote astrogenesis during this early age **(Ahima et al., 1999; Rottkamp et al., 2015)**.

It should be taken into consideration that unlike rodents, the increase in leptin in humans takes place *in utero*, with leptin concentrations increasing dramatically in fetal arterial cord blood by the end of gestation, with newborns with intrauterine growth retardation reported to have lower leptin levels than those with normal growth **(Jaquet et al., 1998)**. Although experiments have shown that leptin appears to be secreted preferentially to the maternal side of the placenta, there is also secretion to the fetal side **(Linnemann et al., 2000; Hoggard et**

al., 2001). The rat placenta is reported to be permeable to leptin **(Smith and Waddell, 2003)**. Both human **(Masuzaki et al., 1997)** and murine **(Hoggard et al., 1997)** placenta express leptin and the leptin receptor **(Hoggard et al., 1997; Masuzaki et al., 1997; Linnemann et al., 2000)**, suggesting that this hormone has an important role during gestation. In addition, during lactation, leptin can pass from the mother to the offspring through the maternal milk **(Teixeira et al., 2002).**

Although insulin does not cross the placenta or in very low amounts, glucose from the mother does, and this glucose acts on the fetal pancreas to stimulate insulin secretion **(Buse et al., 1962; Freinkel et al., 1979).** Studies have demonstrated that obesity in the mother, produced by gestational diabetes or an inadequate diet, is associated with hyperinsulinemia, hyperleptinemia, hyperphagia and increased adiposity in the offspring **(Catalano et al., 2003; Harvey et al., 2007; Samuelsson et al., 2008; Nivoit et al., 2009)**. Thus, the long-term effects on the offspring's metabolism may occur not only through modifications in leptin, but also insulin levels, as this hormone also influences hypothalamic development **(Vogt et al., 2014).**

A well-known and important example of the dramatic influence of early nutritional factors on human offspring is what occurred during the Second World War, when pregnant women suffered from famine. The offspring were affected in different ways depending on which trimester of gestation the mother suffered starvation. Several studies have illustrated that the offspring from mothers affected during the first trimester of pregnancy, had a higher risk for the development of a wider spectrum of metabolic diseases than babies born from mothers affected either in the second or third trimester of pregnancy. However, the offspring from mothers experiencing starvation during the second or third trimester had a higher risk to develop metabolic diseases than a baby born from a healthy mother **(Kyle and Pichard, 2006; Lumey et al., 2011; Roseboom et al., 2011).**

Therefore, nutritional modifications produced during both the intrauterine and the perinatal period should be considered as potential risk factors for metabolic alterations in the adult. Part of these alterations is most likely due to

modifications in hormonal signals at critical moments in the development of hypothalamic metabolic circuits.

3. Differences between the sexes in obesity and metabolism

The importance of taking into consideration the differences between males and females has been recently highlighted by the National Institutes of Health (NIH) in the United States, as the inclusion of both sexes in clinical trials and basic research has become mandatory **(Clayton and Collins, 2014; Health, 2014)**. Indeed, understanding how physiological processes and the responses to metabolic challenges differ between males and females will allow more accurate treatments according to sex.

3.1 Sex differences in the propensity to become obese, adipose tissue physiology and the development of secondary complications

The relation between obesity and associated complications and mortality is well established **(Gundogan et al., 2009; Martos-Moreno and Argente, 2011)**. However, the link between obesity and its secondary complications has been more thoroughly studied in men than in women. Studies indicate that the propensity to become obese is different between the sexes and that this is largely owing to the direct influence of sex steroids. Indeed, estrogens protect from body weight gain, fat mass accumulation and obesity complications **(Stubbins et al., 2012; Dakin et al., 2015)**. They also have inhibitory effects on appetite and increase energy expenditure by increasing the activity of anorexigenic signals and exert the opposite effect on orexigenic signals **(Tarttelin and Gorski, 1971; Clegg et al., 2006; Clegg et al., 2007; Shen et al., 2010; Zhu et al., 2013)**. Declining androgens levels have also been associated with obesity and metabolic disorders such as T2D **(Dhindsa et al., 2010; Grossmann, 2011)**. Differences between men and women in the propensity to become obese also reside in their dissimilarities regarding adipose tissue distribution.

Adipose tissue is an active endocrine organ in constant communication with the CNS by releasing numerous adipokines and responding to hormones and

neuropeptides from the CNS **(Galic et al., 2010; Rosen and Spiegelman, 2014)**. Expansion of fat mass can occur by hypertrophy of the existing adipocytes or by hyperplasia due to recruitment of new preadipocytes **(Jo et al., 2009)**. Some studies relate hyperplasia with subcutaneous adipose tissue (SCAT) and hypertrophy with visceral adipose tissue (VAT) **(Wang et al., 2013)**. An increase in the size of adipocytes is associated with higher metabolic risk **(Lundgren et al., 2007)**. Estrogens are suggested to favor hyperplasia, increasing adipocyte progenitor cells and facilitating vascular supply to adipose tissue **(Gealekman et al., 2011; Kim et al., 2014b)**.

Males and females differ not only as to the amount and distribution of adipose tissue **(Kotani et al., 1994)**, but also with regard to adipose tissue metabolism and function **(Havel et al., 1996; Tran et al., 2008; Macotela et al., 2009)**. It is clear that differences in circulating sex steroids play a critical role in some of the observed sexual dimorphisms, but not all metabolic dissimilarities can be explained by differences in gonadal hormone levels. Indeed, some sex differences in metabolism are present even before puberty **(Taylor et al., 2010)**, although they become more evident in adulthood. In addition, as reported by Chen et al, the number of X chromosomes also influences adiposity in mice **(Chen et al., 2012)**.

Unlike males, which reach their maximum levels of fat accumulation at puberty, females have a continuous increase in fat mass throughout development, with women having higher levels of adiposity compared to men throughout lifespan **(Gallagher et al., 1996)**. Females tend to accumulate more SCAT in the gluteal and femoral zones, acquiring what is often referred to as the typical *pear shape* **(Figure 2)**. In contrast, males accumulate more visceral (abdominal) adipose tissue with the characteristic *apple shape* distribution **(White and Tchoukalova, 2014)**. It is well known that the accumulation of fat mass in the upper zone of the body (abdominal fat) is more related to obesity-associated comorbidities such as cardiovascular diseases or diabetes; on the contrary, fat mass accumulation in the gluteal-femoral region is not and could even be protective **(Wajchenberg, 2000; Tran et al., 2008; Manolopoulos et al., 2010)**.

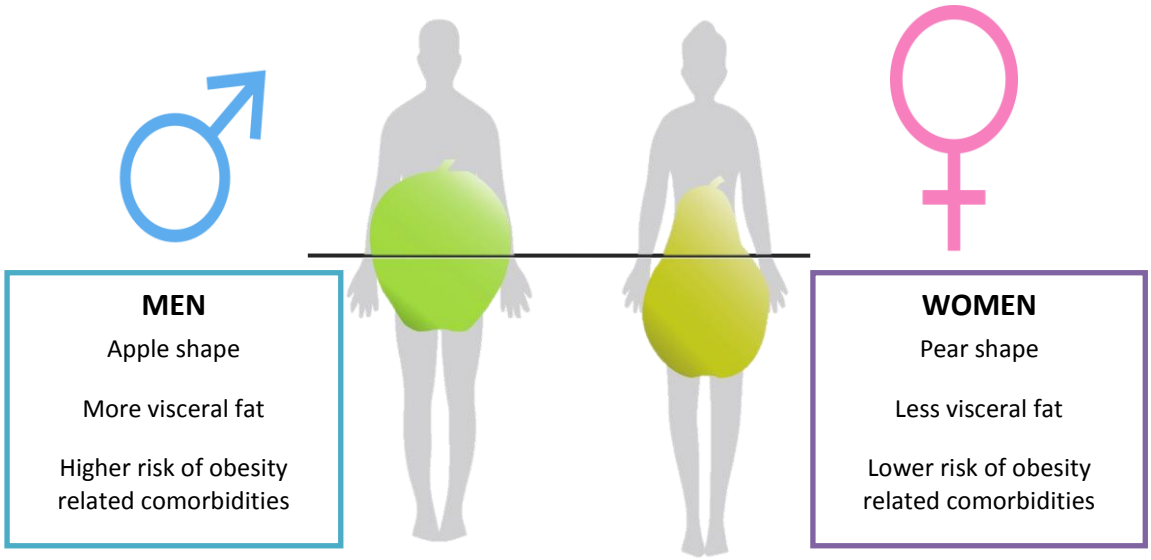


Figure 2. Apple-shaped (male) and pear-shaped (female) distribution of adipose tissue. Men tend to accumulate fat above the waist, whereas women do so below the waist.

Different fat mass localization implies different adipose tissue function, including variations in regards to adipokine production, insulin sensitivity, mitochondrial function, fatty acid release and lipolysis, as well as the inflammatory profile **(Power and Schulkin, 2008; Macotela et al., 2009)**. For example, serum levels of leptin exhibit a sexual dimorphic pattern in both humans and rodents. Women have higher circulating leptin levels than men **(Hickey et al., 1996; Argente et al., 1997)**. Inversely, male rodents have higher serum leptin levels compared to female rodents **(Landt et al., 1998)**. These differences are not only due to the variations in the amount and distribution of adipose tissue, but also to its capacity to produce this adipokine.

The signals that adipose tissue receives from the CNS are also sexually dimorphic. Neurons projecting to visceral fat are more numerous in males than in females, whereas in females the neurons projecting to subcutaneous fat are more abundant than in males **(Adler et al., 2012)**. In addition, female brains appear to be more sensitive to the effects of leptin on the regulation of food intake and energy expenditure, which is suggested to indicate a tight relationship between leptin and estrogens **(Clegg et al., 2006)**.

3.2 Sexual dimorphic responses to perinatal changes

The long-term effects of early nutritional modifications can be sex specific **(Cheverud et al., 2011; Fuente-Martin et al., 2012b; Sanchez-Garrido et al., 2013)**. However, the majority of the experimental studies analyzing these effects have not been performed simultaneously in both sexes. Males and females have metabolically different responses not only to early nutritional changes **(Sardinha et al., 2006; Erhuma et al., 2007; Fuente-Martin et al., 2012b)**, but also to stressful conditions during early life **(Bowman et al., 2004; Garcia-Caceres et al., 2010; Mela et al., 2012; Tibu et al., 2014)**. Moreover, the sexually dimorphic responses to these early interventions often vary according to age **(Fuente-Martin et al., 2012b; Mela et al., 2012)**. Although gonadal steroids are clearly responsible for some of the post-pubertal differences between males and females **(Kanaley et al., 2001; Stubbins et al., 2012; Dakin et al., 2015)**, they cannot explain all of these differences, let alone sex differences observed in early neonatal life. Males experience a surge in testosterone during neonatal life **(Miyachi et al., 1973)**. As alterations in gonadal steroid levels during development have been implicated in metabolic disturbances in adulthood **(Nohara et al., 2013c; Ongaro et al., 2015)**, it is possible that this event is involved in the development of some of the sexually dimorphic characteristics of metabolism.

Administration of testosterone to neonatal females has been classically used as an experimental model to study the mechanisms underlying sexual dimorphism in the brain **(Raisman and Field, 1973)**. This experimental model of androgenization has also been employed to study the long-term effects of increased neonatal androgen levels on the female reproductive axis and the development of polycystic ovary syndrome **(Dunlap et al., 1972; Gellert et al., 1977; Goomer et al., 1977; Ongaro et al., 2015)**. More recently, this experimental model of neonatal androgenization has been used to study metabolic abnormalities in females **(Nohara et al., 2013b; Mauvais-Jarvis, 2014; Ongaro et al., 2015).**

4. Hypothalamic regulation of energy homeostasis

Food intake, energy balance and body weight are regulated by the CNS, which senses the overall metabolic status through the reception of hormonal and nutritional signals. A wide range of circulating peptides from the gut (stomach, small intestine and pancreas) and adipose tissue act on the hypothalamus, brain stem and afferent autonomic nerves through the vagus nerve and the superior cervical ganglion (SCG) to modulate energy metabolism **(Halford and Blundell, 2000; Ring and Zeltser, 2010)**. The CNS then integrates these inputs and executes the appropriate hunger or satiety signal by releasing orexigenic or anorexigenic neuropeptides, respectively.

Although various brain regions such as the solitary nucleus, the amygdala and prefrontal cortex are implicated in metabolic control, the hypothalamus is considered the main integration site for the regulation of feeding behavior **(Schwartz et al., 2000; Horvath, 2005; Abizaid and Horvath, 2008)**. Hypothalamic involvement in the control of food intake was first observed when alterations in food intake were produced in animals with hypothalamic lesions **(Hetherington and Ranson, 1940; Hetherington and Ranson, 1942)** and later, in animals with hypothalamic tumors **(Brobeck, 1946)**.

The hypothalamus is the brain region where peripheral signals, including hormones and metabolites such as glucose and fatty acids, converge and are integrated to achieve appropriate appetite regulation, with the arcuate nucleus being key in this process. This nucleus is located adjacent to the floor of the third ventricle and the median eminence, making it more accessible to incoming peripheral signals. Lesions of the arcuate nucleus in rats have been shown to produce a profound deregulation of appetite that leads to hyperphagia, with some of the first demonstrations of this phenomenon occurring over 4 decades ago **(Olney, 1969; Young et al., 1994)**.

The arcuate nucleus contains two of the main populations of neurons implicated in the regulation of energy balance **(Cone, 2005)**. From the arcuate

nucleus, these neurons send their projections to other hypothalamic nuclei involved in food intake regulation, including the paraventricular nucleus (PVN), the dorsomedial hypothalamus (DMH) and the lateral hypothalamic area (LHA) **(Elias et al., 1998b; Elmquist et al., 1998b) (Figure 3)**.

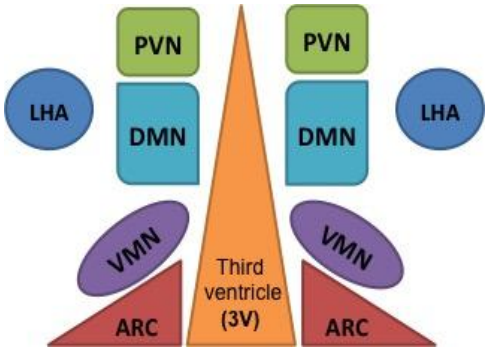
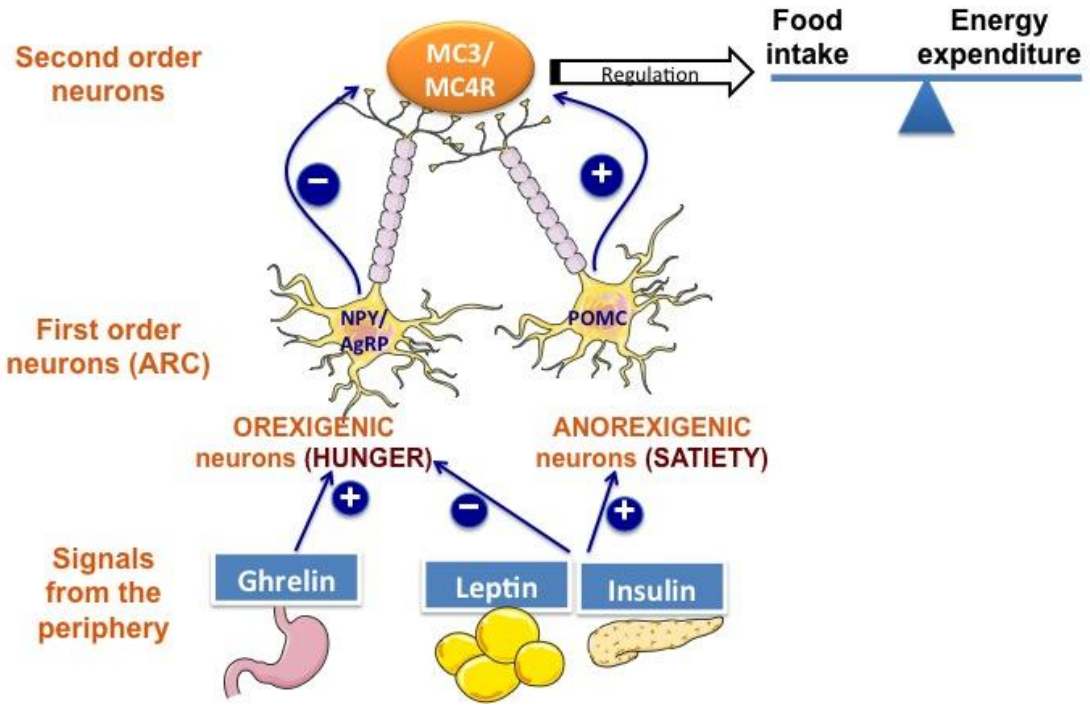


Figure 3. Hypothalamic nuclei

These neuronal populations located in the arcuate nucleus are known as primary order neurons and express neuropeptides that exert opposite actions **(Figure 4).** One neuronal population co-expresses neuropeptide Y (NPY) and agouti-related protein (AgRP) **(Tatemoto et al., 1982; Hahn et al., 1998),** as well as the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) **(Horvath et al., 1997)**. Both neuropeptides exert a potent orexigenic action, which means that their actions will stimulate appetite **(Horvath et al., 1997)**. These neurons are activated in deficient energy states, such as during fasting or when leptin and insulin levels are below normal, to promote food intake and inhibit energy expenditure. On the contrary, high levels of leptin and insulin inhibit this neuronal population **(Schwartz et al., 2000).**

The other important neuronal population is anorexigenic, inhibiting appetite and stimulating energy expenditure, and co-expresses proopiomelanocortin (POMC) and cocaine and amphetamine-related transcript (CART). The precursor POMC protein is processed to produce α and β melanocyte-stimulating hormone (MSH), amongst other peptides **(Cone et al., 1996)**. These melanocortin peptides have anorexigenic effects by acting through the melanocortin receptors 3 (MC3-R) and 4 (MC4-R). A positive energy balance activates these neurons, in part due to the resulting high concentrations of leptin, stimulating melanocortin release and subsequently inhibiting food intake and stimulating energy expenditure **(Elias et al., 1998a)**. These anorexigenic neurons receive innervation from NPY/AgRP neurons, with AgRP having antagonistic effects on MC3-R and MC4-R **(Fan et al., 1997; Ollmann et al., 1997)**. Thus, AgRP is a potent orexigenic signal not only by directly stimulating

food intake, but it also acts indirectly through inhibition of anorexigenic signals at melanocortin receptors. Another POMC-derived peptide is β-endorphin (β-EP), which has orexigenic effects and also antagonizes the effects of MSH on food



intake and body weight **(Grossman et al., 2003; Dutia et al., 2012; Koch et al., 2015)**.

Figure 4. NPY and POMC/AgRP neurons in the ARC nucleus of the hypothalamus are first order neurons that respond to circulating signals from adipose tissue (e.g., leptin), stomach (e.g., ghrelin) and pancreas (e.g., insulin). These neurons release orexigenic (NPY and AgRP) or anorexigenic (CART and the POMC-derived peptide α-MSH) peptides, which impact on second order neurons in other areas of the hypothalamus to regulate food intake and energy homeostasis by stimulating or inhibiting hunger and satiety (B).

5. The control of food intake: communication between the periphery and central nervous system

Communication between peripheral signals and the CNS includes a complex gut-brain-adipose network in which the interaction and actions of hormones and neuropeptides is crucial in the control of metabolic homeostasis and energy balance. Peripheral signals coming from diverse organs or tissues and nutrients are

integrated by the hypothalamus, where, according to the information received, the appropriate signals will be elaborated in order that energy requirements are adequately fulfilled. These hormones come from at least three different sites: adipocytes, the gastrointestinal (GI) tract and the pancreas.

5.1 Signals from adipose tissue

Adipose tissue, once relegated to the category of a passive organ storing triglycerides (TG), is now considered to be an active endocrine organ *per se* **(Antuna-Puente et al., 2008; Coelho et al., 2013)**. There is a constant dialogue between adipose tissue and the CNS **(Figure 5)**, mediated by a variety of adipokines secreted by the adipose tissue, which inform the brain about energy stores. Regarding their actions in the brain, leptin is the most well studied adipokine **(Halaas et al., 1995; Maffei et al., 1995a)**. Although CNS actions of other adipokines such as adiponectin, resistin and apelin have also been reported **(Rajala et al., 2004; Kadowaki et al., 2006; Pope et al., 2012).** Moreover, the brain produces "adipotropins" that act on adipocytes through the activation of specific receptors **(Schaffler et al., 2006).**

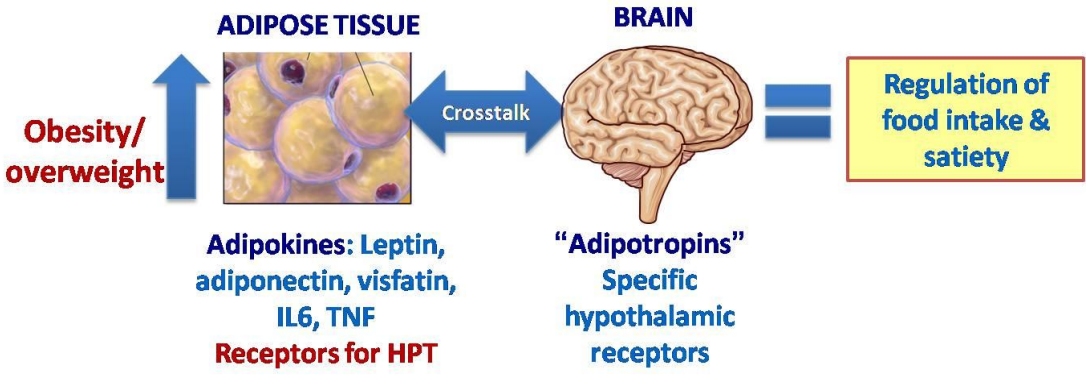


Figure 5. Adipose tissue and brain communication, showing an active role of adipose tissue in the regulation of food intake.

5.1.1 Leptin

In 1994, the cloning and characterization of the Ob gene by Jeffrey Friedman and colleagues **(Zhang et al., 1994b)** resulted in the identification of its protein product, leptin (*leptos* from latin: thin). This discovery was a milestone in the understanding of appetite control since leptin is one of the most important signals secreted by adipose tissue participating in the regulation of energy homeostasis and the pathogenesis of obesity. Leptin is anorexigenic and is secreted almost exclusively by adipose tissue, although to a minor extent by the liver, stomach, hypothalamus, placenta and ovaries **(Hoggard et al., 1997; Bado et al., 1998; Mantzoros, 1999)**. Defects in leptin expression in ob/ob mice **(Drel et al., 2006)**, as well as rare mutations of the leptin gene in humans leading to leptin deficiency, result in profound obesity with affected individuals showing hyperphagia and decreased energy expenditure **(Farooqi et al., 1999)**. Leptin replacement in humans has been shown to normalize these symptoms **(Farooqi et al., 2002).**

Plasma leptin concentrations positively correlate with fat stores and also increase with overfeeding and decrease in starvation states **(Considine et al., 1996; Friedman and Halaas, 1998)**. However, in addition to food intake and BMI, leptin concentrations also vary according to sex, age and circadian rhythms **(Argente et al., 1997; Blum et al., 1997; Mantzoros, 1999).** Leptin circulates either free or bound to a soluble form of its receptor, Ob-R (Ob-Re). Leptin passes through the blood-brain-barrier (BBB) by using a saturable transporter system and reaches one of its main sites of action, the hypothalamus, where it acts to decrease food intake and increase energy expenditure **(Frederich et al., 1995a; Houseknecht et al., 1998)**. Specifically, leptin exerts its anorexigenic effects in the arcuate nucleus by inhibiting NPY/AgRP neurons and stimulating POMC neurons. **(Schwartz et al., 2000; Horvath, 2005; Simpson et al., 2009)**. In 1997, Halaas and colleges showed that intra-cerebral ventricular (ICV) injection of leptin provoked these effects without modifying circulating leptin levels, indicating a central action of leptin **(Halaas et al., 1997)**. Under normal conditions, there is a tight relationship between circulating

leptin levels and its transport into the brain. Thus, fasting states decrease the transport of leptin into the brain due to its low levels in blood, while food consumption produces both an increase in circulating levels and in the transport of leptin across the BBB **(Kastin and Pan, 2000).**

5.1.1.1 Leptin's structure

Leptin, a 167 amino acid peptide hormone with a molecular weight of 16 kilodalton (kDa) is encoded by the *Ob* gene. The human *Ob* gene is located on chromosome 7q31-3 and is composed of three exons separated by two introns **(Isse et al., 1995).** The three-dimensional structure of leptin is organized into four antiparallel α-helices connected by two long crossover links and one short loop. There is an interchain disulphide bond that is key for the stability and biological activity of this hormone **(Zhang et al., 1997; Fruhbeck, 2006)**. Human leptin shares high homology with that of other mammals, sharing up to 84% of homology with mouse leptin and 83% with rat leptin.

5.1.1.2 Leptin receptors and signaling

The leptin receptor, Ob-R, was discovered in 1995 by Tartaglia and coworkers and resembles a class 1 cytokine receptor **(Tartaglia et al., 1995)**. While the CNS accounts for the majority of leptin's target sites **(Elmquist et al., 1998a; Cohen et al., 2001; McMinn et al., 2005; Pan et al., 2008)**, Ob-R is also expressed in other tissues, including pancreatic beta cells, adipose tissue and the testis **(Kielar et al., 1998; Tena-Sempere et al., 2001; Covey et al., 2006)**. This receptor is encoded by the diabetes (db) gene and has at least six-splice variants, denoted Ob-Ra to Ob-Rf. These isoforms can be classified into three classes: short, long and secretory **(Tartaglia et al., 1995)**.

Ob-Ra, Ob-Rc, Ob-Rd and Ob-Rf, the short isoforms of the leptin receptor lacking the intracellular domain, are involved in the internalization and breakdown of leptin **(Tartaglia et al., 1995; Uotani et al., 1999)**. Ob-Ra is the most ubiquitous isoform in most tissues and cells **(Tartaglia et al., 1995)**. Ob-Re is the soluble and

smallest isoform and is involved in transporting circulating leptin to its membrane receptors.

Ob-Rb, the longest leptin receptor isoform with the longest intracellular C-terminal domain, has intracellular signal-transducing capabilities. Ob-Rb is highly expressed in the hypothalamus **(Tartaglia, 1997; Scott et al., 2009)** and is predominantly responsible for mediating leptin's metabolic actions in the CNS. **(Schwartz et al., 2000)**. Both POMC/CART and NPY/AgRP neurons express Ob-Rb **(Cheung et al., 1997; Baskin et al., 1999)**, as do neurons distributed throughout the VMH, DMH and LHA that participate in the regulation of feeding **(Elmquist et al., 1998a; Dhillon et al., 2006; Hommel et al., 2006)**. In addition to neurons, astrocytes also express Ob-Rb **(Pan et al., 2008; Kim et al., 2014a)**, suggesting that these cells also participate in mediating leptin's effects on metabolism.

The amino acid sequences of human and murine leptin receptors also share high homology, including 78% identity in the extracellular and 71% in the intracellular domains **(Chen et al., 1996)**. While the majority of transcripts encoding the short intracellular domain isoforms are found in almost all tissues, the long intracellular domain form is less abundant except for in the hypothalamus, where it is expressed at high levels **(Ghilardi et al., 1996)**. Similar to leptin deficient humans and mice, leptin receptor deficient humans and mutant mice (db/db) display hyperphagia and decreased energy expenditure, presenting early onset obesity **(Chua et al., 1996)**.

5.1.1.3 Mechanism of action of leptin

Leptin binds to Ob-Rb to activate intracellular signaling transduction through the Janus Activated Kinases (JAK)/Signal Transducer and Activators of Transcription (STAT) pathway. Although this is not the only pathway activated by leptin, it is the most studied with regards to food intake control. Leptin binds Ob-Rb causing a conformational change and inducing auto-phosphorylation and activation of JAK family proteins, in particular JAK-2 tyrosine kinase, which in turn produces phosphorylation of STAT family proteins **(Figure 6) (Vaisse et al., 1996; Banks et al.,**

2000), which have two important phosphorylation sites. First, STAT-3 is phosphorylated on tyrosine-705 (Tyr705), which causes dimerization, translocation to the nucleus and DNA binding **(Guschin et al., 1995)**. Then, a second phosphorylation takes place at serine-727 (Ser727). Whereas Tyr705 phosphorylation is mediated by Janus kinases, Ser727 activation may be mediated by several kinases and has a role in transcriptional activation **(Zhang et al., 1998; Decker and Kovarik, 2000)**. This signaling cascade results in the activation of the key genes involved in mediating leptin's actions **(Ghilardi et al., 1996; Harvey and Ashford, 2003; Allison and Myers, 2014).** The phosphorylation of the STAT family proteins also induces the expression of genes such as suppressor of cytokine signaling 3 (SOCS3), which mediates feedback inhibition of the leptin pathway **(Bjorbaek et al., 1998)**. Of note, leptin suppresses AMP-protein kinase (AMPK) stimulating food intake **(Minokoshi et al., 2004)**.

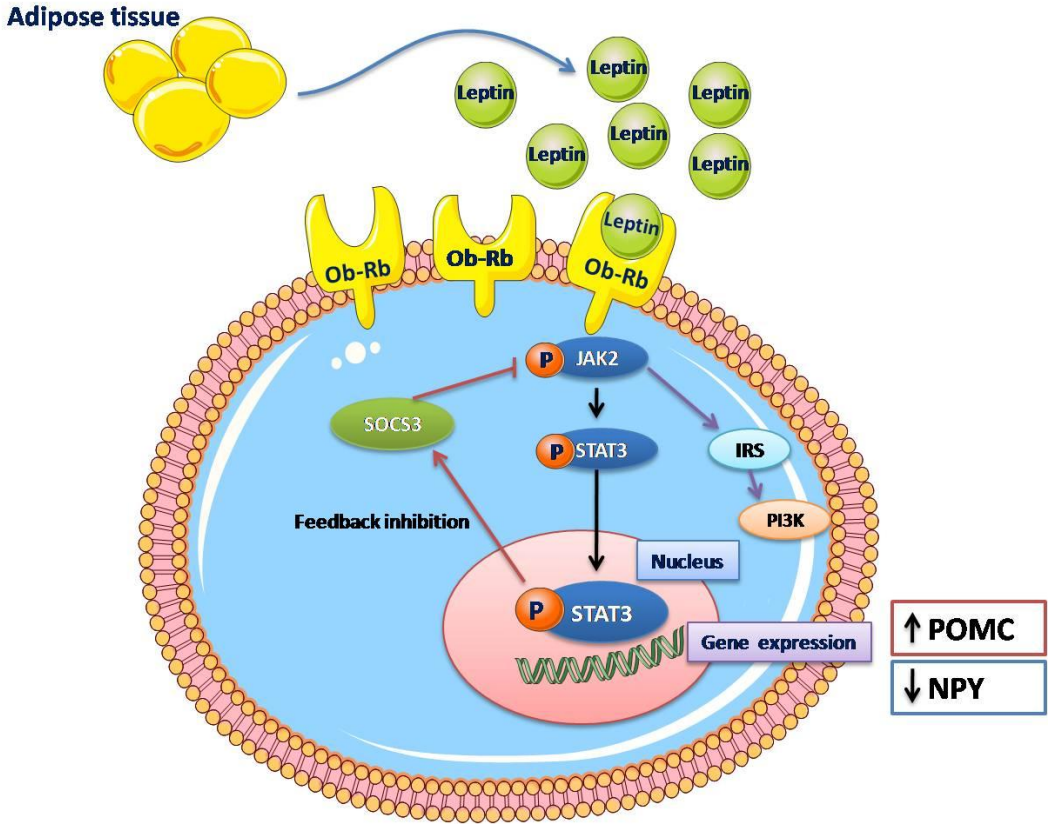


Figure 6. **Simplification of the leptin signaling pathway**. Leptin is secreted by adipose tissue to the bloodsteam postpandrially. Leptin binds to Ob-Rb to activate JAK2 and then STAT3, which translocates to the nucleus to activate the expression of the key genes involved in leptin's satiety effects. STAT3 also induces the expression of SOCS3 that has an inhibitory effect on JAK2, suppressing leptin's actions. The leptin pathway converges with the insulin intracellular signaling pathway at the level of PI3K activation.

5.1.1.4 Leptin's functions

As stated above, leptin is an anorectic hormone secreted to the bloodstream accordingly to the amount of stored adipose tissue an individual has **(Frederich et al., 1995a; Argente et al., 1997).** Although leptin has been used as an efficient treatment in obese leptin deficient patients to reduce the amount of adipose tissue **(Farooqi et al., 2002; Mantzoros et al., 2011)**, high leptin levels are usually found in obese individuals. This implies that there is a resistance to leptin's effects on weight loss **(Frederich et al., 1995b; Maffei et al., 1995b)**. In addition, obese individuals are reported to have reduced leptin transport across the BBB, which would contribute to the leptin resistance observed in obese subjects **(El-Haschimi et al., 2000)**. Indeed, diverse studies exposing mice to high fat diet (HFD) showed an increase in food intake probably due to the development of leptin resistance **(Lin et al., 2000; Enriori et al., 2007; Knight et al., 2010).**

Leptin also influences brain development and participates in the maturation of neuronal circuits implicated in appetite control and food intake behavior **(Bouret et al., 2004b)**. As previously stated, either a deficiency or an excess of leptin during this perinatal period can have long-term effects on the metabolic control **(Steppan and Swick, 1999; Bouret, 2010a; Granado et al., 2014; Mela et al., 2015)** and this is at least in part due to modifications in the development of metabolic circuits. In rodents, the projections from the arcuate nucleus to other important hypothalamic nuclei implicated in feeding behavior are immature at birth, becoming fully mature around PND20 **(Bouret et al., 2004a)**. Mice genetically lacking leptin (ob/ob mice) have modifications in the number of projections and synaptic inputs to NPY and POMC neurons. This synaptic connectivity can be rapidly restored to that seen in wild type (WT) mice by administration of leptin **(Pinto et al., 2004)**. Bouret and colleges demonstrated that ob/ob mice have permanent alterations in the neuronal projections from the arcuate nucleus. Leptin treatment during the neonatal period rescues the development of these projections, while no effect is found in adult animals, indicating that there is a critical window when leptin is

capable of influencing the development of hypothalamic feeding circuits **(Bouret et al., 2004b)**.

Leptin modulates lipid and glucose metabolism, stimulating lipolysis and inhibiting lipogenesis in liver and adipose tissue **(Hynes and Jones, 2001)**. It also has antagonic effects on insulin, decreasing glucose-stimulated insulin secretion **(Hynes and Jones, 2001; Muzumdar et al., 2003)**. Leptin is also implicated in numerous other physiological functions such as reproduction, immunity and adipogenesis **(Friedman and Halaas, 1998).**

5.1.1.5 Leptin and astrocytes

Astrocytes express receptors for leptin **(Pan et al., 2008; Hsuchou et al., 2009; Kim et al., 2014a)** and leptin also plays an important role in astrocyte development **(Pan et al., 2012; Rottkamp et al., 2015)**. Calcium waves are induced in astrocytes in response to leptin, indicating that the leptin receptors in these glial cells are functional **(Hsuchou et al., 2009)**. Increased expression of leptin receptors in astrocytes has been reported in obese animals **(Pan et al., 2008; Hsuchou et al., 2009).** Moreover, we have previously shown that leptin induces morphological changes in astrocytes, both *in vivo* and *in vitro* **(Garcia-Caceres et al., 2011; Kim et al., 2014a)**, and these morphological changes are involved in leptin-induced modifications in synaptic inputs to NPY and POMC neurons **(Kim et al. 2014).** Leptin also modifies the ability of hypothalamic astrocytes to transport both glutamate and glucose, with the time of leptin exposure producing different responses **(Fuente-Martin et al., 2012a)**.

5.1.2 Adiponectin

Adiponectin is a 30kDa protein composed of 247 amino acids. It is exclusively and abundantly secreted by adipose tissue and is thought to play important roles in glucose and lipid metabolism **(Hu et al., 1996; Brochu-Gaudreau et al., 2010)**. Adiponectin is released into the bloodstream in three different isoforms that differ in molecular weight, with the activity of adiponectin in tissues

depending on the amount of each isoform **(Waki et al., 2003)**.

At least in the adult, adiponectin is associated with beneficial effects and it is reported to be an anti-diabetic hormone. This is due to its activation of AMPK **(Yamauchi et al., 2002)** and the peroxisome proliferator activated receptor α (PPARα) **(Kersten et al., 2000)** in liver and skeletal muscle. The adiponectin receptor AdipoR1, is abundantly expressed in skeletal muscle, while AdipoR2 is more highly expressed in liver. Although the neuroendocrine function of adiponectin has been less studied, receptors for this adipokine are also expressed in the arcuate nucleus of the hypothalamus **(Koch et al., 2014).** Binding of adiponectin to these receptors stimulates the activation of AMPK and PPARα, respectively **(Yamauchi et al., 2003)**. Oxidative stress in adipose tissue is associated with decreased plasma adiponectin levels **(Kadowaki and Yamauchi, 2005; Yamauchi et al., 2007)**. Adiponectin has been associated with anti-inflammatory effects, with increased plasma adiponectin levels reducing adipose tissue inflammation in obesity through PPARα activation **(Tsuchida et al., 2005)**. Adiponectin can also up-regulate interleukin 10 (IL-10) expression in macrophages and leukocytes, resulting in a potent anti-inflammatory activity **(Wolf et al., 2004)**.

The effect of adiponectin on hypothalamic metabolic circuit development remains unknown; however, levels of this adipokine in cord blood are positively correlated with leptin levels and adiposity and it is suggested to play a role in early growth **(Tsai et al., 2004; Ballesteros et al., 2011)**. Although adiponectin is reported to increase insulin sensitivity in adults **(Yamauchi et al., 2001)**, in neonates adiponectin levels do not correlate with insulin sensitivity **(Meral et al., 2011)**.

Adiponectin expression by white adipose tissue (WAT) is reported to be sexually dimorphic, with females having higher adiponectin production **(Amengual-Cladera et al., 2012; Capllonch-Amer et al., 2014)**. Secretion of this adipokine is influenced by testosterone, as castrated rodents and hypogonadal men have higher serum total and high molecular weight adiponectin concentrations, while testosterone treatments decreases its levels **(Nishizawa et al., 2002; Xu et al., 2005).**

5.2 Signals from the pancreas

5.2.1 Insulin

Insulin was isolated for the first time by Banting and Best in 1922 **(Banting, 1924; Raju, 2006)**. It is almost exclusively secreted by the pancreas and, once in the circulation, it crosses the BBB by a saturable transport system **(Baura et al., 1993)**. In the CNS, insulin also communicates the metabolic status to the hypothalamus, thus being another key molecule for the control of energy homeostasis **(Vogt and Bruning, 2013)**. Pancreatic beta cells secrete this protein in response to elevated blood glucose levels, after consumption of a meal for example, with insulin thus being the major regulator of glycemia. It stimulates the uptake of glucose by liver, muscle and adipose tissue and suppresses hepatic glucose production **(Kahn, 1994)**. Importantly, and like leptin, circulating insulin levels are correlated to body adiposity **(Polonsky et al., 1988).**

Insulin exerts its actions in both the periphery and the CNS after binding to its receptor (IR), a tyrosine kinase receptor **(Kahn, 1994)**. Insulin receptors are present throughout the brain, including the hypothalamus **(Wozniak et al., 1993)** where they are highly expressed in the arcuate nucleus **(van Houten et al., 1979)**. Insulin activation of the phosphoinositide-3 kinase pathway (PI3/Akt), where AKT triggers forkhead-O transcription factor (FOXO) phosphorylation which is translocated to the nucleus and degraded, is essential for maintenance of energy homeostasis by the CNS **(Plum et al., 2006)**, and the mitogen-activated protein kinase (MAPK) signaling pathway **(White, 2003)**, which negatively regulates FOXO activity **(Biggs et al., 1999).** There is crosstalk between insulin and leptin signaling pathways in the hypothalamus and specifically, insulin signaling converges with leptin signaling at the level of PI3K activation to increase the firing of POMC neurons **(Niswender et al., 2003; Belgardt et al., 2009).** Blockade of PI3K inhibits the anorexic effects on food intake of both leptin and insulin **(Niswender and Schwartz, 2003).**

5.2.1.1 Peripheral and central effects of insulin

Glucose-stimulated insulin secretion regulates appetite through direct activation of POMC and inhibition of AgRP expression **(van Houten et al., 1979; Bruning et al., 2000)**, causing inhibition of food intake and increased energy expenditure **(Woods et al., 1979; Qiu et al., 2014)**. Correct IR signaling is indeed important for body weight control, as IR deficiency in mice leads to increased fat mass and leptin levels **(Bruning et al., 2000)**. It is important to note that insulin exerts independent actions in the periphery and centrally, with this hormone having important functions on metabolism, neural plasticity and cognition **(Banks et al., 2012).** In the brain, IR expression is reported to be higher in neurons than glial cells **(Unger et al., 1989)**. Moreover, aging is associated to decreased IR density in the brain **(Frolich et al., 1998; Bosco et al., 2011)**, which is related with decreased cognitive function and could also have a role in the increased propensity to gain weight with age.

Hyperinsulinemia as a consequence of obesity can lead to insulin resistance and eventually type 2 diabetes **(Moller and Flier, 1991).** Hyperinsulinemia and decreased insulin sensitivity have been proposed to be perpetuated by the inflammatory state that occurs in obesity, due to an abnormal accumulation of lipids in adipose tissue and the production of proinflammatory cytokines **(De Souza et al., 2005; Shoelson et al., 2006)**, with serine kinases such as c-Jun N-terminal kinases (JNKs) mediating the proinflammatory signals and impairing insulin signaling **(Hirosumi et al., 2002; Hotamisligil, 2003)**. Indeed, TNFα and IL6 production by adipose tissue of obese individuals can induce peripheral insulin resistance **(Hotamisligil et al., 1993; Rotter et al., 2003)**.

5.2.1.2 Insulin and brain development

Insulin is essential for adequate CNS development and function, including neuronal differentiation, maturation and survival **(Recio-Pinto et al., 1986; Valenciano et al., 2006)**, synaptic plasticity, learning and memory **(Dou et al., 2005)** and neuronal circuitry formation **(Chiu et al., 2008; Chong et al., 2015)**. Equilibrated insulin levels during prenatal and neonatal life are fundamental, as insulin actively participates in the normal development of metabolic neuronal circuits **(Dorner and Plagemann, 1994;**

Plagemann et al., 1999c; Konner et al., 2009). Indeed, the formation of POMC projections from the arcuate nucleus is disrupted as a consequence of hyperinsulinemia in the offspring due to maternal HFD intake during lactation **(Vogt et al., 2014)**.

5.3 Gastrointestinal (GI) hormones

There are numerous other hormones and factors that have been implicated in metabolic control [for a review see **(Maric et al., 2014)**] by targeting specific neurons in the brain and/or by modulating vagal nerve function **(Cummings and Overduin, 2007**). Some of the most studied hormones participating in food intake control and secreted mainly in the gastrointestinal tract are ghrelin, cholecystokinin (CCK), peptide YY (PYY), glucagon and amylin.

Ghrelin is mainly secreted in the oxyntic cells of the stomach **(Kojima et al., 1999; Nakazato et al., 2001)** and exerts its actions through the ghrelin receptor (GHS-R1a). Ghrelin is the only GI hormone that stimulates food intake. It also stimulates adipose tissue accumulation and modulates energy expenditure and glucose homeostasis **(Tschop et al., 2000; Wren et al., 2001; Muller et al., 2015).** Its secretion is induced in fasting conditions to stimulate NPY/AgRP neurons and to inhibit POMC neurons in the hypothalamic arcuate nucleus, which express GHS-R1a **(Kamegai et al., 2001; Riediger et al., 2003; Wang et al., 2014b).** The stimulatory effect of ghrelin on food intake is also mediated by GABA release from AgRP neurons, impeding melanocortin signaling by inhibiting POMC neurons **(Cowley et al., 2001; Wu et al., 2008)**. Contrary to leptin, ghrelin stimulates AMPK activity in the hypothalamus, consequently increasing food intake **(Andersson et al., 2004)**.

CCK is synthesized in the intestine and acts as potent satiety signal, decreasing meal size by acting in vagal afferents and the hindbrain **(Moran and Kinzig, 2004).** Disorders in food intake and obesity have been found in rats lacking CCK receptors **(Miyasaka et al., 1994; Moran et al., 1998).** Leptin interacts with CCK, increasing its actions **(Peters et al., 2006; Grill, 2010).**

PYY is secreted in the lower intestine and regulates appetite, mediating its effects through the NPY receptors. Serum PYY levels rise postpandrially, in response to calorie intake, resulting in the inhibition of food intake **(Batterham et al., 2002; Batterham and Bloom, 2003).**

The peptide hormone glucagon is secreted in the alpha cells of the pancreas. It is released to the bloodstream when glucose levels are low in order to restore glycemia levels by converting glycogen stored in the liver into glucose **(Woods et al., 2006)**. It is also secreted postpandrially, providing a satiety signal **(Geary, 1990).**

Amylin is synthesized by pancreatic β-cells to decrease food intake. Amylin can interact with leptin to regulate metabolism **(Lutz, 2010).**

5.4 Sex steroids in metabolism

5.4.1 Estrogens

Estrogens are an important group of hormones produced mainly by the ovaries in females and the testes in males, although they are also produced to a lesser extent by other tissues including adipose tissue **(Simpson, 2003)**. In addition to their role in the reproductive system **(Christensen et al., 2012)**, estrogens also modulate the neuroendocrine, skeletal, adipogenic, and cardiovascular systems **(Hughes et al., 2009; Xiao et al., 2010).** Estrogens mediate their actions through estrogen receptors (ERs), including estrogen receptor alpha (ER-α) and estrogen receptor beta (ER-β) which are located throughout the CNS, including the arcuate nucleus of the hypothalamus **(Simerly et al., 1990; Shughrue et al., 1997; Osterlund et al., 1998; Mitra et al., 2003)** and are encoded by distinct genes and located in different chromosomes **(Kong et al., 2003)**. Of note, ERβ expression in the hypothalamus is lower than ERα and, importantly, ERα is the isoform that is most implicated in energy homeostasis **(Heine et al., 2000)**.

ERs belong to the steroid hormone superfamily of nuclear receptors (NRs), which act as transcription factors after binding to estrogen **(Osz et al., 2012)**. ERα and ERβ are expressed in adipose tissue of both males and females and influence adiposity

(Mizutani et al., 1994; Shimizu et al., 1997; Davis et al., 2013). Estrogens are suggested to increase the sympathetic tone to adipose tissue depots, in a different manner in men and women, favoring lipid deposition in the subcutaneous depot in women and visceral in men, as shown by increased abdominal adiposity in females as a consequence of ERα deletion in certain neurons **(Xu et al., 2011)**. In the brain, estrogens play an important role in neuronal development and circuit formation in the fetus and neonate **(Arai et al., 1986; McCarthy, 2008)** and also modulate dendritic morphology, synaptic inputs, neurotransmitter and neuropeptide secretion and nuclear volume in the post-pubertal animal **(DeVoogd and Nottebohm, 1981; Garcia-Segura et al., 1986; Carrer et al., 2005)**. They also regulate the expression of BDNF in the hypothalamus that can be related with the browning of fat **(Cao et al., 2011; Nookaew et al., 2013).**

Not only neurons, but also astrocytes are involved in the neuroprotective actions of estradiol **(Acaz-Fonseca et al., 2014; Arevalo et al., 2015)**. Indeed, ERs are also expressed in astroglial cells **(Jung-Testas et al., 1991; Langub and Watson, 1992),** with glial cells participating in the metabolism of gonadal hormones and the synthesis of endogenous steroids **(Kabbadj et al., 1993; Melcangi et al., 1993; Garcia-Segura et al., 1996a).**

5.4.1.1 Estrogens in food intake regulation

Estrogens have protective effects against weight gain, increased adiposity and obesity associated disorders **(Stubbins et al., 2012; Dakin et al., 2015)**, with 17β-estradiol being the most potent endogenous estrogen in appetite regulation **(Geary, 2000),** functioning at least in part by decreasing food intake and increasing leptin sensitivity **(Palmer and Gray, 1986)**. Activation of ERα in the CNS has been shown to regulate food intake, glucose homeostasis and energy expenditure **(Musatov et al., 2007; Xu et al., 2011).** Estrogens have been shown to exert their "protection" on metabolism due to their inhibitory effects on appetite by increasing the activity of anorexigenic signals such as leptin, cholecystokinin, BDNF or apolipoprotein A-IV and decreasing orexigenic signals like ghrelin or melanin-concentrating hormone

(Tarttelin and Gorski, 1971; Clegg et al., 2006; Clegg et al., 2007; Shen et al., 2010; Zhu et al., 2013).

The menstrual cycle in women is reported to induce fluctuations in calorie intake, with a decrease during the periovulatory phase when estradiol reaches its peak **(Barr et al., 1995; Buffenstein et al., 1995; Davidsen et al., 2007)**. This is also seen in female rodents, which consume less during diestrous (right after the preovulatory rise in estradiol secretion) and increase food intake during estrus when estradiol levels are lower, reinforcing the association between estradiol levels and food intake **(Tarttelin and Gorski, 1971; Asarian and Geary, 2013)**. In addition, female rats are reported to gain less weight compared to males when exposed to a HFD, but this sex difference is no longer observed after ovariectomy **(Stubbins et al., 2012)**.

Estrogens also increase energy expenditure **(Gambacciani et al., 1997)**. Indeed, activation of ERs in the VMN of the hypothalamus in rodent models increases energy expenditure **(Musatov et al., 2007; Xu et al., 2011)**. Post-menupausal women generally tend to increase body weight due to decreased estradiol levels and they are also reported to have a lower rate of energy expenditure during exercise and sleep **(Lovejoy et al., 2008)**. Therefore, estrogens protect against weight gain through both inhibition of food intake and stimulation of energy expenditure. Estrogens are also important for men as blockade of androgen conversion to estrogen leads to impaired insulin sensitivity and metabolism **(Finkelstein et al., 2013).**

5.5 Nutrient derived signals: Glucose and fatty acids

Control of energy homeostasis by the hypothalamus is modulated not only by peripheral hormones, but also by nutrients such as glucose or fatty acids.

5.5.1 Hypothalamic glucose sensing

Claude Bernard observed that hypothalamic lesions produced hyperglycemia in dogs, with this being the first observation that the brain is

implicated in the control of blood glucose levels **(Bernard, 1855).** Later, specific cells in the hypothalamus that monitor glucose concentrations and translate the variations in glucose levels into chemical signals to control feeding were identified **(Mayer, 1953)**. Subsequently, numerous studies have illustrated that glucose acts on hypothalamic sites that are involved in the control of energy homeostasis **(Jordan et al., 2010; Levin et al., 2011)**. Due to its location close to the median eminence, the arcuate nucleus is one of the first central sites to be exposed to circulating glucose levels.

Neurons with the ability to sense blood glucose levels alter their action potential frequency in response to changes in interstitial glucose levels **(Ashford et al., 1990)**. Of special interest are glucose-sensing neurons found in the ventromedial, lateral, paraventricular and arcuate nuclei **(Oomura et al., 1964; Kow and Pfaff, 1989; Song et al., 2001; Wang et al., 2004)**. In the arcuate nucleus, in addition to glucose-sensing neurons **(Wang et al., 2004; Fioramonti et al., 2007)**, astrocytes **(Morgello et al., 1995; Kacem et al., 1998; Fuente-Martin et al., 2012a)** and other glial cells like tanycytes **(García et al., 2003; Salgado et al., 2014)** surrounding the third ventricle are also involved in sensing glucose levels.

Glucose transport and sensing in the brain is carried-out through glucose transporters (GLUT) 1, 2, 3 and 4, expressed in the vasculature, glial cells and neurons. GLUT1 is highly expressed in the astrocyte endfeet that envelop capillaries and is the principal glucose transporter in the BBB **(Morgello et al., 1995; Kacem et al., 1998)**. GLUT2 is expressed in hypothalamic astrocytes, ependymal-glial cells, tanycytes and glucose-sensitive neurons **(García et al., 2003; Guillod-Maximin et al., 2004; Kang et al., 2004)** and is especially important for the glucose-sensing process, actively participating in the homeostatic control of circulating glucose levels **(Bady et al., 2006; Stolarczyk et al., 2010)**. GLUT3 is mainly expressed in neurons **(Gerhart et al., 1995).** AMPK is a fuel sensor **(Kemp et al., 1999)** and is suggested to be a mediator of hypothalamic glucose-sensing, as it is activated when energy stores are low (low generation of ATP) and stimulates food intake **(Claret et al., 2007; Jordan et al., 2010).**

Direct *icv* administration of glucose inhibits feeding through modulation of glucose-sensing neurons **(Kurata et al., 1986; Chang et al., 2005)**. However, this central hypophagic effect of glucose is abolished when an individual is exposed to chronic high fat diet during 8 weeks, with glucose-excited (POMC) and glucose-inhibited (NPY) neurons being less responsive to glucose fluctuations **(Levin et al., 1998; de Andrade et al., 2015)**, suggesting defective neuronal activation by glucose caused by high fat diet.

5.5.2 Hypothalamic fatty acid sensing

Although glucose is the primary source of energy for neurons and acts as a signaling molecule, some hypothalamic neurons also sense long-chain fatty acids (LCFAs) **(Oomura et al., 1975)**. Moreover, enzymes responsible for fatty acid synthesis are also present in neurons of the arcuate nucleus **(Kim et al., 2002)**. Like insulin and leptin, levels of fatty acids and glycerol are proportional to adiposity and thus are also signals of nutrient abundance that modulate hypothalamic function to maintain homeostasis **(Kennedy, 1953; Friedman, 2000)**.

The intake of fat rich diets has increased alarmingly and, although certain levels of fatty acids are necessary, elevated levels of free fatty acids (FFAs) can be harmful. Serum FFAs are increased in obesity **(Reaven et al., 1988; Boden, 2008)** and their elevation is associated with adipocyte dysfunction and the development of insulin resistance **(Boden et al., 2001; Guilherme et al., 2008)**. Moreover, elevated levels of FFAs in perinatal life may also affect hypothalamic neurocircuit development **(Sullivan et al., 2011)**. In addition, FFAs, especially saturated fatty acids, can activate an inflammatory response in different cell types **(Boden and Shulman, 2002; Iyer et al., 2010)**, as well as induce endoplasmic reticulum (ER) stress **(Gregor and Hotamisligil, 2007)**. Excess FFAs derived from the diet accumulate in the adipocyte and form TGs that are stored as energy (one TG is composed of three fatty acids and one molecule of glycerol) in fat droplets in the adipocyte **(Gregor and Hotamisligil, 2007)**. The main features of the most abundant fatty acids in diet are summarized in **Figure 7.**

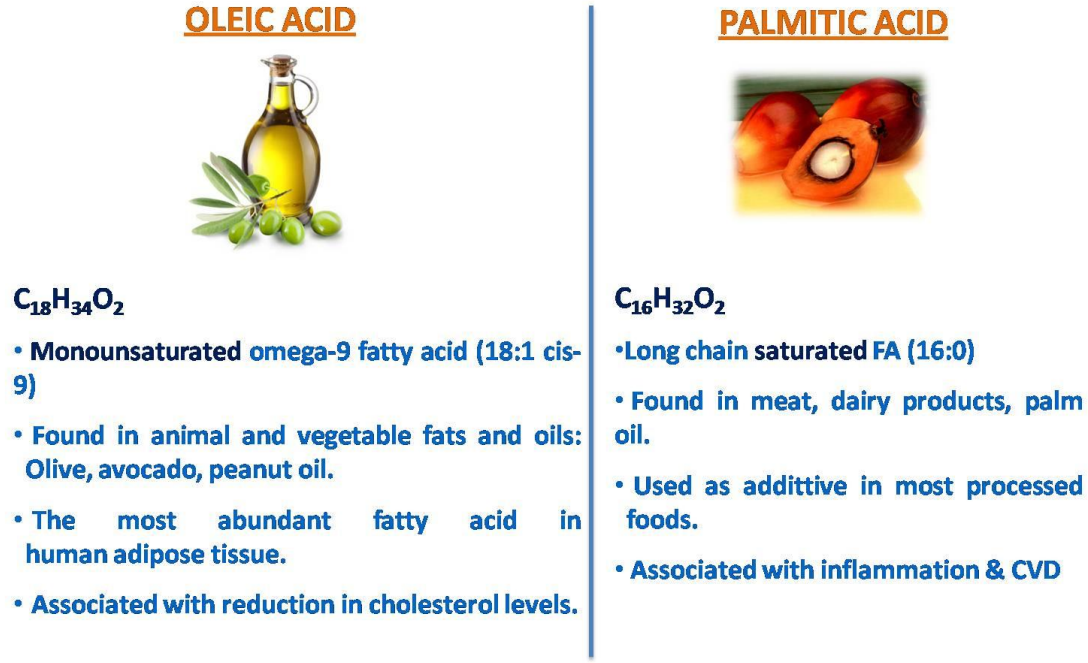


Figure 7. Main characteristics of the most abundant unsaturated (oleic acid) and saturated (palmitic acid) fatty acids in the diet.

Circulating fatty acids access the brain through passive diffusion **(Hamilton and Brunaldi, 2007)** or by active transport **(Mitchell et al., 2011)** and are reported to access the CNS in proportion to their concentrations in plasma (Miller et al., 1987; Rapoport, 1996), although Goto and Spitzer quantified around 6% in the cerebro spinal fluid of their plasma concentration in fasting dogs (Goto and Spitzer, 1971). Once LCFAs enter into the brain and then into hypothalamic glial cells and neurons, they are esterified by acetyl-coA synthase (ACS) to long chain fatty acyl-CoA (LCFA-CoA), whose accumulation will induce inhibitory effects on food intake and glucose production by the liver. Thus, LCFA-CoA accumulation is essential for lipid sensing in the hypothalamus and consequent inhibition of glucose production in the liver **(Woods et al., 1984; Chu et al., 2002; Yue and Lam, 2012)**. LCFA-CoA enters the mitochondria for beta oxidation by carnitine palmitoyl transferase 1 (CPT-1) **(Caspi**

et al., 2007). This oxidation is regulated by malonyl-CoA that inhibits CPT-1 activity **(McGarry et al., 1983)**. Hence, circulating lipids also act as signals to the hypothalamus regarding the nutritional status and modulate neuronal activity to control energy homeostasis **(Woods et al., 1984; Matzinger et al., 2000).**

LCFAs are able to excite or inhibit neurons in the arcuate nucleus **(Morgan et al., 2004; Migrenne et al., 2006; Le Foll et al., 2014)**. Centrally infused oleic acid inhibits food intake and glucose production by the liver, at least in part through inhibiting the expression of the orexigenic neuropeptides NPY and AgRP **(Obici, 2002; Morgan et al., 2004)**. In addition, oleic acid has been reported to stimulate neurogenesis of metabolic neurons **(Nascimento et al., 2015)** and to improve insulin resistance in obese mice **(Oliveira et al., 2015)**. In contrast, exposure to saturated LCFAs, like palmitic acid, leads to resistance to leptin's anorexigenic actions **(Milanski et al., 2009)** and appears to exacerbate the inflammatory responses observed in obesity and to cause lipotoxicity **(Listenberger et al., 2001; Beeharry et al., 2003)**. Palmitic acid is reported to cause inflammatory responses in both astrocytes and microglia **(Gupta et al., 2012; Valdearcos et al., 2014)**, which most likely participates in the disruption of appropriate metabolic sensing in neurons.

6. Inflammation in obesity

Inflammation is a host response to protect against an insult and is necessary to repair the tissue and restore homeostasis **(Calder et al., 2009)**. Obesity is characterized by a chronic, low-grade inflammatory state **(Gregor and Hotamisligil, 2011)**. However, the inflammation associated with obesity is different from the classical inflammation in which pain, redness, swelling and heat are typical and a rapid immune response eliminates the insult and resolves the inflammation in the site of injury **(Medzhitov, 2008)**. The first report of systemic inflammation related to obesity was published by Hotamisligil and co-workers, where they reported an elevation in TNFα levels in obese compared to lean mice **(Hotamisligil et al., 1993; Hotamisligil et al., 1995)**. In addition to TNFα, the expression of other inflammatory

cytokines, such as IL1β and IL6, has also been shown to be elevated in obesity **(Berg and Scherer, 2005; Shoelson et al., 2006)**.

In obesity, adipose tissue is the main site of increased systemic cytokine production, including IL6, TNFα and IL1β amongst others, with excess nutrient intake suggested to activate this inflammatory response **(Fried et al., 1998).** The increased secretion of inflammatory cytokines such as TNFα can impair insulin signaling and thus contribute to the development of insulin resistance **(Stephens et al., 1997)**. The inflammatory response in adipose tissue is associated with adipocyte hypertrophy, but not adipocyte proliferation **(Weisberg et al., 2003)**. Indeed, adipocyte hypertrophy is associated with immune cell, macrophage and T cell infiltration in addition to the local proinflammatory environment **(Minihane et al., 2015)**. The distribution of adipose tissue in overweight and obese subjects seems to be important in determining the inflammatory milieu, with VAT appearing to contribute to a greater extent than SCAT to the inflammatory process and cytokine production **(Weiss, 2007a).**

Importantly, the cytokines released by adipose tissue are also metabolic signals that can cross the BBB and reach the metabolic control units, including both neurons and astrocytes within the hypothalamus **(Banks et al., 1995; Benveniste, 1998).** Moreover, these cytokines can also be locally produced in the brain **(Benveniste, 1998).** Indeed, in addition to adipose tissue, other tissues involved in metabolism such as the brain **(De Souza et al., 2005)**, liver **(Cai et al., 2005)**, pancreas **(Nicol et al., 2013)** and muscle **(Saghizadeh et al., 1996)** may also participate in the inflammatory state in obesity.

There is evidence that inflammatory responses can be directly evoked by nutrients **(Aljada et al., 2004; Cani et al., 2007; Erridge et al., 2007)**. Nutrients and/or inflammatory signals can activate JNK, a major intracellular contributor to the induction of inflammation in metabolic tissues, inhibitor of nuclear factor kappa-B kinase (IKK) and/or other kinases, which can interfere with the insulin-signaling

cascade by targeting IRS-1 for inhibitory phosphorylation **(Boura-Halfon and Zick, 2009; Tanti and Jager, 2009).**

Sex differences in inflammatory markers have been investigated in a few studies, but the reported studies are contradictory. Some studies report no differences between male and female humans and mice **(Forsey et al., 2003; Medrikova et al., 2012)**, while others indicate a sex difference in humans and chimpanzees **(Bouman et al., 2004; Obanda et al., 2014).** Hence, further investigation is required where both sexes are compared in order to achieve clear conclusions in this field.

7. Glial cells in metabolic control

7.1 Astrocytes in metabolic control

The contribution of astrocytes, the most abundant glial cells in the CNS, to the physiological and pathophysiological control of metabolism is increasingly evident **(Garcia-Caceres et al., 2012; Argente-Arizón et al., 2015)**, although further investigation is needed to completely understand their role in both physiological and pathophysiological metabolic control.

7.1.1 Astrocyte functions

Astrocytes are essential for the communication between the periphery and the CNS **(Tsacopoulos and Magistretti, 1996)**. These glial cells are strategically positioned to sense the nutritional status, being part of the BBB and in intimate contact with vascular and synaptic elements **(Abbott et al., 2010; Wang et al., 2014c).** They are therefore capable of providing blood-borne nutrients to neurons, promoting neuronal survival and contributing to CNS homeostasis.

Astrocytes regulate neuronal differentiation, proliferation, and synaptogenesis during development **(Nedergaard et al., 2003; Clarke and Barres, 2013)**. These glial cells highly express the glutamate transporters GLT-1 and GLAST and

modulate synaptic transmission by up-taking glutamate from the synaptic cleft **(Pines et al., 1992; Schmitt et al., 1997)**. This not only terminates excitatory transmission, but also protects against excitotoxicity **(Araque et al., 1999; Ransom et al., 2003).** One single astrocyte contacts various neurons and thousands of synapses **(Bushong et al., 2002; Theodosis et al., 2008)**, highlighting the importance of how changes in astrocytes can extensively influence neuronal activity.

In response to CNS injuries, infections or foreign substances, astrocytes become activated and respond by producing cytokines, thus participating in central immune responses **(Aschner, 1998a; b)**. Depending on the type of stimulus and its intensity or time of exposure, the cytokines produced from astrocytes can have protective or beneficial effects or, in contrast, detrimental inflammatory effects often associated with scar tissue formation **(Choi et al., 2014).** Astrocytes also participate in diverse processes that could affect metabolism, such as glucose and lipid sensing, and they express receptors for various hormones that participate in the control of food intake, including leptin, insulin, ghrelin and glucocorticoid receptors **(Vielkind et al., 1990; Zhu et al., 1990; Hsuchou et al., 2009; Baquedano et al., 2013).**

7.1.2 Astrocytes in glucose and fatty acids sensing

The astrocyte endfeet surrounding blood vessels express glucose transporters GLUT1 and GLUT2 to transport glucose from the circulation into the brain **(Morgello et al., 1995; Vannucci et al., 1997; Marty et al., 2005; Stolarczyk et al., 2010)**. Glucose can be stored inside astrocytes as glycogen or be metabolized to lactate, which can be released to serve as an energy substrate for neurons **(Levin et al., 2011)**. Notably, astrocytes also secrete endozepines, which in addition to exerting anorexigenic effects **(do Rego et al., 2007)**, participate in glucose-sensing, possible through the melanocortin pathway **(Lanfray et al., 2013).**

Astrocytes are the sole cells in the CNS able to β-oxidize FAs to produce ketone bodies; thus, these cells are the main source of FA oxidation in the brain. Ketone bodies produced by astrocytes serve as a fuel to neurons, especially in

situations of intense neuronal activity or fasting **(Auestad et al., 1991; Guzman and Blazquez, 2001)**. However, ketone bodies are also produced during chronic HFD intake, with this possibly disrupting FA signaling mechanisms involved in metabolic control **(Le Foll et al., 2014).**

Astrocytes express PPARγ, an important sensor of lipids and regulator of metabolism **(Cristiano et al., 2005),** and apolipoprotein E (ApoE), the major lipid transporter in the CNS **(Boyles et al., 1985; Krul and Tang, 1992)**. ApoE can also act as a satiety factor, possibly by mediating some of the inhibitory effects of leptin **(Shen et al., 2008; Shen et al., 2009a)**, and may play a protective role against apoptosis of astrocytes **(Zhou et al., 2013).**

7.1.3 Influence of metabolic hormones on astrocytes

Astrocytes express receptors for leptin **(Hsuchou et al., 2009; Kim et al., 2014a)**, as well as receptors for insulin, ghrelin and glucocorticoids **(Vielkind et al., 1990; Zhu et al., 1990; Baquedano et al., 2013)**. Different isoforms of Ob-R are expressed in hypothalamic astrocytes **(Pan et al., 2008; Hsuchou et al., 2009)**, with an increase in their expression in obese rodents **(Pan et al., 2008)**. Leptin modifies astrocyte morphology by changing primary projections length and/or number **(Garcia-Caceres et al., 2011; Kim et al., 2014a)**, affecting synaptic inputs onto POMC and NPY neurons and thus their function **(Kim et al., 2014a)**. These changes in morphology, for example as a consequence of HFD, may be an adaptation to the new metabolic condition; however, if this situation is maintained, these responses could become pathophysiological. Indeed, the glial response to leptin is time dependent, and prolonged exposure to this hormone stimulates astroglial production of cytokines **(Garcia-Caceres et al., 2011)**. Leptin also modifies the ability of hypothalamic astrocytes to transport both glutamate and glucose, with the time of leptin exposure producing different responses **(Fuente-Martin et al., 2012a)**. Leptin's effect on glucose uptake by astrocytes could possibly change the local transport of glucose and its metabolites to neurons, which could in turn modify glucose sensing mechanisms and neuronal control of energy homeostasis.

As demonstrated by Kim and colleges **(Kim et al., 2014a)**, loss of Ob-R specifically in GFAP positive cells, affects the synaptic organization of the melanocortin system, modifying the satiety response to leptin and increasing the ghrelin response to fasting. The number and length of astrocyte projections in the arcuate nucleus is reduced in the absence of Ob-R. This is associated with decreased astrocytic coverage of POMC neurons and an increase in the number of synaptic inputs to these neurons in addition to modification in the electrical activity of both POMC and AgRP neurons.

Astrocytes also express receptors for estrogens, androgens and progesterone **(Pfaff and Keiner, 1973; Garcia-Segura et al., 1996b; Melcangi et al., 2001; Garcia-Ovejero et al., 2005)** with gonadal steroid modulation of these glial cells possibly participating in the reported sexual dimorphism in the inflammatory response to HFD **(Louwe et al., 2012; Morselli et al., 2014)**. Indeed, females are suggested to be more resistant than males to obesity associated comorbidities, at least in part, due to the expression of ERα in astrocytes **(Morselli et al., 2014)**.

Although how leptin and estrogens affect astrocytes has begun to be elucidated, little is known regarding how other metabolic hormones or nutrients modulate astrocyte function and participation in metabolic control. For instance, glucocorticoids modulate astrocytes in the suprachiasmatic nucleus **(Maurel et al., 2000)**, but whether this affects circadian rhythms of food intake remains to be elucidated. The orexigenic hormone ghrelin has also been reported to have direct effects on hypothalamic astrocytes **(Garcia-Caceres et al., 2014)**, although how this affects overall metabolism is still unknown. Although most studies on the role of astrocytes in obesity have been performed in models of HFD-induced obesity, fructose is also able to induce astrogliosis **(Li et al., 2014)**. However, a high sucrose diet, which causes increased adiposity, does not produce the classical hypothalamic astrogliosis reported in HFD-induced obesity **(Fuente-Martin et al., 2013b)**. Hence, although astrocytes appear to be involved in mediating central metabolic signals, there is still much to be learned regarding the mechanisms involved. Illustration of

hypothalamic astrocytes in a physiological and a pathophysiological state is shown in **Figure 8**.

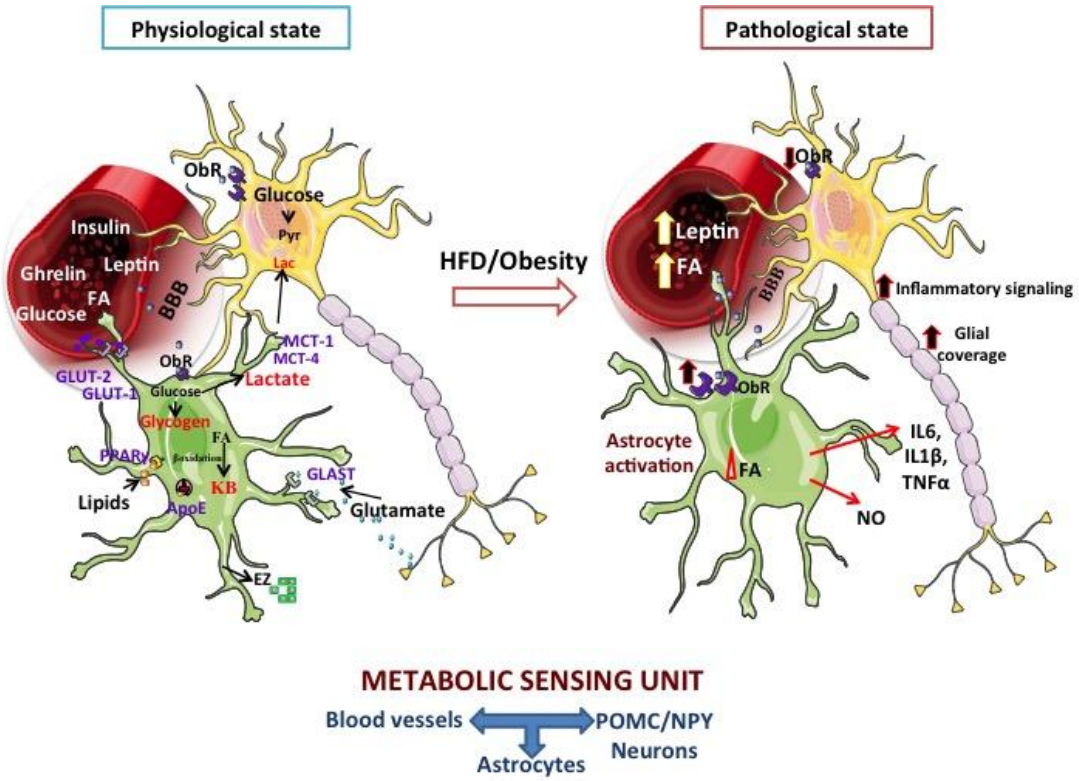


Figure 8. Hypothalamic astrocytes in a normal physiological and in a pathological state in response to HFD or obesity.

A. Astrocytes are sensors of the general metabolic status due to its privileged location between the periphery and the central nervous system. They are the main energy source in brain by providing energy to neurons from circulation supplying them with glucose, lactate or ketone bodies. Only astrocytes can βoxidize fatty acids to produce ketone bodies. The major lipid carrier protein in the CNS, ApoE is produced by astrocytes. Astrogial cells express receptors for important metabolic hormones, such as leptin and insulin, and thus may participate in their signaling effects. They also release endozepines, which exert anorexigenic effects and act as gliotransmitters by modulation of neuronal activity through GABA.

Together these functions make astrocytes key elements for neuronal support and survival, regulation of synaptic transmission, maintenance of the BBB and players in the central immune response.

B. High fat diet consumption and in consequence, obesity, leads to disruption of metabolic status and to an increase in important metabolic hormones as leptin. Astrocytes can be activated, changing their morphology, affecting their contact with blood vessels and neuronal coverage, as well as the number of

synaptic inputs to metabolic neurons. Astrocytes also increase the number of receptors for leptin, possibly causing changes in leptin transport.

If the elevated exposition to leptin is prolonged and, in consequence astrocyte activation, production of cytokines and neurotoxic substances by astrocytes can occur, leading eventually, to detrimental effects. Ketone bodies produced by astrocytes from fatty acids are a source of energy for neurons in cases of high neuronal activity or fasting. However, in chronic HFD, they are also synthesized, impairing FA signaling.

Figure legend: EZ: Endozepines; **FA**: Fatty acids; **KB:** Ketone bodies; **BBB**: blood brain barrier; **IL-6**: Interleukin 6; **IL1β**: Interleukin 1 β; **TNFα**: Tumor necrosis factor α; **MCT-1**: Monocarboxylate transporter 1; **MCT-4**: Monocarboxylate transporter 4; **GLAST**: Glutamate aspartate transporter; **GLUT-1**: Glucose transporter 1; **GLUT-2**: Glucose transporter 2**; ApoE**: Apolipoprotein E; **PPARγ**: Peroxisome proliferator activated receptor γ; **NO:** Nitric oxide.

7.2 Microglia in metabolic control

Microglia are the immune cells of the CNS **(Rivest, 2009)**. In physiological conditions, these cells are continuously maintaining the cellular environment by cleaning debris **(Aloisi, 2001; Nimmerjahn et al., 2005)**, modulating synapses **(Batchelor et al., 1999; Zhong et al., 2010)**, as well as through the production of diverse substances that include cytokines when necessary **(Shoelson et al., 2006)**. It has been proposed that microglia and astrocytes cooperate to support and modulate neural communication **(Schafer et al., 2013).**

Microglia can become reactive as a consequence of HFD or obesity, resulting in morphological changes and the release of diverse substances. Saturated fatty acids can directly active these glial cells **(Milanski et al., 2009),** and this activation occurs specifically in the hypothalamus after HFD intake **(Thaler et al., 2012)**. This microglial response can involve the production of cytokines, reactive oxygen species (ROS) and nitric oxide (NO) that, upon becoming chronic, can exacerbate microglia activation and may cause neuronal impairment and toxicity, with POMC neurons being particularly vulnerable **(Block and Hong, 2007)**. In turn, NPY and α-MSH can modulate the release of cytokines and NO by microglia **(Delgado et al., 1998;**

Ferreira et al., 2011; Gonçalves et al., 2012), suggesting that there is direct communication between metabolic neurons and microglia and that this cross-talk may participate in the inflammatory process.

Leptin can directly active microglia **(Tang et al., 2007; Lafrance et al., 2010; Gao et al., 2014)**, suggesting that this hormone participates in the activation of these cells in obese subjects. Ob/Ob mice that lack leptin have lower levels of hypothalamic microglia activation than control mice **(Gao et al., 2014)**. Despite the fact that HFD intake by these mice increases microglia activation in the hypothalamus, it does not reach control levels. Moreover, leptin administration increases microglia number and ramification even though these animals lose weight **(Gao et al., 2014)**. Thus, independently of body weight, metabolic hormones and nutrients control hypothalamic microglial activity.

Pro-inflammatory cytokines released by activated microglia may also participate in the development of insulin resistance that can accompany central inflammation **(De Souza et al., 2005; Shoelson et al., 2006)**. Of note, exercise counteracts microglia activation produced by HFD, as well as improves glucose tolerance **(Yi et al., 2012)**. Thus, exercise not only has beneficial effects by increasing energy expenditure, but it also ameliorates central inflammation.

The early nutritional environment influences the maturation of these immune cells, causing long lasting effects in the response to neuroimmune challenges **(Bilbo et al., 2010; Clarke et al., 2012)**. Indeed, HFD intake during gestation and the release of proinflammatory cytokines by microglia has been shown to affect the development of the melanocortin circuit in nonhuman primates **(Grayson et al., 2006)**. In rodent models, the offspring of mothers fed with a HFD during gestation had increased hippocampal microglial activation at birth, as well as increased microglial density in adulthood **(Bilbo and Tsang, 2010)**. Moreover, neonatal overnutrition in rats results in microglial activation in hypothalamic areas and other brain areas, which is accompanied by an increase in the expression of inflammatory genes in the adult hypothalamus **(Tapia-González et al., 2011; Tu et al., 2011; Ziko et al., 2014).**

The number of microglia and their morphology is reported to differ between the sexes in some brain areas **(Schwarz et al., 2012)**. However, there is little information in the literature as to whether males and females differ in their microglial response to metabolic challenges.

8. The question in context

As stated above, it is clear that the early nutritional status has long-term effects on energy homeostasis, with specific metabolic outcomes possibly being affected differently depending on age. Moreover, although males and females are known to respond differently to metabolic challenges, the mechanisms underlying this dimorphism are not fully elucidated. Differences in gonadal steroid production are obviously involved in postpubertal sex differences, but they cannot explain what occurs in early development. As the neonatal hormonal/nutritional environment modulates the development of hypothalamic neuronal circuits involved in the control of energy homeostasis, it is possible that differences between males and females exist even during this early developmental stage and this impacts on the development of these circuits. Moreover, the response to metabolic challenges during perinatal life could also affect males and females differently.

Studies analyzing the effect of early nutritional/hormonal changes on the development of hypothalamic metabolic circuits have focused on neurons. However, glial cells are involved not only in the development, but also the correct functioning of these circuits in adulthood. Indeed, the involvement of astrocytes and microglia in the physiological and pathophysiological neuroendocrine control of metabolism has become an area of increasing interest in the investigation of obesity, but little is known regarding the effect of perinatal nutritional changes on glial cell development or their involvement in later metabolic abnormalities. Moreover, how astrocytes differ between males and females remains to be fully explore.

Obesity courses with a chronic mild inflammatory state that includes the hypothalamus, a process in which astrocytes and microglia participate. These glial cells also respond to specific hormonal and nutritional signals, becoming activated and producing cytokines and other factors, with hypothalmic gliosis and inflammation being implicated in the development of secondary complications of obesity. Thus, if hypothalamic astrocytes differ between males and females, they could participate not only in the physiological differences between the sexes in metabolic control, but also the pathophysiologial consequeces of a poor diet and obesity.

Together, these observations have lead us to the following hypothesis and objectives.

II. HYPOTHESIS

Males and females respond differently to nutritional challenges and this could be in part due to sex differences in hypothalamic astrocytes.

III. OBJECTIVES

Objective 1. To evaluate the changes in peripheral hormones, body weight and adipose tissue in response to overnutrition due to a reduction in litter size of male and female rats at different stages of postnatal development.

Objective 2. To correlate the peripheral changes as a consequence of the reduction in litter size, with central changes in the hypothalamus and specifically in astrocytes, throughout development of male and female rats.

Objective 3. To analyze whether males and females respond differently to neonatal over-nutrition.

Objective 4. To investigate whether hypothalamic astrocytes from male and female rats respond differently to free fatty acids.

Objective 5. To determine if estrogens affect the response of hypothalamic astrocytes to free fatty acids.

IV. MATERIALS AND METHODS

1. Experimental Models

All experiments were designed according to the European Communities Council Directive (86/609/EEC; 2010/63/UE) and NIH guidelines for animal care. Experiments in these studies complied with the Royal Decree 53/2013 pertaining to the protection of experimental animals. The studies were approved by the Commission of Investigation of the Hospital Infantil Universitario Niño Jesús and the corresponding university ethics committee where the experiments were performed, including the Ethical Committee of Animal Experimentation of the Universidad Complutense de Madrid, Ethical Committee of Animal Experimentation of the Universidad Autónoma de Madrid, Ethical Committee of Animal Experimentation of the Universidad de Córdoba or the Institutional Animal Care and Use Committee of Yale University. The animals were always treated respectfully and the least possible number of animals was used in all experiments.

1.1*IN VIVO* STUDIES

1.1.1 Neonatal overnutrition throughout development

Male and female adult Wistar rats were purchased from Harlan Interfauna Ibérica S.A., (Barcelona, Spain) and allowed to acclimate for two weeks before mating. One male was placed in a cage with three virgin females (all weighing approximately 150 g) females. Each dam was then housed individually and fed *ad libitum*. Rats were maintained at a constant temperature (22-24ºC) and humidity (50-55%) in a 12 hour light-dark cycle, with lights on at 07:30. Animals were given free access to chow (commercial diet for rodents A04-10/15022, Panlab, Barcelona, Spain) and tap water.

a) Experimental design

Only mothers that gave birth to between 8 and 12 pups were employed for the study (mean litter size 10.3 ± 0.2 pups born/litter). All litters were arranged on the day of birth, postnatal day (PND) 0. At this time, pups were weighed, measured

and organized into two different litter sizes: litters of 12 pups per dam (L12: 6 males and 6 females), as control (CT) litters or litters of 4 pups per dam (L4: 2 males and 2 females) as neonatal overnutrition (NON) litters. A total of 12 litters of 12 pups per dam and 36 litters of 4 pups per dam were employed in these studies. Cross-fostering was used and every litter contained a balanced number of males (M) and females (F). Litters were arranged such that the mean initial weights did not differ between groups (ML4: 5.8 ± 0.1 g, ML12: 6 ± 0.1 g; FL4: 5.7 ± 0.1 g, FL12: 5.7 ± 0.1 g). Rats were raised in these litters from birth to weaning, which took place at PND21.

This procedure is a relatively physiological way of inducing overnutrition by increasing milk availability due to the reduction in the number of pups/dam (Fiorotto et al., 1991; Fink et al., 2001). This procedure may also affect maternal nurturing (Fiorotto et al., 1991; Koskela et al., 2009), which can also have long-term effects on metabolism.

After weaning, the rats were housed 2 per cage, according to sex and experimental litter size. From this point on, the animals were given free access to normal rat chow and tap water. Body weight, length and food intake were monitored once a week until sacrifice. Daily food intake was measured once a week by placing a known amount of food in the cage and weighing the remaining amount of food at the same time the following day. To account for spillage, food was retrieved from the bedding before weighing. The number of cages was used as the n for statistical analysis. The amount of chow ingested per animal was calculated by dividing the total amount of food eaten by the number of rats per cage. Body length was monitored by placing the animal on a ruler and measuring the longitude from the snout to the base of the tail. Rats older than 90 days were lightly anesthetized with isoflurane in order to immobilize the animal and obtain a more accurate measurement. This anesthesia was not applied on the day of sacrifice.

Rats were sacrificed at different time points throughout development. These time-points were chosen as they represent key developmental periods (Figure 9. For a review on how a rat's age relates to that in humans see (Sengupta, 2013).

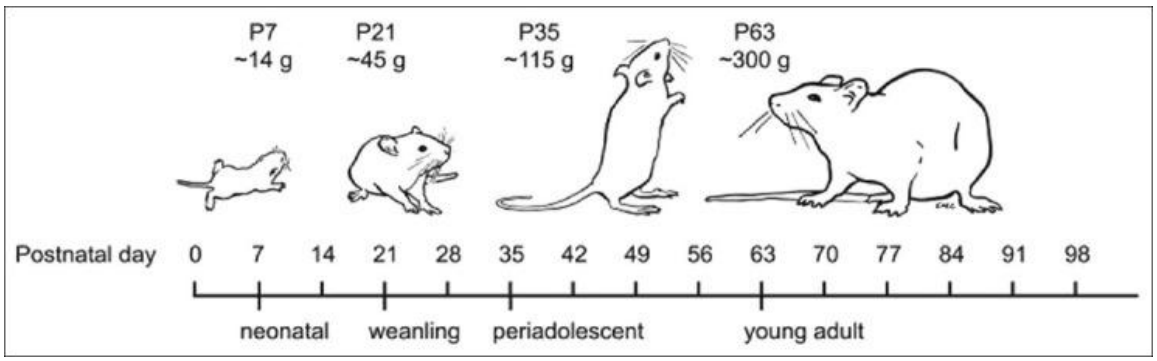


Figure 9: "The laboratory rat: relating rat's age to that in humans". Taken from *Sengupta, 2013.*

In the present study, rats were sacrificed on the following days:

- **Postnatal day 10**: Day 10 is a crucial stage in rat development due to the leptin surge that takes place around this period of time. Serum leptin levels start to increase around PND 5 and peak at PND 9-10 (Ahima et al., 1998; Delahaye et al., 2008).
- **Postnatal day 21:** Rats were weaned at PND 21, thus concluding the period of exposure to different nutritional environments due to litter size. From this point on, all rats were allowed to eat and drink *ad libitum*.
- **Posnatal day 30:** Rats are still pre-pubertal on day 30 after birth, but approaching pubertal onset. In our facilities, males reach sexual maturation around PND 42-44 and females around PND 32-35 (Castellano et al., 2011).
- **Postnatal day 50:** At this age rats are post-pubertal, but still in the periadolescent period, approaching adulthood.
- **Postnatal day 85:** At PND85 rats are adult, with numerous published studies using rats between PND60-PND90 for metabolic analysis.
- **Postnatal day 150:** This stage allows the study of older adult rat where some effects of aging on metabolism may begin to appear.

The experimental design of neonatal overnutrition protocol is shown in Figure 10.

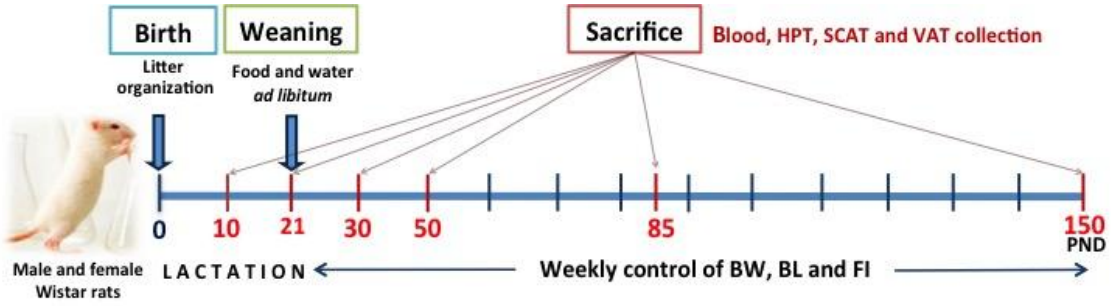


Figure 10. Diagram of the experimental design for neonatal overnutrition (NON) throughout development in male and female Wistar rats. N= 12 for each experimental group; BW: body weight, BL: body length, FI: food intake, PND= Postnatal day. HPT = Hypothamamus, SCAT = Subcutaneous adipose tissue, VAT = Visceral adipose tissue.

b) Sacrifice and sample collection

Animals were placed under fasting conditions 12 hours before sacrifice, except for the rats sacrificed on PNDs 10 and 21, which were allowed to nurse until sacrificed in order to not induce additional stress on being removed from their mothers. On the day of sacrifice, body weight and length were measured. Blood glucose levels were determined in a drop of blood from the tip of the tail by using a glucometer (Optimum Xceed, Abbot). A quick vaginal swap was performed in the female rats to determine the estrous cycle stage at the moment of sacrifice.

A total of twelve animals per group were sacrificed between 09:00 and 11:00. Six rats were processed to obtain fresh tissue and the other six were perfused as described below. Of note, PND10 and PND21 rats were not transcardially perfused, but their brains and fat pads were directly fixed by placing the brains and fat pads in 4% paraformaldehyde and left overnight (O/N). To obtain fresh tissue, rats were killed by rapid decapitation. Blood was collected from the trunk, allowed to clot and preserved on ice before being centrifuged at 3000 RPM during 20 minutes at 4°C to obtain serum. Serum samples were kept at -80°C until processed.

After decapitation, the brain was rapidly removed and the hypothalamus, defined rostrally by the optic chiasm and caudally by the anterior margin of the mammillary bodies, was dissected out with a depth of approximately 2 mm. Subcutaneous (inguinal) and visceral (perigonadal) adipose depots were removed, weighed, frozen on dry ice and stored at -80°C until processed. Six animals per group were perfused transcardially and the tissue then collected as described below.

Serum samples were processed for the analysis of leptin, insulin, interleukin (IL) 1β, 6, tumor necrosis factor alpha (TNFα) and adiponectin, as described below. Testosterone and 17β-estradiol were measured in males and females, respectively.

Rats that were killed at PND150 were subjected to an oral glucose tolerance test (OGTT) one week before sacrifice. Rats were fasted over-night (from 20:00 to 8:00 h) and at 8:00 h basal glycemia was measured with a glucometer (Optium Plus, Abbot Diabetes Care, Witney Oxon, UK). A bolus of 3 mg/g-bw of glucose was then orally administered to the rats by means of a lavage tube. Glycemia was measured at 30, 60 and 120 min after glucose administration by venous tail puncture. The total area under the curve (AUC) for the glucose response was calculated by using the following formula: AUC = 0.25 × (fasting value) + 0.5 × (30-min value) + 0.75 × (1-h value) + 0.5 × (2-h value).

Proteins involved in gliosis and intracellular signaling pathways were analyzed in the hypothalamus by Western blotting. Relative levels of mRNA of various cytokines and adipokines were quantified in the hypothalamus and adipose tissue by real time PCR.

Perfused brains from PND150 rats were processed for GFAP and Iba1 immunohistochemistry to determine the number and morphology of GFAP and number of Iba1 positive cells in the hypothalamus. Double immunohistochemistry was also performed to analyze the expression of the leptin receptor (ObR) in GFAP positive cells.

Table 1 and 2 outline the experimental groups and ages of sacrifice, as well as the description of the samples collected at each age and the analysis performed in each of them.

EXPERIMENTAL GROUPS	
Males from litters of 12 pups	ML12
Males from litters of 4 pups	ML4
Females from litters of 12 pups	FL12
Females from litters of 4 pups	FL4

	AGE OF SACRIFICE					
	PND 10	PND 21	PND 30	PND 50	PND 85	PND 150
Samples						
BLOOD	X	X	X	X	X	X
HYPOTHALAMUS	X	X	X	X	X	X
SCAT	X	X			X	X
VAT		X			X	X
Perfused brain						X
Analysis						
Serum hormones and cytokines	X	X	X	X	X	X
Serum lipids						X
Cytokine expression in AT	X	X			X	X
Proteins involved in gliosis	X	X	X	X	X	X
IHC						X

Table 1 & 2. Description of the experimental groups, the ages at which they were sacrificed as well as the samples collected and the analysis performed. PND: postnatal day, AT: adipose tissue, IHC: Immuohistochemistry.

1.1.2 Neonatal androgenization

Wistar rats raised in the animal facility of the Medical School of University of Córdoba, Spain, were used for this study. The rats were kept under conditions of constant temperature (22-24ºC) and humidity (50-55%) and 12 hour light-dark

cycles. They were allowed *ad libitum* access to chow diet (A04-10/15022 Panlab, Barcelona, Spain) and tap water.

c) Experimental design

In order to better understand the effect of neonatal sex steroids on metabolism, three groups of animals were generated. On PND1, female rats received a subcutaneous injection of 1.25 mg of testosterone propionate (SIGMA T-1875) dissolved in 100 µL of olive oil [approximately 208 mg/kg; androgenized females (AF)] or 100 µL of olive oil, as vehicle (control females, F). Males (M) also received vehicle (100 µL of olive oil). Rats were sacrifice on PND10 or PND90. Vehicle treated adult females sacrificed at PND90 were monitored to control the estrus cycle by performing vaginal swabs two weeks before sacrifice being all of them sacrificed on the morning of diestrus. Androgenized females did not show external signs of puberty or regular cycles. The experimental design is shown in Figure 11.

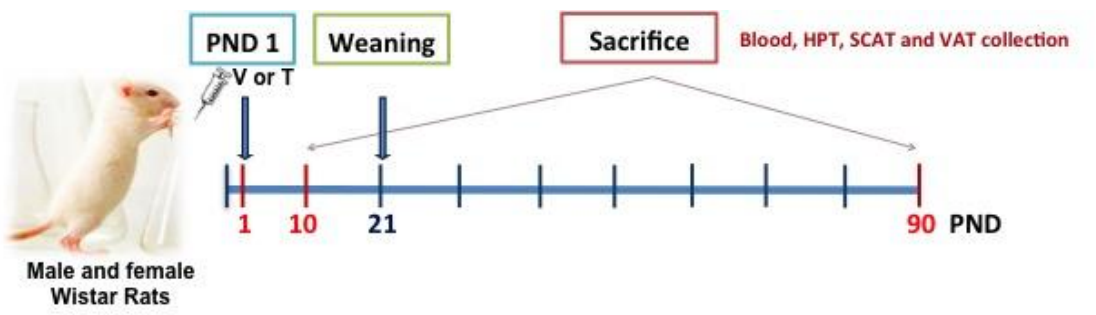


Figure 11. Schematic representation of the neonatal androgenization protocol in male (M), female (F) and androgenized female (AF) Wistar rats. T: testosterone; V: vehicle; PND: postnatal day.

d) Sacrifice and sample collection

Half of each group was killed on PND10 and half on PND90. PND 90 animals were fasted for 12 hours before sacrifice. At PND 90, 3-4 rats of each group were perfused transcardially as described below. The number of animals in each group is

shown in Table 3. Body weight, length and glycemia were determined on the day of sacrifice.

	PND 10		PND 90
	Fresh	**Perfused**	**Fresh**
Males	M (n=10)	X	M (n=6)
Females	F (n=10)	X	F (n=8)
Androgenized females	AF (n=10)	X	AF (n=8)

Table 3. Description of the experimental groups in the neonatal androgenization study and the total number of animals in each group.

The hypothalamus was removed as described above. Fat pads (subcutaneous and visceral) were collected and weighed.

Serum levels of testosterone, 17β-estradiol, leptin, insulin, IL6, IL1β, TNFα and adiponectin were measured by ELISA or multiplex arrays in all samples.

Vimentin and GFAP levels were analyzed by Western blotting in the hypothalamus of PND10 and 90 rats.

IL6, TNFα, IL1β, leptin and adiponectin, mRNA levels were determined in subcutaneous (PND 10 and PND 90) and visceral (PND 90) adipose tissues by RT-PCR.

Perfused visceral fat was processed for analysis of adipocyte morphology after hematoxilin-eosin staining at PND 90.

1.2 IN VITRO STUDIES

All plastic materials used to perform primary astrocyte cultures were acquired from Falcon (Becton Dickinson, Franklin Lakes, NJ, United States). The culture media was from Gibco (Invitrogen Co., Auckland, New Zeeland).

1.2.1 Primary cultures of hypothalamic astrocytes

Primary cultures of hypothalamic astrocytes were prepared from PND2 Wistar rat pups. Both sexes were used and male and female cells were cultured separately. Ano-genital distance was used to determine the sex of the animal. 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 carefully dissected and the meninges carefully removed. Hypothalami were then triturated in the same media. 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 minutes. The resulting pellet was resuspended in DMEM/F12 enriched with 10% fetal bovine serum (FBS) plus 1% Ab/Am solution. Cells (1 mL) were then seeded in 75 cm^2 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% CO_2. 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% confluence, flasks were placed in a 37 °C shaking incubator (SI-300, Jeoi Tech; Medline Scientific) at 280 rpm for 6 hours.

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 minutes 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 $4.35X10^5$ or $1X10^6$ cells/plate, respectively. Cells were then grown for 24 hours. After 24 hours, 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 to 4). Figure 12 resumes the procedure followed to obtain cultures of primary hypothalamic astrocytes and Figure 13 the protocol followed after purification.

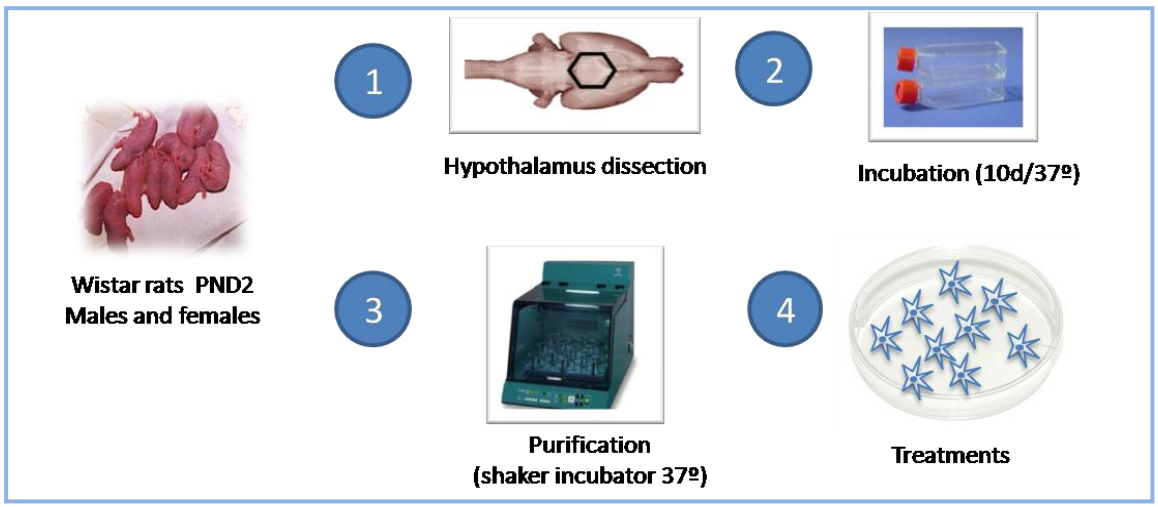


Figure 12. Schematic representation of the main steps carried out to obtain primary astrocyte cultures.

This protocol results in astrocyte enriched cultures. The purity was assessed by immunocytochemistry for glial fibrilary acidic protein (GFAP), a marker of astrocytes, and ionized calcium-binding adapter molecule 1 (Iba1), a marker of microglia. No staining with the Iba-1 antibody was found, as shown in figure 14. In addition, the expression of Iba1, galactocerebroside (GalC) a marker for oligodendrocytes and S100 calcium binding protein A4 (S100A4) as a marker of fibroblast were analyzed by RT-PCR. The expression of these markers decreased inversely with the time of shaking before plating the cells.

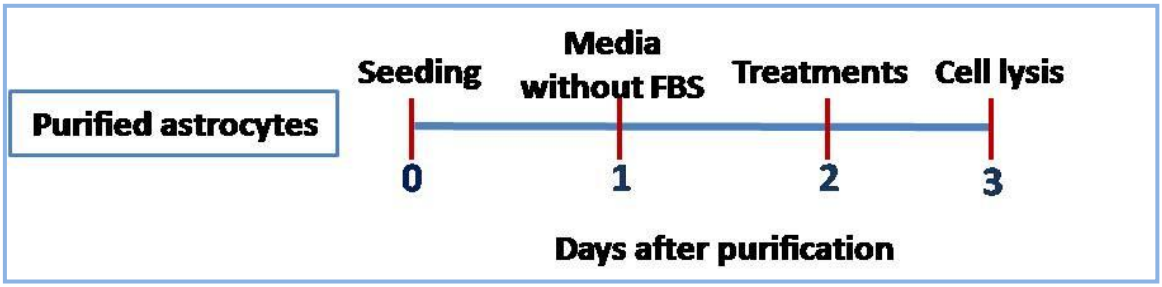


Figure 13. Protocol followed after purification to obtain astrocyte enriched primary cultures. FBS: fetal bovine serum.

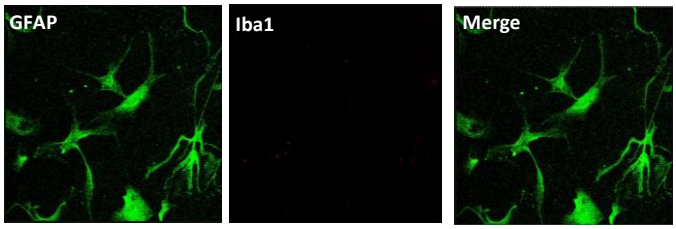


Figure 14. Double immunocytochemistry for GFAP (green) and Iba1 (red) to verify astrocyte primary cultures purity. No immunolabeling of Iba1 can be observed.

1.2.1.1 Treatments

Astrocytes from males and females received the following treatments:

a) Fatty acids

All reagents were purchased from Sigma-Aldrich, Inc. (Saint Louis, MO, USA). Fatty acid supplemented medium was prepared according to *previously published* protocols **(Huynh et al., 2014)**.

Palmitic acid (PA) and oleic acid (OA) were used to treat astrocytes. Fatty acid-free bovine serum albumin (BSA) was added to ensure lipid solubility in the aqueous solution. A dose-response curve of increasing concentrations (0.05mM, 0.1mM, 0.25mM and 0.5mM) was performed for PA. When used in combination, PA and OA were used at a concentration of 0.5mM each [0.5mM oleate:palmitate (1:1)]. Control plates received an equivalent amount of vehicle solution.

Stock solutions of sodium oleate (Sigma, O-3880), palmitic acid (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.

A stock solution of 100 mM sodium oleate was prepared in H_2O. To facilitate solubilization of the fatty acid, 25 µL of 1N NaOH were added. The solution was incubated at 37°C in a water bath during approximately 30 minutes and vortexed occasionally until the solution was clear. The PA stock solution was prepared at 200 mM in ethanol and vortexed until the solution was clear. The BSA stock solution was prepared at 30% and L-carnitine at 200 mM; both of them were dissolved in H_2O.

Fatty acids were 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) medium to achieve the desired final fatty acid concentration. The L-carnitine (1 mM) was added to the final fatty acid concentration solution before being added to cultures, as this appears to be an essential component of the lipid-induced insulin resistance with certain fatty acids.

b) β-estradiol

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

When 17β-estradiol was used in combination with PA, astrocytes were "pre-treated" with 10^{-9} M β-estradiol for 3 hours previous to PA addition.

c) Ultrapure lipopolysaccharide from Rhodobacter sphaeroides (LPS-RS) - TLR4 antagonist

LPS-RS Ultrapure was purchased from InvivoGen (San Diego, CA, USA) as a purified preparation of the TLR4 antagonist LPS-RS. A stock solution of 1mg/mL was

prepared in water. Serial dilutions were prepared to obtain a working concentration of 300ng/mL according to the commercial source recommendations and literature **(Lu et al., 2013).** Primary astrocytes were pretreated with LPS-RS for 2 hours followed by treatment with PA 0.1mM.

2. Techniques and Protocols

Reagents were acquired from Merck KGaA (Darmstadt, Germany) or Sigma-Aldrich, Inc. (Saint Louis, MO, USA), unless stated otherwise.

2.1 Determination of glycemia and serum hormone, cytokine and lipid concentrations

2.1.1. Blood glucose levels

Glycemia was determined by using a glucose meter and test strips (Optium Plus, Abbot Diabetes Care, Witney Oxon, UK) in blood obtained from a lateral tail vein puncture.

2.1.2 Determination of serum levels of leptin, adiponectin, estradiol and testosterone by enzyme-linked immunosorbent assay (ELISA)

Serum leptin and adiponectin levels were quantified by using ELISAs purchased from Millipore (Millipore, Billerica, MA, USA) and performed according to the manufacturer's instructions.

Briefly, assay buffer and samples were added to the corresponding wells and incubated for 2 hours at room temperature while shaking at 400-500 rpm. All subsequent incubations were done under the same conditions of temperature and shaking. As instructed, for the adiponectin assay serum samples were diluted (1:500) with assay buffer. Molecules of adiponectin in the samples were captured by the microtiter plate coated with a monoclonal adiponectin antibody. After the 2 hour incubation, the wells were washed to remove unbound antibody. In contrast, the leptin assay requires that the antiserum solution be added to the sample and then

the resulting complexes are added and bound to the wells that were previously coated with another anti-leptin antibody (sandwich). The following steps were similar for both assays. A purified biotinylated antibody (anti-rat adiponectin or anti-rat leptin) was added to detect the immobilized adiponectin or leptin molecules and incubated for 1 hour. After the corresponding washes were carried out in a HydroFlex (Tecan) microplate washer, streptavidin-horseradish peroxidase conjugate was added for during 30 minutes to bind to the immobilized biotinylated antibody and washed afterwards to remove the excess of free enzyme conjugates. Lastly, peroxidase substrate was added and horseradish peroxidase (HRP) activity monitored until the stop solution was added. Quantification was measured in a spectrophotometer (Tecan Infinite M200, Grödig, Austria) at a wavelength of 450 nm and corrected at 590 nm. The increase in absorbency is directly proportional to the amount of captured rat leptin or adiponectin in the unknown samples. The concentrations were calculated from the standard curve supplied with the kits. Sensitivities of the method for leptin and adiponectin were 0.08 and 0.4 ng/ml, respectively. All samples were run in duplicate. The intra-assay coefficient of variation (CV) was 2.2 % for leptin and 1.3% for adiponectin and the inter-assay CV was 3.4 % for leptina and 6.9 % for adiponectin.

Serum estradiol and testosterone levels were determined by using an ELISA kit from Cusabio Biotech CO., LTD (Wuhan, P.R. China) according to the manufacturer's protocol. Microtiter plates pre-coated with a goat-anti-rabbit antibody towards the substance to be analyzed were used. Standards or samples were added in duplicate to the appropriate wells followed by addition of HRP-conjugated testosterone or estradiol. Then, a specific antibody was added to each well. The plates were incubated for 1 hour for the testosterone assay and 2 hours for estradiol. After incubation and the appropriate washes, substrate solutions were added to each well and incubated for 15 minutes at 37ºC. Stop solution was then added to terminate the enzyme-substrate reaction. Finally, the color change was measured at a wavelength of 450 nm (Tecan Infinite M200). Estradiol and

testosterone concentrations in the samples were calculated comparing the O.D. of the samples to the standard curve. The estradiol assay has a sensitivity of 25 pg/ml and the testosterone assay 0.06 ng/mL. Inter- and intra-assay coefficients of variation are <15% for both assays. All samples were run in duplicate.

2.1.3 Determination of serum levels of insulin, interleukin 6, interleukin 1β and tumor necrosis factor α with a multiplex bead immunoassay

Circulating levels of insulin, IL6, IL1β and TNFα were determined in duplicate by using a multiplexed bead immunoassay kit according to manufacturer's specifications (Millipore). Briefly, beads conjugated to the appropriate antibodies and serum samples (25 µl each) were incubated overnight at 4°C and under moderate shaking. The next morning, plate wells were washed three times with the provided wash buffer. Then, the biotinylated detection antibody (50 µl) was added and incubated for 2 hours at RT while shaking at 500 rpm. Finally, 50 µl of the reporter dye, streptavidin-conjugated phycoerythrin, was added and incubated for 30 minutes. After washing, the beads were analyzed in a Bio-Plex suspension array system 200 (Bio-Rad Laboratories, Hercules, CA, USA) and mean fluorescence intensity was analyzed using Bio-Plex Manager Software 4.1.

	Insulin	**IL-6**	**IL1-β**	**TNF-α**
Sensitivity (pg/mL)	52.5	8.8	1.2	3.2
Intra-assay CV (%)	1.7-4.2	1.7-4.2	1.7-4.2	1.7-4.2
Inter-assay CV (%)	2.8-13.6	2.8-13.6	2.8-13.6	2.8-13.6

Table 4. Assay sensitivities (minimum detectable concentrations, pg/mL), intra- and inter-assay coefficient of variation for multiplex bead immunoassay. CV=coefficient of variation

2.1.4 Determination of triglycerides in serum samples

Total triglycerides were measured in serum samples of PND150 rats by using a commercial kit purchased from *Randox Laboratories Limited (United Kingdom).* Serum samples were diluted 1:2 in saline. Triglycerides in the samples are quantified after enzymatic hydrolysis with lipases. The colorimetric indicator is quinoneimine

formed from hydrogen-peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase. The reaction mix was incubated for 10 minutes at room temperature and the absorbance measured at a wavelength of 500 nm (Tecan Infinite M200). Triglyceride concentrations (mg/dl) in the samples were calculated comparing the O.D. of the samples to the standard curve.

2.1.5 Determination of non-esterified free fatty acids in serum samples

The kits for the quantitative determination of non-esterified free fatty acids (NEFA) were purchased from Wako (Neuss, Germany) and followed the ACS-ACOD (Acyl-CoA synthethase – Acyl-CoA oxidase) method. The concentrations of NEFA were determined by a reaction of the serum sample with reactive A (0.3 kU/l acyl-CoA-synthetase, 3 kU/l ascorbate oxydase; 06 g/l coenzyma A, 5 mmol/l ATP, 1.5 mmol/l 4-aminophenazone, 50 mmol/l phosphate buffer, pH = 6.9; 3 mmol/l magnesium chloride). After an incubation of 10 minutes at 37 ºC reactive B (6.6 kU/l acylCoA oxidase; 7,5 kU/l peroxidase; 1.2 mmol/l methyl-N-ethyl-N-hydroxyethyl aniline) was added. The mixture was incubated again for 10 minutes at at 37 ºC, after which the absorbance was measured at 550 nm. The concentration of NEFA was than determined by comparison with a standard curve prepared from serial dilutions of oleic acid in saline.

2.2 Protein analysis

2.2.1 Total protein extraction

a) Protein extraction from tissue

Individual hypothalami were homogenized on ice in 300-500 ul of lysis buffer, with the volume of buffer varying according to the size of the hypothalamus. The lysis buffer, radioimmunoprecipitation assay buffer (RIPA), contained sodium phosphate buffer (PBS, 0.1 M, pH 7.4), 1 % Triton X-100, sodium dodecyl sulphate (SDS, 0.1 %), sodium azide (0.5 %), ethylenediaminetetraacetic acid (EDTA; 2 mM), sodium deoxycholate (0.5 %), phenylmethanesulfonyl fluoride (PMSF; 1 mM) and a

protease inhibitor cocktail (Roche Diagnostics, GmbH). Lysates were incubated overnight at -80°C and then centrifuged at 14000 rpm for 10 minutes at 4°C. Supernatants were collected and stored at -80°C for later use. Total protein concentration was determined by the method of Bradford (Protein Assay; Bio-Rad Laboratories).

b) Protein extraction from hypothalamic astrocytes primary cultures

To extract protein from primary astrocyte cultures, the cells were first washed twice with PBS. The PBS was then totally removed and the cells lysed with 200 µl of lysis buffer (25 mM HEPES, 150 mM KCl, 2 mM EDTA, 0.1% Igepal, 1 mM PMSF, 10 uM benzamidine and leupeptin and 0.5 mM DTT) in each dish. The samples were frozen at - 80°C to boost cell lysis. Next, samples were centrifuged for 20 minutes at 14000 rpm (4°C). Following centrifugation, supernatants were removed and frozen over night. Finally, the samples were lyophilized (Cryodos. TELSTAR. Tarragona, Spain) and the resulting pellet resuspended in 40 µl of sterile water and stored at –80°C.

Total protein concentration was determined by the method of Bradford (Protein Assay; Bio-Rad Laboratories).

2.2.2 Quantification of protein concentration

Total protein concentration was determined by the method of Bradford (Protein Assay; Bio-Rad Laboratories), developed by Marion M. Bradford in 1976 (Bradford, 1976). This colorimetric method is based on the principle of protein-dye binding and the shifts caused in the absorbance that is measured by using a spectrophotometer.

Samples were diluted in distilled water (primary cultures) or lysis buffer (tissue) and mixed with Bradford reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA), following the manufacturer's instructions and incubated for 5 minutes at room temperature (RT). For each assay, a standard curve was prepared with increasing concentrations, from 0 to 25 µg/µl of BSA. The standard curve and unknown samples

were pipetted into a 96-well plate and the absorbance measured at 595 nm in an TECAN Infinite M200 spectrophotometer.

2.2.3 Western blotting

The reagents used for western blotting analysis were purchased from Bio-Rad Laboratories, Inc. except when indicated.

An appropriate concentration of total protein (10, 40 or 60 µg depending on the protein to be analyzed) was mixed with an equal volume of Laemmli buffer (2x), containing Tris-HCl (1 M, pH 6.8), glycerol (25 %), SDS (2 %) and bromophenol blue (0.01 %) and denatured in a thermoblock for 5 minutes at 100 ºC.

The protein was resolved on SDS-polyacrylamide gels under denaturing conditions and electro-transferred to polyvinylidene difluoride (PVDF) membranes. Transfer efficiency was determined by Ponceau red dyeing. Membranes were blocked with Tris-buffered saline (TBS, 20 mM) containing 0.1% Tween 20 and 5% nonfat dried milk or 5% BSA for 2 hrs and incubated overnight at 4°C under agitation with the primary antibody at a concentration of 1:1000 unless otherwise stated. Primary antibodies used were against glial structural proteins and indicators of glial activation: GFAP, vimentin, Iba1; intra- cellular signaling proteins: pAKT (1:500), AKT, pIKB (1:500), STAT3, pSTAT3$^{(tyr705)}$ (1:500), pSTAT3$^{(Ser634)}$ (1:500), pIRS (1:500), pPTEN (1:500), PTEN, SOCS3 and the housekeeping protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and actin. Membranes were washed with TBS and incubated with the corresponding secondary antibodies conjugated with peroxidase (Pierce Biotechnology, Rockford, IL, USA). Peroxidase activity was visualized by chemiluminescence (Perkin Elmer Life Science, Boston, MA, USA) and quantified with a Kodak Gel Logic 1500 Image Analysis system and Molecular Imaging Software, version 4.0 (Rochester, NY, USA) and ImageQuant Las 4000 Software (GE Healthcare Life Sciences, Barcelona, Spain). Gel loading variability was normalized with either the non-phosphorylated form of the protein, GAPDH or actin. Data were normalized to control values on each gel.

2.2.4 Determination of hypothalamic levels of leptin, IL1β, IL6, IL10 and TNFα by using a multiplex magnetic bead immunoassay

Hypothalamic levels of leptin, IL6, IL1β, IL10 and TNFα were determined in duplicated by a multiplexed magnetic bead immunoassay kit according to the manufacturer's specifications (Millipore). Beads conjugated to the appropriate antibodies and 5 µg of hypothalamic lysate in assay buffer (25 µl each) were incubated for 2 hours at RT and under moderate shaking. Afterwards, plate wells were washed two times with the provided wash buffer and 25 µl of the biotinylated detection antibody was added and incubated for 1 hour at RT while shaking at 500 rpm. Finally, 25 µl of the reporter dye, streptavidin-conjugated phycoerythrin, was added and incubated for 30 minutes. After washing, the beads were analyzed in a Bio-Plex suspension array system 200 (Bio-Rad Laboratories) and mean fluorescence intensity was analyzed using Bio-Plex Manager Software 4.1.

	Leptin	IL-6	IL1-β	IL10	TNF-α
Sensitivity (pg/mL)	10.2	30.7	2.8	2.7	1.9
Intra-assay CV (%)	3.4	2.3	3.6	3.8	2.7
Inter-assay CV (%)	14.3	12.7	11.3	9	10.8

Table 5. Assay sensitivities (minimum detectable concentrations, pg/mL), intra- and inter-assay coefficient of variation for rat cytokine magnetic bead immunoassay. CV = coefficient of variation

2.2.5 Multi-pathway magnetic bead kit primary cultures

A Multi-Pathway Signaling Magnetic Bead kit, was used to detect changes in phosphorylated ERK/MAP kinase 1/2 (Thr185/Tyr187), Akt (Ser473), STAT3 (Ser727), JNK (Thr183/Tyr185), p70 S6 kinase (Thr412), NFkB (Ser536), STAT5A/B (Tyr694/699), CREB (Ser133), and p38 (Thr180/Tyr182) and the corresponding total protein levels in astrocyte cell lysates using the Luminex® xMAP® technology.

After corresponding treatments, astrocytes were lysed in the supplied lysis buffer with protease inhibitors added. The amount of protein/well was adjusted to a concentration of 15 µg by diluting with the provided assay buffer. The protocol was followed according to the manufacturer's specifications (Millipore). Beads

conjugated to the appropriate antibodies and astrocytes cell lysates (25 µl each) were incubated O/N at 4°C with shaking and protected from light. The next morning, plate wells were washed two times with the provided assay buffer and 25 µl of the biotinylated detection antibody was added and incubated for 1 hour at RT with shaking and in dark. Then, 25 µl of the reporter dye, streptavidin-conjugated phycoerythrin, was added and incubated for 15 minutes after which 25 µl amplification buffer was added for 15 minutes. Finally, the beads were analyzed in a Bio-Plex suspension array system 200 and mean fluorescence intensity was analyzed using Bio-Plex Manager Software 4.1.

2.3 Quantification of relative levels of mRNA

2.3.1 Total RNA isolation

Total mRNA was isolated from the entire hypothalamus, subcutaneous and visceral fat pads, and astrocyte cultured cells using the TRIzol® Reagent method (Invitrogen) and according to manufacturer's instructions. Primary astrocytes grown in petri dishes were rinsed with ice-cold PBS before 1 mL of TRIzol® was added in order to lyse and collect the cells. For the hypothalamus or adipose tissue, 1 mL of TRI-Reagent was used.

Total mRNA concentration was quantified using the spectrophotometer Nanodrop 1000, from Thermo Scientific.

2.3.2 Quantitative real-time polymerase chain reaction assay (qRT-PCR)

Relative mRNA levels were quantified in subcutaneous and visceral adipose tissue and primary cultured astrocytes by qRT-PCR. Complementary DNA (cDNA) was synthesized from 1 or 2 µg of total mRNA by using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA).

Quantitative RT-PCR was performed by using assay-on-demand kits (Applied Biosystems, Foster City, CA, USA). Taq-Man Universal PCR Master Mix (Applied

Biosystems) was used for amplification according to the manufacturer's protocol in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Values were normalized to the housekeeping genes 18S and Rpl13a. According to manufacturer's guidelines, the ΔΔCT method was used to determine relative expression levels. Statistics were performed using ΔΔCT values. All data are expressed as % control at each age.

In adipose tissue, leptin, adiponectin, (IL) 1β, 6 and TNFα genes were analyzed. In adipose tissue, various housekeeping genes were analyzed to find an internal control that did not vary between groups. However, we were unable to find suitable housekeeping genes that did not vary with age, sex and litter size; thus, the mRNA levels of the genes analyzed in adipose tissue were not compared between ages. Results are compared at each age to determine the effects of sex and litter size and were normalized to two of the following: phosphoglycerate kinase 1 (Pgk-1), cyclophilin A (PPIA) or ribosomal protein S 18-like (Rps18). The housekeeping genes used did not change between groups at a given age. All data are expressed as the percent of the values of L12 males at each age.

In primary cultures the following genes were analyzed: GFAP, vimentin, (IL) 1β, 6, TNFα DDIT, IGF-1, Esr1, Esr2, NFκb, Iκbb Results were normalized to Rps18 or ribosomal protein L13a (Rpl13a).

The primers (Applied Biosystems) employed for the analysis of gene expression by RT-PCR are shown in Table 6.

Gene	Reference
Adiponectin	Rn00595250_m1
CHOP	Rn00492098_g1
GFAP	Rn00566603_m1
IGF-1	Rn99999087_m1
IKBKB	Rn00584379_m1
IL-1β	Rn01336189_m1
IL-6	Rn01410330_m1
IL-10	Rn00563409_m1
Leptin	Rn00565158_m1
NFKBIA	Rn01473657_m1
PPIA	Rn00690933
Pgk-1	Rn00821429
Rpl13a	Rn00821946_g1
TNFα	Rn01525859_g1
Vimentin	Rn00579738_m1
18s	Rn01428915_g1

Table 6. List of primers used in adipose tissue and astrocyte primary cultures.

2.4 Transcardial perfusion

Tissue fixation was carried out by transcardial perfusion *in vivo* with 0.1 M phosphate buffer (PB) and then PB-buffered 4% paraformaldehyde (PFA; pH 7.4) and 1% glutaraldehyde (GA) in PB, perfusing approximately 300 mL/rat. The animals were previously profoundly anesthetized with an intraperitoneal (IP) injection of pentobarbital (1 mg/kg; Braun Vetcare, Barcelona. Spain).

When perfusion was completed, brains and subcutaneous and visceral fat pads were collected and post-fixed overnight in 4% PFA overnight at 4°C. After three washes with PB 0.1 M, they were stored in cryoprotection solution (30% sucrose, 30% ethylene glycol in PB) at -18°C until used. PND 10 and 21 rats were not perfused *in vivo.* At sacrifice, the brains and fat pads were directly placed into 4% PFA (pH 7.4) and fixed in this solution overnight at 4°C.

2.4.1 Histological sections

2.4.1.1 Perfused brain sections

Perfused brains were used to obtain 40 µm coronal sections by using a vibratome (Leica VT1200S, Leica Biosystems Nussloch GmbH, Nussloch, Germany). According to Paxinos and Watson atlas "The rat brain in stereotaxic coordinates", all sections that contained the arcuate nucleus (between 2.3 and 3.3 mm from Bregma), were collected and preserved in cryoprotecion solution and stored at -18ºC until used for immunohistochemistry.

2.4.1.2 Visceral adipose tissue sections

Visceral adipose tissue was sectioned for subsequent histological study of adipocyte morphology. Sections of 15 µm were obtained with a cryostat (Leica, Madrid, España).

Samples were equilibrated to -15ºC inside the cryostat workstation for 45 minutes. A block of frozen visceral fat was then carefully attached to a microtome chuck with aspecial tissue glue that allows frozen sectioning at the optimal cutting temperature (OCT media; Tissue Tek, Electron Microscopy Sciences, Hatfield, PA, USA).

The 15 µm sections were mounted onto poly-lysine treated microscope slides and stored at -20ºC until stained with hematoxilyn-eosin for examination.

2.5 Staining techniques for histological studies

2.5.1 Immunohistochemistry in the hypothalamus

Immunohistochemistry was performed on coronal free-floating brain sections (40 µm). Six sections per animal were selected throughout the arcuate nucleus. All experimental groups were assayed in parallel.

All washes and antibody incubations were done under mild shaking on a rotating platform. The wash solution (PBT) consisted of PB (0.1M; pH 7.4), 0.3% Triton X-100 and 0.3% BSA. This buffer was used in all washes and incubations.

Free-floating sections were first washed several times with PB to completely remove the cryoprotection solution. Next, suppression of endogenous peroxidase activity was carried-out by incubating the sections in 30% methanol containing 3% hydrogen peroxide for 30 minutes at room temperature. The sections were rinsed with wash solution (3 times, 10 minutes each) and incubated in blocking solution (3% triton X-100 and 3% BSA in PB) for 2 hours at room temperature to reduce non-specific binding.

The sections were then incubated with a primary antibody that recognizes either GFAP (Sigma, 1:1000) or Iba1 (Wako, 1:500) overnight at 4ºC. The following day, the sections were washed (3 times for ten minutes each) and incubated in biotinylated anti-mouse secondary antibody (1:2000 for 2 hours at RT) or biotinylated anti-rabbit secondary antibody (1:1000 for 2 hours at RT). Afterwards, 3 washes were done and sections were placed in avidin-biotin complex (ABC 32020; Pierce Biotechnology) for signal amplification during 2 hours. The ABC complex was diluted 1:500 in washing solution plus 5% BSA. The slices were then washed 3 times.

Finally, peroxidase activity was revealed by incubating sections with 0.03% 3-3’ diaminobenzidine (DAB; Sigma) and 0.01% hydrogen peroxide in PB. The reaction was monitored until the signal was clearly developed (approximately 2-3minutes). The sections were then placed in 0.1 M PB and mounted on poly-L-lysine hydro-bromide (50 µg/ml; Sigma) coated glass slides. Lastly, and after dehydration in increasing concentrations of ethanol (70%, 96%, 100%) and then xylol, samples were covered with Depex (BDH Laboratory Supplies, United Kingdom) and a coverslip applied. The slides were examined by using an optical microscope (ZEISS. Thornwood, NY, USA). Images were captured at 40X magnification by using a digital camera and Image Pro-Plus software. Immunostaining was absent when the primary antibody was omitted.

2.5.2 Double immunohistofluorescence

Double labeling for GFAP (Sigma) and leptin receptor, Ob-R (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was performed on coronal free-floating brain sections (40 µm). Six sections per animal were selected throughout the arcuate nucleus. All experimental groups were assayed in parallel.

The same solution for washes, blocking and antibody incubation as described in 2.3.1 section were used for immunohistofluorescence. Free-floating sections were washed several times with PB to completely remove the cryoprotection solution followed by one wash with PBT and then incubated for 1.5 h in the blocking solution. Primary antibodies were then added, GFAP (mouse, 1:1000) and leptin receptor Ob-R (goat, 1:250)., and incubated at 4ºC under mild shaking for 48 hours.

After primary antibody incubation, the sections were washed (3 times for ten minutes each) and incubated in biotinylated anti-goat secondary antibody (1:1000 for 5 hours at RT) for Ob-R signal amplification. Afterwards, 3 washes were done and sections were placed in the secondary antibody Alexa Fluor® 633 goat anti-mouse IgG (Molecular Probes. Eugene, OR, USA, 1:2000) and streptavidin, Alexa Fluor® 488 conjugate (Molecular Probes, 1:2000) and incubated in the dark for 1.5 h. The slices were then washed 3 times with 0.1 M PB and carefully mounted while being protected from the light and then a coverslip was placed over the tissue using Clear Mount.

Images were captured using a confocal fluorescence microscope (Leica) with wavelengths of 495 nm and 632 nm being used for the excitation of Alexa-Fluor-488 and Alexa-Fluor-633 fluorophores, respectively. Negative controls, where the primary antibody was omitted, were included in every assay and in which immunofluorescence signal was absent.

2.5.3 Hematoxylin and eosin staining of visceral adipose tissue (VAT)

Visceral adipose tissue slices (15 µm thick) were stained with hematoxylin-eosin. First, sections were fixed in a 10% buffered formalin solution for 15 minutes. Following a rinse with distilled water, sections are soaked in hematoxylin (Panreac,

Barcelona, Spain) for 10 minutes to stain the nuclei. Next, eosin (Leica Biosystems, Barcelona, Spain) staining was carried out during 1 minute and the slides then rinsed with water. Sections were dehydrated in increasing concentrations of ethanol (70°, 96°, 100°) for 1 minute each and then 3 minutes in xylol. Depex was used to place a coverslip over the tissue. Sections were imaged with an optical microscope (ZEISS) using a 40X objective and the digital images were captured with a digital camera (JAI Corporation, CV-S3200, Japan) and analyzed with Image-Pro Plus software (version 5.0; Media Cybernetics Inc., Silver Spring, MD, USA). The mean perimeter and area of the adipocytes were calculated from visceral adipose tissue slices from 4 animals per group, analyzing 4 sections per animal and measuring 5-10 cells per section.

2.6 Quantification of GFAP+ cells and morphological analysis

For the quantitative evaluation of astrocytes, six sections per animal throughout the arcuate nucleus were analyzed. In each section, images of a single focal plane from twenty rectangular fields of the arcuate nucleus (area of 19.5 mm^2 per field), ten on each side of the third ventricle, were captured using 40X magnification with an optical microscope attached to a digital camera. Images were processed using Image-Pro Plus software.

The number of GFAP immunoreactive (GFAP+) cells per field was counted, as well as the number of primary projections (those emerging from the soma) of each GFAP positive cell determined with ImageJ software. All morphometric analyses were performed without previous knowledge of the experimental group (samples were blinded for analysis).

Analysis of the mean levels of Ob-R immunoreactivity in GFAP positive cells in the arcuate nucleus was performed using ImageJ software. The red and green signals over individual somas were separated, measured and recorded in a mean of 10 cells per field and 10 fields/animal throughout the arcuate nucleus. From these measurements, the mean intensity of Ob-R immunoreactivity in the soma of GFAP positive cells was determined.

2.7 Double immunocytofluorescence for GFAP and Iba1 in primary astrocyte cultures

Double immunocytofluorescence was performed in hypothalamic astrocytes primary cultures from postnatal day 2 Wistar rats in order to determine if microglia can be detected in the enriched astrocyte cultures. An antibody against GFAP was used to label astrocytes, whereas the Iba 1 antibody was used to identify microglia.

Cells were seeded on round coverslips that had been placed in 24-well plates and previously treated with poly-L-lysine hydrobromide (10 µg/ml; Sigma-Aldrich). All steps were performed in the well plate until coverslips were removed to be mounted.

Forty-eight hours after the cells were seeded the wells were washed three times with cool PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature. The wells were washed 3 times and then equilibrated with buffer consisting of 0.1 M PB (pH 7.4), 0.1% BSA and 0.3% Triton-X100 for 15 minutes. Blocking solution [0.1 M PBS, (pH 7.4), 0.3% BSA and 0.3% Triton-X100] was added to the samples for 1.5 hours. The two primary antibodies, mouse anti-GFAP (Sigma, 1:1000) and rabbit anti-Iba1 (Wako, 1:1000) were added to the corresponding wells and incubation was then carried out overnight at 4ºC.

The following morning, the primary antibody solution was removed and the wells rinsed with PB 0.1M, three times, 5 minutes each wash. The two secondary antibodies (diluted in blocking solution) were added and incubated during 1 hour in the dark and at room temperature. The secondary antibodies (1:1000) used were goat anti-mouse and goat anti-rabbit IgG conjugated with Alexa Fluor® 488 and Alexa Fluor® 633 (Molecular Probes. Eugene, OR, USA), respectively. The final three washes (10 minutes each) were done in the dark and the coverslips were carefully mounted on microscope slides cover glasses using a drop of Clear Mount mounting medium and stored in the dark at 4ºC until examination.

Images were captured using a confocal fluorescence microscope. Negative controls, where the primary antibody was omitted, were included in every assay and in which immunofluorescence signal was absent.

Antigen	Host	Working dilution	Commercial source
GFAP	Mouse	1:1000	Sigma
Iba 1	Mouse	1:1000	Wako
Ob-R	Goat	1:500	Santa Cruz

Table 7. Antibodies used for immunohistochemistry and immunocytochemistry

2.8 Detection of nitrites and nitrates in culture media

Culture media was collected and kept frozen at -80ºC until used. Quantification of nitrites and nitrates (NO_3^- and NO_2^-) was performed according to *Miranda and colleagues* (Miranda et al., 2001). The assay is based on the simultaneous detection of nitrate and nitrite concentrations by reducing nitrates with vanadium (III) and the combined detection by using the acidic Griess reaction **(Griess, 1879)**. The resulting color is spectrophotometrically quantified at a wavelength of 540nm.

Briefly, in a 96-well plate, 100 µL of vanadium (III) chloride (VCl_3) was added to 100 µL of each cell culture media sample and, immediately, 100 µL of Griess reagent was added. The Griess reagent was prepared just before use by mixing equal volume of ethylenediamine dihydrochloride (NEDD) and sulfonamide. The plate was then incubated for 30 minutes at RT and the absorbance read (Tecan Infinite M200). The results were compared to a standard curve consisting of sodium nitrate ($NaNO_2$) in concentrations from 1 to 200 µM.

2.9 Estimation of the number of cells

The cristal violet dye elution (CVDE) method was employed to determine cell viability in response to each *in vitro* cell treatment. The cristal violet dye binds to cell nuclei, which is then solubilized and quantified spectrophotometrically. The resulting absorbance is proportional to DNA content and therefore, cell number.

For this assay, cells were grown in 24 well plates. The cells were washed twice with PBS and then 1 mL of 1% GT was added in order to fix the cells. After 10 minutes, the GT was removed and the cells were washed twice with PBS. The cristal violet solution (0.1%) was then added (1 mL) and the cells incubated at RT for 20 minutes. The cristal violet solution was removed and the cells rinsed under running tap water for 20 minutes. The plate was then left at RT for 2 hours or at 37°C for 30 minutes to dry completely. The cristal violet was then dissolved with 10% acetic acid, mixed well and 200 µL added to a 96 well plate and the absorbance read at 590 nm. The number of cells is calculated according to the following equation: Number of cells = (265030* absorbance)-1950.

2.10 Statistical analysis

The program SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. To determine the effect and/or interaction of two or more factors two or three-way ANOVAs were used. When significant effects were found with the two or three-way analyses, a subsequent two-way and/or one-way ANOVA followed by Scheffé's f test was used to determine whether specific differences existed amongst the experimental groups. Bonferroni correction was used for multiple comparisions in case of primary astrocyte cultures studies. Two-tailed Student's t tests were used to compare differences between two independent groups. 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 or Student's t test when indicated.

V. RESULTS

1. Effects of neonatal overnutrition throughout development in male and female rats

1.1 Effects of sex, litter size and age on body weight, body length and food intake.

1.1.1 Body weight

On the day of birth (PND0), all litters were organized and cross-fostered such that the mean starting weight did not differ between groups, as shown in Figure 15A. It is of note that body weight at birth was higher in males than females ($F_{(1,284)}$ = 34.1, p<0.0001; Figure 15B).

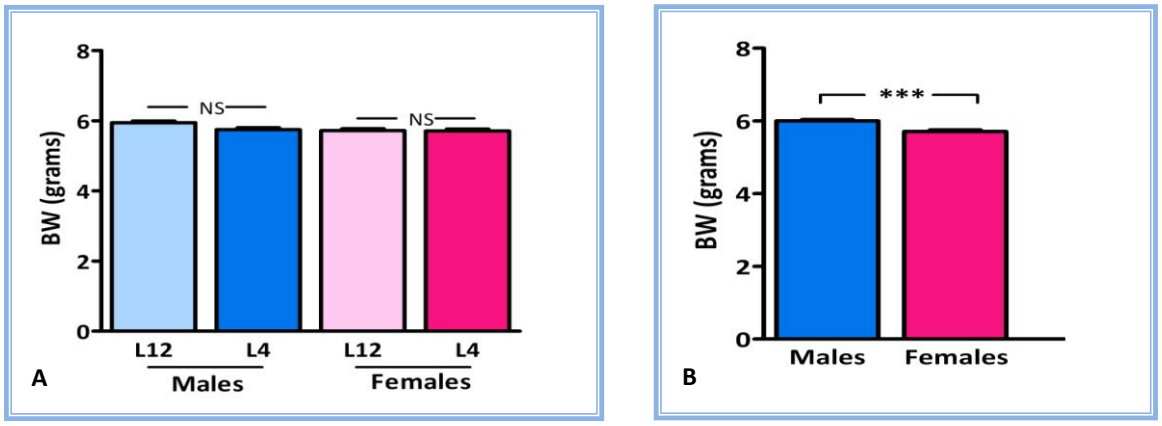


Figure 15. A. Mean body weight (BW) of each experimental group on the day of birth. N = 62-66 for each experimental group. NS = not significant. **B.** Mean BW of males and females on the day of birth n =124-126. Student's T test: *** = p<0.0001.

The changes in body weight, body length and food intake were studied from birth to PND150. There was an effect of sex ($F_{(1,1923)}$ = 12539.4, p<0.0001), litter size ($F_{(1,1923)}$ = 210.05, p<0.0001) and age ($F_{(20,1923)}$ = 4546.4, p<0.0001) on BW, with interactions between sex and litter size ($F_{(1,1923)}$ = 20.9, p<0.0001), sex and age ($F_{(20,1923)}$ = 381.1, p<0.0001) and litter size and age ($F_{(20,1923)}$ = 2.8, p<0.0001). Figure 16 shows the evolution of BW from birth to PND150.

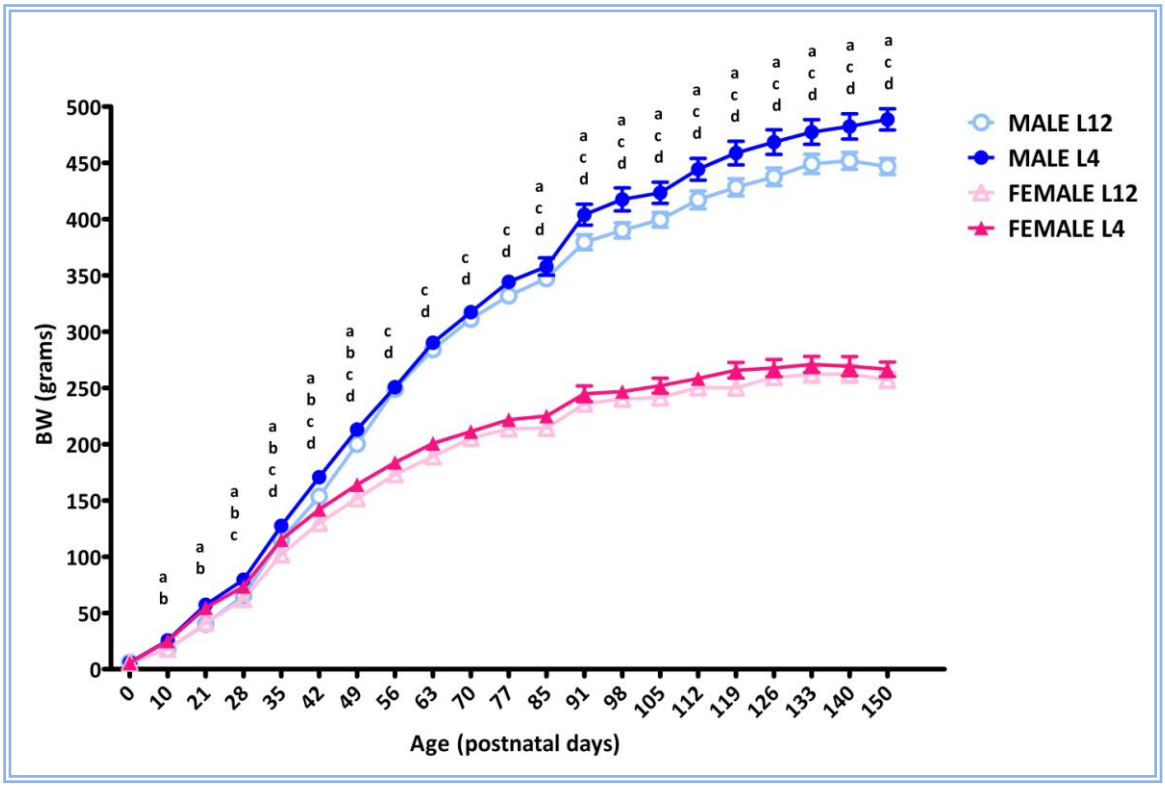


Figure 16. Change in body weight (BW) throughout development in male and female rats raised in control litters (L12) and small litters (L4). Data are expressed as mean ± SEM. ($F_{(83,2006)}$ = 1253.8); p<0.0001. **a**= Effect of litter size on BW in males; **b**= Effect of litter size on BW in females; **c**= Effect of sex on BW in small litters (L4); **d**= Effect of sex on BW in control litters (L12). ANOVA repeated measures: Sex effect ($F_{(1, 42)}$: 496.15, p<0.0001) and Litter size effect: ($F_{(1, 42)}$: 10.1, p<0.003).

Neonatal overnutrition produced changes in body weight as early as PND10. At this early postnatal age, both males and females from L4 weighed more than those from L12, ($F_{(1, 48)}$ = 128.0, p<0.0001), with no differences between sexes (Figure 17).

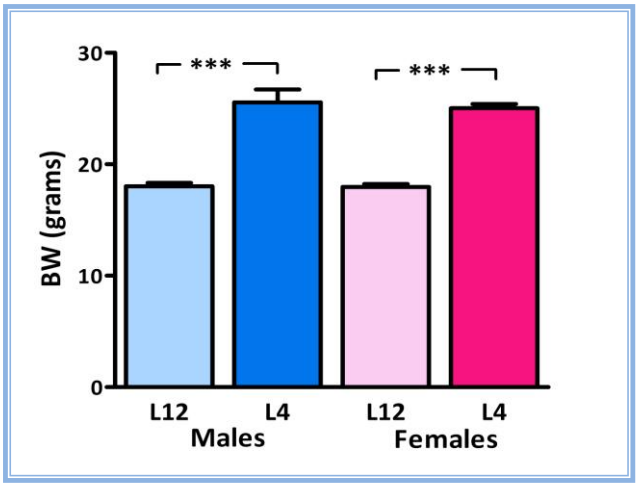


Figure 17. Mean body weight (BW) at PND10. There was an increase in BW as a consequence of neonatal overnutrition in both males and females. L12= litter of 12 pups. L4 = litter of 4 pups. ($F_{(3,47)}$ = 42.8); *** = p<0.0001.

The effect of neonatal overnutrition on BW continued at weaning, PND21, ($F_{(1,223)}$ =146.9, p<0.0001), with no difference between males and females (Figure 18A).

At PND28, both males and females raised in small litters continued to weigh more than control litters ($F_{(1,170)}$ = 73.9, p<0.0001). In addition, even at this prepubertal stage there was an effect of sex on BW ($F_{(1,170)}$: 10.2, p<0.003), with L4 males weighing more than L4 females (Figure 18B).

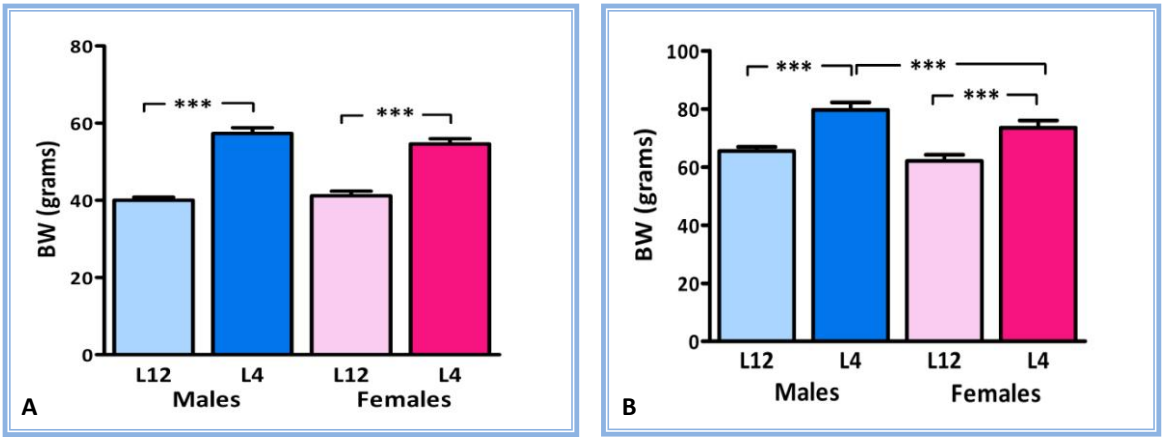


Figure 18. Mean body weight (BW) at PND21 **(A)** and at PND28 **(B)** in rats raised in litters of 12 (L12) or 4 (L4) pups. PND21 ($F_{(3, 226)}$ = 50.1); PND28 ($F_{(3, 173)}$ = 27.9); *** = p<0.0001.

As can be observed in Figure 19, at PND56 males weighed more than females regardless of their experimental group and this effect remained until the end of the study. However, the effect of litter size on body weight ceased to be observed at approximately PND56 in both sexes.

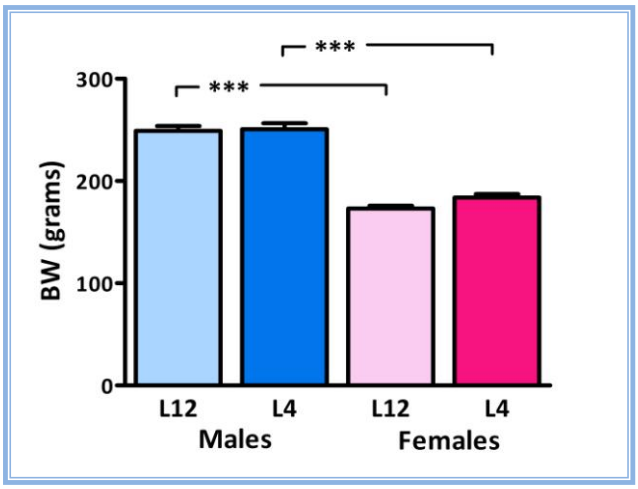


Figure 19. Mean body weight (BW) at PND56 in rats reared in litters of 12 (L12) or 4 (L4) pups when the effect of litter size was no longer observed in either sex. ($F_{(3,95)}$ = 89.2; *** = p<0.0001.

Although the effect of litter size on BW ceased to be observed at approximately PND56, it was again manifest at approximately PND90, but only in males (Figure 20A). Male rats from L4 weighed significantly more than those from L12 throughout the rest of the study, with no further effect seen in females (Figure 20 B).

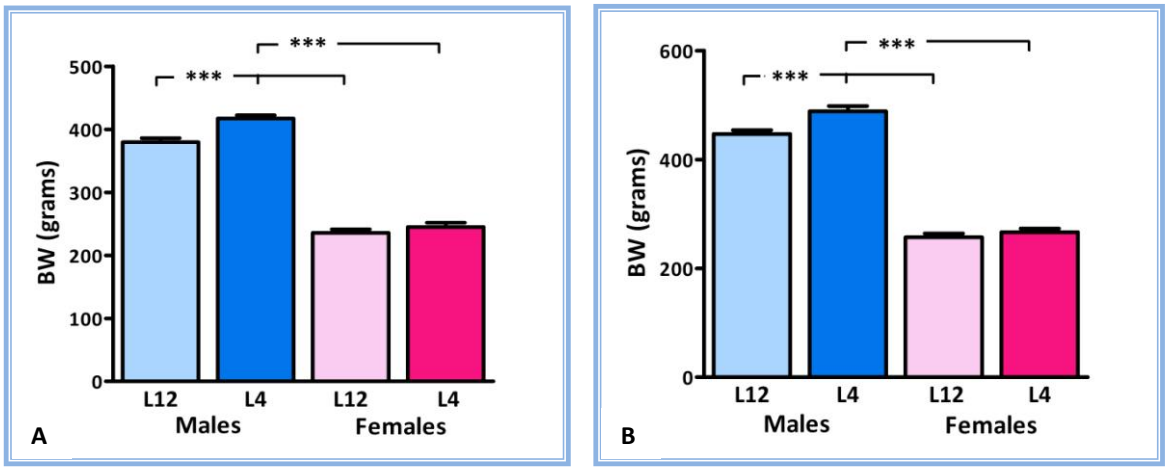


Figure 20. Body weight at PND91 **(A)**, when males from litters of 4 pups (L4) again weighed more than those from litters of 12 pups (L12). Males continued to weigh more than females regardless of litter size.

($F_{(3,46)}$ = 165.7; *** = p<0.0001. At PND150 **(B)** the effect of litter size in males continued to be observed, while no effect in females was found.

1.1.2 Body length

There was an effect of sex ($F_{(1,1144)}$ = 1284.8, p<0.0001), litter size ($F_{(1,1144)}$ = 27.8, p<0.0001) and age ($F_{(18,1144)}$ = 6242.7, p<0.0001) on body length with interactions between sex and age ($F_{(18,1144)}$ = 53.3, p<0.0001) and litter size and age ($F_{(18,1144)}$ = 11.1; p<0.0001). The changes in body length throughout development can be seen in Figure 21.

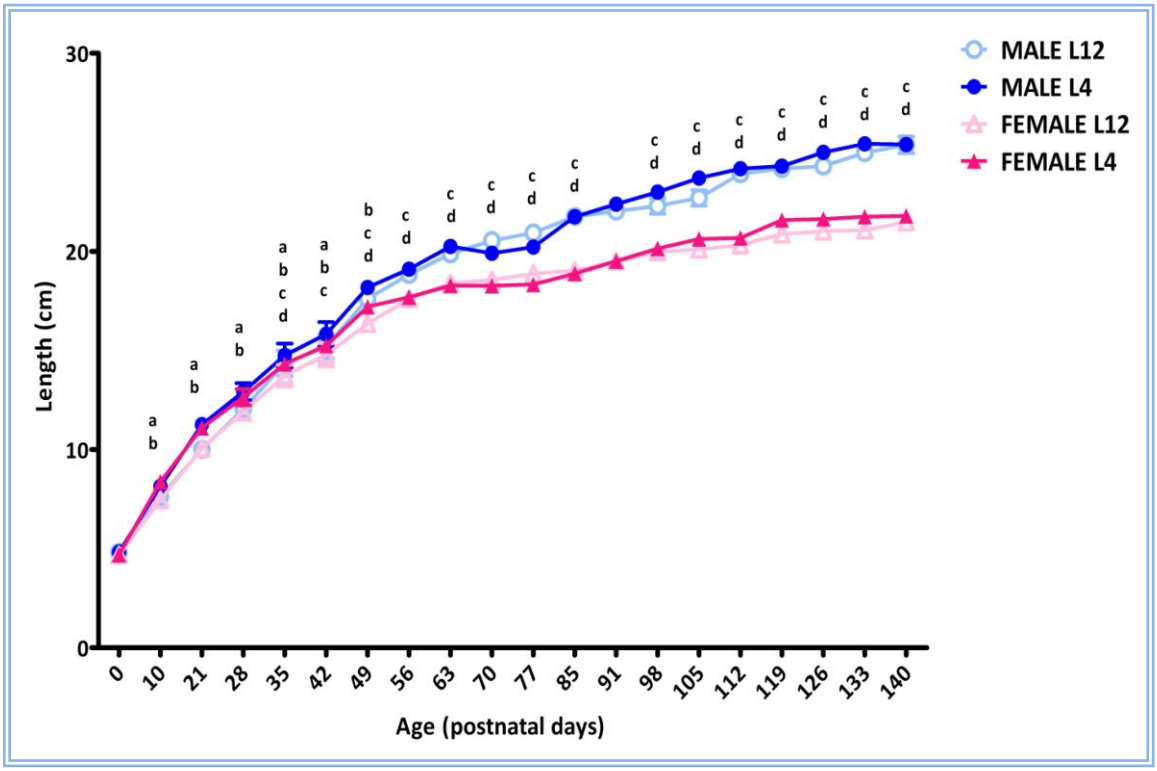


Figure 21. Changes in body length throughout development in male and female rats from control litters of 12 pups (L12) and overnourished litters of 4 pups (L4). Data are expressed as mean ± SEM. ($F_{(72,1183)}$ = 1538.4, p<0.0001). **a**= Effect of litter size on BW in males; b= Effect of litter size on BW in females; c= Effect of sex on BW in small litters (L4); d= Effect of sex on BW in control litters (L12). ANOVA repeated measures: sex ($F_{(1, 44)}$: 168.91, p<0.0001); litter size ($F_{(1, 44)}$: 24.73 p<0.0001).

On the day of birth, the mean body length did not differ according to litter size (Figure 22A; however, there was an effect of sex with males being longer than females ($F_{(1,245)}$ = 27.6, p<0.0001; Figure 22B).

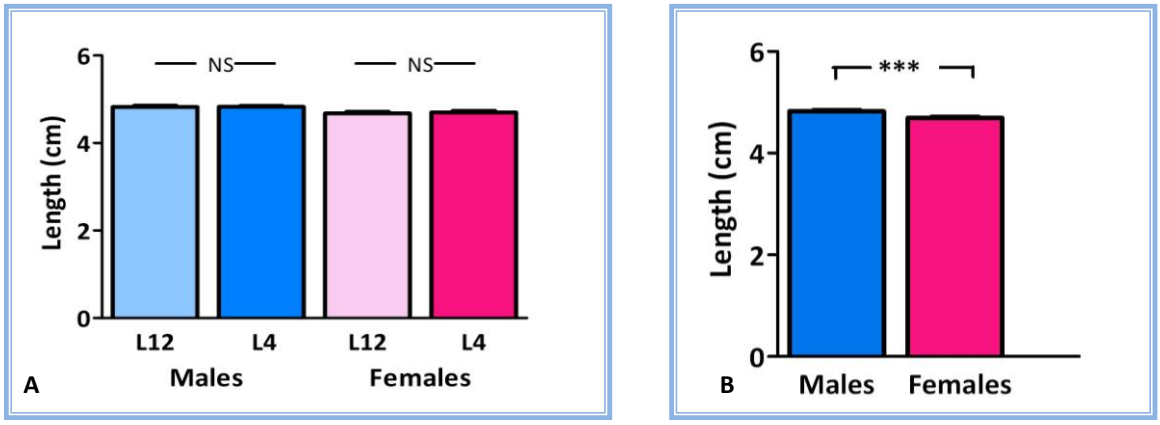


Figure 22. **A**. Mean body length of each experimental group on the day of birth N = 64-66, NS = not significant. **B.** Mean body length of male and female rats showing differences between the sexes on the day of birth. N=124-126. *** = p <0.0001.

By PND10, both males and females with neonatal overnutrition (L4) were longer than those from control litters (L12) ($F_{(1,48)}$ = 50.3, p<0.0001), as can be seen in Figure 23. The litter size effect continued at PND21 ($F_{(1,179)}$ = 85.4, p<0.0001) and PND28 ($F_{(1,128)}$ = 39.8; p<0.0001).

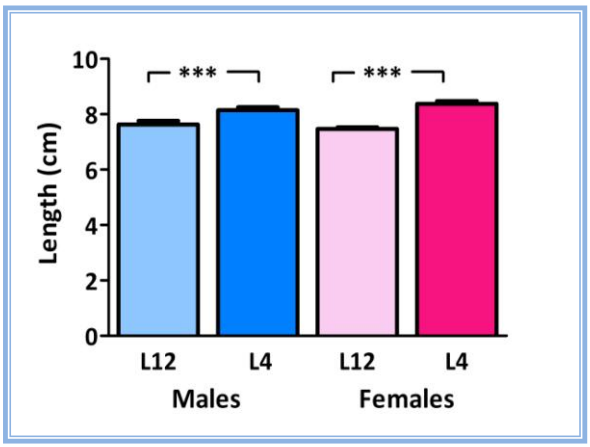


Figure 23. On postnatal day 10, both males and females from litters of 4 pups (L4) were longer than those from litters of 12 pups (L12). There was no effect of sex observed at this time. ($F_{(3, 47)}$ = 18.1, *** = p<0.0001, n =12.

At PND35, males were found to be longer than females ($F_{(1,94)}$ = 27.2; p<0.0001) and remained so at all subsequent ages. A litter size effect ($F_{(1,94)}$ = 24.6;

p<0.0001) was also found at PND35, but was no longer observed in either sex after PND56 (Figure 24).

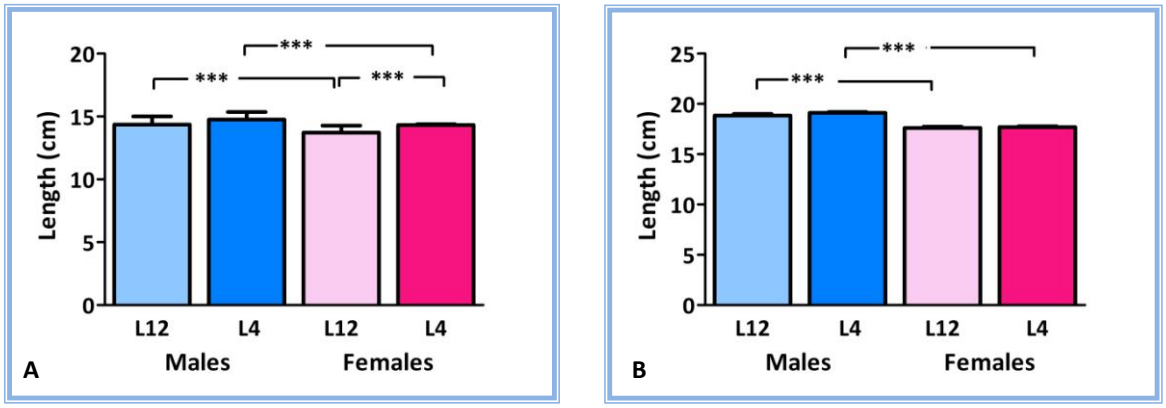


Figure 24. Males began to be longer than females around PND 35 **(A)** and at PND 56 **(B)** the litter size effect on body length was no longer observed. PND35: ($F_{(3,93)}$ = 18.1); PND56: ($F_{(3,47)}$ = 38.1) ; *** = p <0.0001.

1.1.3 Mean food intake per day

The mean food intake per day throughout the study, was affected by sex being greater in males than in females ($F_{(1,321)}$ = 591.7, p<0.0001), by litter size, with L4 rats eating more than L12 rats ($F_{(1,320)}$ = 14.6, p<0.0001) and increased with age ($F_{(15,321)}$ = 73.6, p<0.0001), with an interaction between sex and age ($F_{(15,321)}$ = 9.4, p<0.0001) and between litter size and age ($F_{(15,321)}$ = 1.9, p<0.03) . Males ate more than females even during the first week after weaning (PND28, sex effect: $F_{(1,58)}$ = 6.5, p<0.02). At this time both male and female rats from L4 ate more than those from L12 (ML12: 11.3 ± 0.2, ML4: 12.7 ± 0.3, FL12: 10.1 ± 0.3, FL4: 11.7 ± 0.3 g/day; litter size: $F_{(1,58)}$ = 13.3, p<0.002). Rats from L4 continued to eat more than those from L12 until approximately PND50 after which there was no effect of litter size. At approximately PND120 (week 14 post-weaning), L4 males ate again significantly more than L12 males $F_{(3,11)}$ = 46.1, p<0.0001) . The mean daily food intake from weaning until the end of the study was greater in males than in females. However, there was no affect of litter size in either sex at the end point of the study, PND150

(ML12: 21.3 ± 0.9, ML4: 23.2 ± 0.5, FL12: 15.3 ± 0.3, FL4: 16.1 ± 1.1 g/rat/day; p<0.0001).

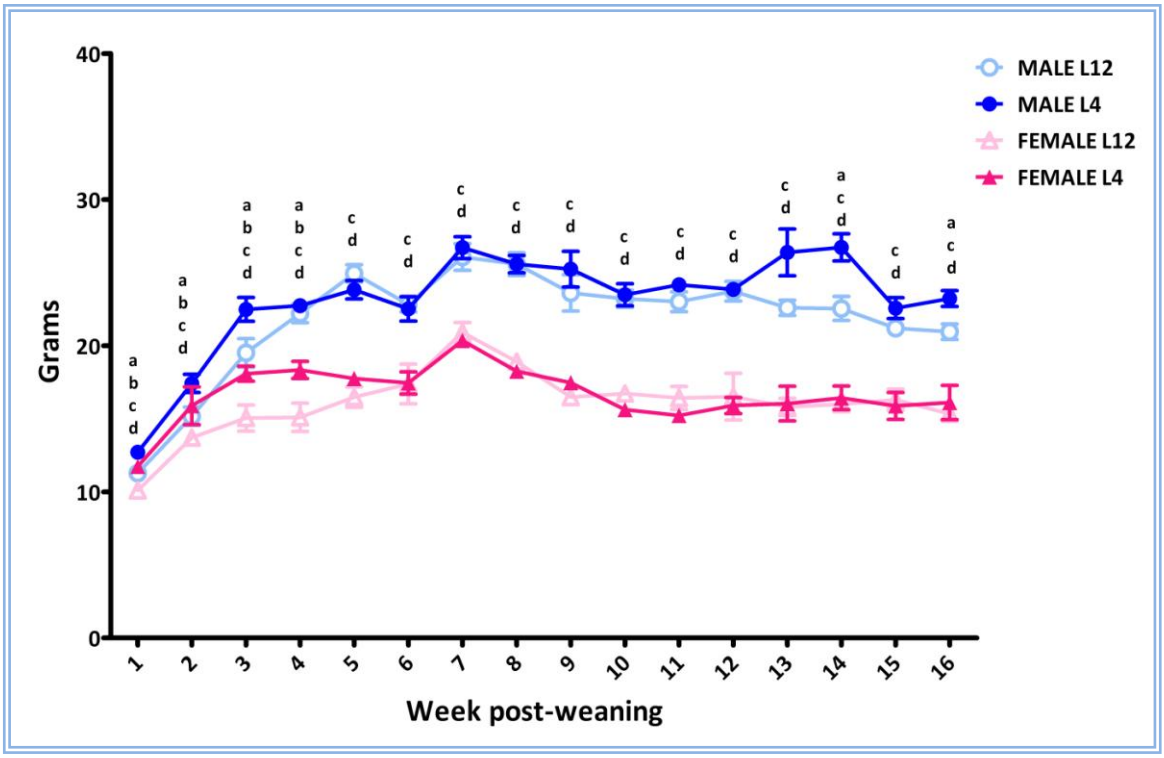


Figure 25. Mean daily food intake measured at each week post-weaning in male and female rats from control litters of 12 pups (L12) and overnourished litters of 4 pups (L4). Data are expressed as mean ± SEM. ($F_{(63, 384)}$ = 29.4 , p<0.001). **a**= Effect of litter size on food intake in males (M12 different from M4); **b**= Effect of litter size on food intake in females; **c**= Effect of sex on food intake in small litters (L4); d= Effect of sex on food intake in control litters (L12).

1.2Effects of litter size and sex on adipose tissue quantity and distribution

As described above, over-nutrition during early postnatal life resulted in rapid weight gain. However, this effect was attenuated in early adulthood, only to appear again around PND90 selectively in males. Although no litter size effect was observed on body weight in early adulthood, body composition could be affected. Moreover, it is well established that adult males and females have different adipose tissue distribution both in rodents **(Grove et al., 2010)** and in humans **(Schreiner et al., 1996; Jackson et al., 2002; Goodpaster et al., 2005; Shen et al., 2009b)**, but little is known regarding this sex difference during prepubertal life.

1.2.1 Subcutaneous adipose tissue

The amount of SCAT was influenced by sex ($F_{(1,164)}$ = 152.5, p<0.0001), litter size ($F_{(1,164)}$ = 78.3, p<0.0001) and age ($F_{(5,164)}$ = 214.8, p<0.0001) with interactions between sex and litter size ($F_{(1,164)}$ = 5.2, p<0.05), sex and age ($F_{(5,164)}$ = 57.3, p<0.0001), litter size and age ($F_{(5,164)}$ = 3.2, p<0.009), and sex, litter size and age ($F_{(4,164)}$ = 6.6, p<0.0001). When the amount of SCAT was normalized to body weight (g/100 g BW), these effects remained [sex ($F_{(1,164)}$: 74.0, p<0.0001), litter size ($F_{(1,164)}$ = 23.8, p<0.0001) and age ($F_{(5,164)}$ = 85.2, p<0.0001)] with interactions between sex and age ($F_{(5,164)}$ = 87.8; p<0.0001, litter size and age ($F_{(5,164)}$ = 13.6; p<0.0001) and sex, litter size and age ($F_{(5,164)}$ = 3.7; p<0.01).

At PND10, the amount of SCAT was affected by sex ($F_{(1,47)}$ = 20.5, p<0.0001) and litter size ($F_{(1,47)}$ = 150.5, p<0.0001), with animals from small litters having more SCAT than those from control litters in both sexes. In addition, L4 females had a greater increase in % SCAT than L4 males ($F_{(3,46)}$ = 59.3), p<0.0001.

Upon weaning at PND21, the influence of litter size in the amount of SCAT remained ($F_{(1,46)}$ = 54.3, p<0.0001), as well as the sex effect ($F_{(1,46)}$ = 19.3, p<0.0001). The amount of SCAT continued to be higher in L4 rats of both sexes, with females having more than males of their corresponding litter size (Figure 27).

The effect of litter size ($F_{(1, 24)}$ = 10.5, p<0.005) continued to be present at PND30, as well as an overall influence of sex ($F_{(1, 24)}$ = 4.5, p<0.05).

At PND 50, no differences between males and females were observed; however, the effect of litter size remained ($F_{(1, 24)}$ = 6.5, p<0.03).

The effect of litter size ($F_{(1, 24)}$ = 4.6, p<0.05) was still present at PND85, when males had an overall higher percentage of SCAT than females ($F_{(1, 24)}$ = 8.61, p<0.01).

At PND150, there was no significant effect of litter size, but L4 males tended to have higher levels compared to control males, with an overall effect of sex observed ($F_{(1,23)}$ = 5.2, p<0.05).

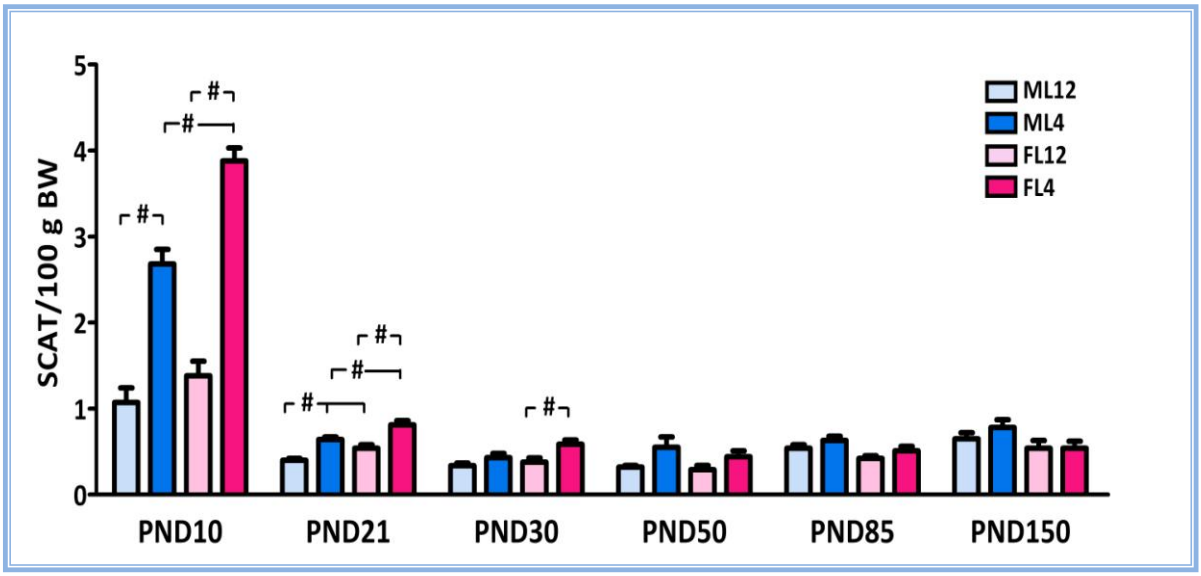


Figure 26. Relative amount of subcutaneous adipose tissue (SCAT) throughout development in male (M) and female (F) rats from small litters (L4) and control litters (L12). **#** = p<0.0001.

1.2.2 Visceral adipose tissue

Visceral adipose tissue was not collected at PND10 due to the fact that there was insufficient tissue for analysis. Throughout the study, the absolute weight of VAT depots was affected by sex ($F_{(1,118)}$ = 46.1, p<0.0001), and age ($F_{(4,118)}$ = 146.1, p<0.0001) and a litter size effect of p= 0.052, with an interaction between sex and age ($F_{(4,118)}$ = 18.5; p<0.0001) and sex, litter size and age ($F_{(4,118)}$ = 3.0, p<0.05). After normalizing the amount of VAT to body weight, an effect of sex ($F_{(1,117)}$ = 5.2, p<0.05), litter size ($F_{(1,117)}$ = 4.4, p<0.05) and age ($F_{(4,117)}$ = 137.1, p<0.0001) was found.

At PND21, both sex ($F_{(1,39)}$ = 42.5, p<0.0001) and litter size ($F_{(1,39)}$ = 37.7, p<0.0001) influenced the relative amount of VAT. Rats raised in small litters had a higher percentage of VAT compared to control rats. In addition, this percentage was higher in males than in females of the same litter size.

At PND30, no effect of litter size was observed, but males had a greater percentage of VAT than females regardless of litter size ($F_{(1,20)}$ = 46.2, p<0.0001).

In postpubertal rats (PND50, PND85 and PND150) the amount of VAT tended to be greater in males than females and in those animals raised in small litters compared to control litters, but these effects were not statistically significant, most likely due to the high variability within the groups.

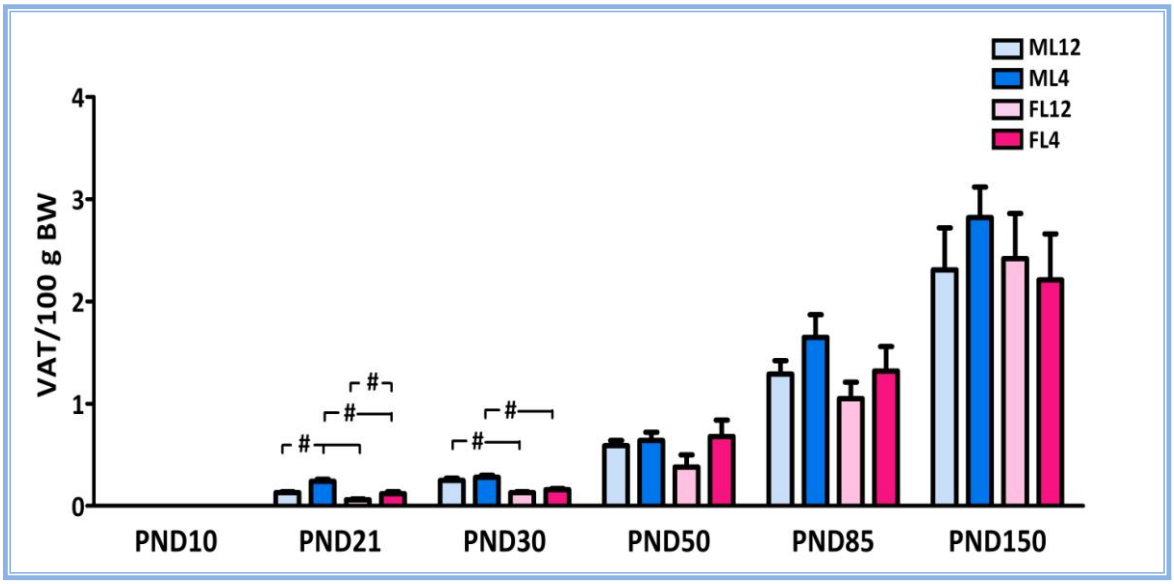


Figure 27. Relative amount of visceral adipose tissue (VAT) throughout development in male (M) and female (F) rats from small litters (L4) and control litters (L12). **#** = p<0.0001.

1.3Effects of litter size and sex on circulating hormones and cytokines.

To determine how overnutrition during early life influences circulating metabolic hormones and cytokines in males and females throughout development glucose, insulin, leptin, adiponectin, IL6, IL1β and TNFα, as well as testosterone and 17β-estradiol levels were measured.

1.3.1 Glycemia

Through development, glucose levels were affected by litter size ($F_{(1,248)}$ = 10.4, p<0.002) and age ($F_{(5,248)}$ = 139.4, p<0.0001) with interactions between these two factors ($F_{(5,248)}$ = 6.2, p<0.0001; Table 8).

At PND10, when rats were still nursing, with both L4 males and females had higher levels of glucose than L12 rats ($F_{(1, 44)}$ = 9.6, p<0.001;

The effect of litter size remained at weaning ($F_{(1, 42)}$ = 25.9, p<0.0001) .

At PND30, glycemia decreased in rats of all groups. Between PND85 and PND150 glycemia rose significantly in all groups except L12 females, where this rise was more gradual starting at PND50.

1.3.2 Circulating insulin levels

Throughout the study, insulin levels (Table 8) were affected by sex ($F_{(1,117)}$ = 12.8, p<0.003) and age ($F_{(5,117)}$ = 23.0, p<0.0001), with an interaction between these two factors ($F_{(1,117)}$ = 5.8, p<0.0001). Circulating levels of this hormone were significantly increased at PND150 in all groups.

At PND10, sex ($F_{(1,20)}$ = 9.5, p<0.006) and litter size ($F_{(1, 20)}$ = 29.5, p<0.0001) affected insulin levels, with males from L4 having higher levels compared to those from L12 ($F_{(3,23)}$ = 13.8, p<0.0001). Females from smaller litters also tended to have higher levels than control females, although this difference was not significant. In addition, L4 males had higher insulin levels than L4 females.

At PND21, the litter size effect continued ($F_{(1,20)}$ = 10.0, p<0.005), although there was no difference between the sexes.

The effects of sex and litter size were not observed between PND21 and PND150. However, at the end of the study the effect of sex on insulin levels was again present ($F_{(1,23)}$ = 11.9, p<0.002).

1.3.3 Homeostatic model assessment-insulin resistance (HOMA-IR)

As an index of insulin sensitivity, homeostatic model assessment (HOMA; Table 8) was calculated (insulin (mIU/ml) x glucose mg/dl/405) (Table 8). There was an effect of sex ($F_{(1,132)}$ = 13.0, p<0.0005), litter size $F_{(1,132)}$ =7.4, p<0.008 and age ($F_{(5,132)}$ =16.3, p<0.0001), with an interaction between sex and age ($F_{(5,132)}$ = 5.4, p<0.0002).

Males had a higher HOMA than females at PND10 (sex effect: $F_{(1,20)}$ = 6.5, p<0.02) and PND150 ($F_{(1,22)}$ = 4.8, p<0.01). At PND10, L4 rats of both sexes had a higher HOMA index than L12 animals (litter effect: $F_{(1,20)}$ = 23.7, p<0.0001, with males L4 having a higher index than L4 females ($F_{(3,20)}$ = 10.9, p<0.0002). The effect of litter

size was maintained in both sexes at PND21 ($F_{(1,20)}$ = 10.9, p<0.004). There were no differences between groups at P30, 50 or 85. In females, HOMA decreased between PND21 and PND30, and then rose again at PND150 regardless of litter size ($F_{(11,65)}$ = 3.02 p<0.003). In males, HOMA decreased between PND10 and PND30 and also increased between PND85 and PND150 ($F_{(11,68)}$ = 7.0, p<0.0001).

		GLUCOSE (mg/dl)	INSULIN (ng/ml)	HOMA
PND 10*	ML12	114.4±4.1	0.44±0.07	3.2±0.5
	ML4	130.9±5.9[a]	0.92±0.10[a]	7.7±1.2[a]
	FL12	121.6±5.1	0.34±0.02	2.4±0.2
	FL4	135.8±4.5[a]	0.60±0.06[b]	4.8±0.5[a,b]
PND 21*	ML12	103.6±5.3	0.32±0.05	2.1±0.4
	ML4	131.7±5.5	0.69±0.24[a]	5.4±1.9[a]
	FL12	109.3±2.9	0.22±0.04	1.5±0.3
	FL4	125.6±3.1	0.80±0.16[a]	6.0±1.3[a]
PND 30	ML12	68.2±2.9[c]	0.19±0.03	0.8±0.1[c]
	ML4	76.9±4.7[c]	0.23±0.05	1.1±0.2[c]
	FL12	72.7±4.8[c]	0.13±0.01	0.6±0.1[c]
	FL4	75.8±4.0[c]	0.57±0.37	2.6±1.8[c]
PND 50	ML12	65.7±3.3	0.89±0.44	3.5±1.8
	ML4	63.8±3.8	0.73±0.36	3.1±1.7
	FL12	63.8±2.4	0.71±0.31	2.6±1.1
	FL4	69.7±3.4	0.37±0.07	1.6±0.3
PND 85	ML12	68.8±3.7	0.70±0.18	3.1±0.9
	ML4	61.0±2.4	0.94±0.37	3.5±1.3
	FL12	73.3±3.4	0.15±0.03	0.7±0.2
	FL4	66.3±2.9	0.60±0.22	2.2±0.9
PND 150	ML12	85.7±4.0[c]	3.46±0.99[c]	13.5±4.4[c]
	ML4	78.4±4.0[c]	2.82±0.25[c]	12.1±1.1[c]
	FL12	81.3±8.7	1.03±0.35[c]	3.8±1.4[b,c]
	FL4	84.9±6.3[c]	1.55±0.40[c]	6.1±1.7[b,c]

Table 8. Circulating glucose and insulin levels at 10, 21, 30, 50, 85 and 150 days of age, from small (L4) and control litters (L12). **Glycemia:** $F_{(23, 271)}$= 32.2, p<0.0001; **Insulin:** $F_{(23, 140)}$= 7.1, p<0.0001; **HOMA:** $F_{(23, 150)}$= 7.1, p<0.0001 [a]different from rats of same age and sex (litter size effect),[b]different from males of same age and litter size, [c]different from preceding age (same sex and litter size). * non-fasting samples.

1.3.4 Oral glucose tolerance test (OGTT)

To further investigate insulin resistance, we performed an oral glucose tolerance test (OGTT) in the animals scarified at PND 150 one week before sacrifice as described in 1.1.1 b section.

Blood glucose levels were measured 30, 60 and 120 minutes after glucose administration in all groups. No differences were found after 30 or 60 minutes. However, there was an effect of litter size on blood glucose levels at 120 min after glucose oral administration (F(1,24) = 6.5, p<0.02), with L4 rats of both sexes having higher blood glucose levels (ML12: 98.3 ± 3.8, ML4: 114.5 ± 4, FL12: 98.7 ± 5.2, FL4: 115.2 ± 10.3). There were no significant differences in the area under the curve (AUC).

1.3.5 Circulating leptin levels

Circulating leptin levels (Table 9) were dependent on age ($F_{(5,129)}$ = 21.7, p<0.0001) and litter size ($F_{(1,129)}$ = 4.9, p<0.03), with an interaction between these two factors ($F_{(1,129)}$ = 3.0, p<0.02).

At PND10, there was an effect of litter size ($F_{(1,28)}$ = 24.0, p<0.0001) with both L4 males and females having higher levels of circulating leptin than their controls. This effect remained at PND21 ($F_{(1,18)}$ = 9.3, p<0.007).

Serum leptin levels increased in all groups between PND85 and PND150. In addition, at PND150 males had higher leptin concentrations than females regardless of litter size and males from small litters had significantly higher leptin levels than their controls ($F_{(3,33)}$ = 23.0, p<0.0001).

1.3.6 Circulating adiponectin levels

Throughout development, circulating adiponectin levels (Table 9) were influenced by litter size ($F_{(1,113)}$ = 7.1, p<0.009) and age ($F_{(5,113)}$ = 71.8, p<0.0001), with an interaction between sex and age ($F_{(5, 113)}$ = 4.21; p<0.002) and sex, litter size and age ($F_{(5,113)}$ = 2.4, p<0.05). Levels of this adipokine increased with age.

At 10 days of age, both males and females from smaller litters had higher levels of circulating adiponectin than control litters ($F_{(1,20)}$ = 108.4, p<0.0001). When weaned, the effect of litter size continued ($F_{(1,20)}$ = 16.2, p<0.003), but only in females ($F_{(3,23)}$ = 10.9, p<0.0001). At PND21, there was also an effect of sex ($F_{(1,20)}$ = 11.9, p<0.001), with L4 females having higher levels than L4males.

At PND30, there was an effect of sex effect on serum adiponectin levels ($F_{(1,20)}$ = 4.8, p<0.05), while at PND 50, no effect of either sex or litter size was observed.

Females had higher levels than their corresponding male groups at PND85 [sex effect ($F_{(1,16)}$ = 6.2, p<0.05)]. At PND150, L12 female rats had higher adiponectin levels than L12 males. Adiponectin levels were lower in L4 than L12 females, but the inverse occurred in males [litter size and sex interaction: ($F_{(1,23)}$ = 6.9, p<0.02)]).

		LEPTIN (ng/ml)	ADIPONECTIN (ng/ml)
PND 10*	**ML12**	1.25±0.21	21.5±2.8
	ML4	2.61±0.32[a]	40.0±2.3 [a]
	FL12	1.24±0.16	17.5±2.3
	FL4	2.43±0.322[a]	41.9±1.5 [a]
PND 21*	**ML12**	1.12±0.18	27.6±1.8
	ML4	1.80±0.21	31.9±1.6
	FL12	0.99±0.22	30.3±1.7
	FL4	1.91±0.48[a]	43.9±3.2 [a,b]
PND 30	**ML12**	0.43±0.12	62.1±6.0[c]
	ML4	0.97±0.44	58.4±6.3 [c]
	FL12	0.71±0.32	42.6±3.0 [b]
	FL4	1.04±0.54	54.2±5.7
PND 50	**ML12**	0.96±0.32	55.2±4.2
	ML4	0.69±0.29	76.2±5.9 [a]
	FL12	0.77±0.29	62.2±10.1
	FL4	0.64±0.31	60.9±9.7
PND 85	**ML12**	1.65±0.20	71.6±14.0
	ML4	1.42±0.21	78.0±19.1
	FL12	1.22±0.44	108.2±9.1 [b]
	FL4	1.04±0.18	105.2±2.5 [b]
PND 150	**ML12**	9.57±1.05[c]	80.6±11.1
	ML4	14.46±1.23[c]	109.4±7.4
	FL12	4.33±0.80[b,c]	108.2±7.5
	FL4	4.56±0.90[b,c]	86.4±12.3

Table 9. Circulating leptin and adiponectin levels at 10, 21, 30, 50, 85 and 150 days of age, in rats from small (L4) and control litters (L12). **Leptin:** $F_{(23, 146)}= 5.7$, $p<0.0001$; **Adiponectin:** $F_{(23,136)}= 15.7$, $p<0.0001$. [a]different from rats of same age and sex (litter size effect),[b]different from males of same age and litter size, [c]different from preceding age (same sex and litter size). * non-fasting samples.

1.3.7 Serum IL1β levels

The levels of the inflammatory cytokine IL1β (Table 10) were affected by age ($F_{(5,124)}$ = 5.1, p<0.0001) with an interaction between sex and litter size ($F_{(1,124)}$ = 5.1, p<0.03).

At PND10, there was an effect of litter size on circulating IL1β levels ($F_{(1,28)}$ = 7.5, p<0.02) with males and females from L4 tending to have lower levels of this cytokine; however, this effect was not significant between specific groups in the post hoc analysis. At weaning, males from L4 tend to have higher levels of IL1β than L12 males, with females having the opposite tendency.

This pattern (L4 males having higher levels than L12 males and L4 females having lower levels than L12 females) was repeated at most ages, but these differences did not reach statistical significance.

1.3.8 Serum IL6 levels

There was an overall effect of age on IL6 levels ($F_{(5,125)}$ = 6.2, p<0.0001; Table 10). At PND 10, there was an overall effect of sex with males having overall higher levels of this cytokine than females ($F_{(1,26)}$ = 4.3, p<0.05). Circulating IL6 levels did not vary between groups at PND21 or 30.

At PND50, there was an effect of sex on circulating IL6 levels ($F_{(1,19)}$ = 4.6, p<0.05). There was no effect of sex or litter size at PND85 or PND150, with many animals having values below the limit of detection.

1.3.9 Serum TNFα levels

There was an effect of age ($F_{(5,131)}$ = 5.5, p<0.0001) on circulating TNFα levels (Table 10).

At PND 10, there were overall effects of sex ($F_{(1,28)}$ = 6.9, p<0.02) and litter size ($F_{(1,28)}$ = 10.9, p<0.003) on circulating TNFα levels. Females from small litters had lower levels than control females and lower levels than males from small litters.

There was no effect of either sex or litter size at PND21 or 30. After PND50, most of the animals had circulating TNFα levels at the limit of detection of the assay.

		IL-1β (pg/dl)	IL-6 (pg/ml)	TNFα (ng/ml)
PND 10*	ML12	14.9±3.1	126.1±38.5	5.1±0.3
	ML4	5.1±1.2[a]	89.2±44.6	3.4±0.7[a]
	FL12	15.0±4.1	16.4±12.2	4.0±0.6
	FL4	7.9±2.0[a]	40.9±29.7	1.3±0.4 [a,b]
PND 21*	ML12	19.6±6.8	56.5±44.6	4.6±1.0
	ML4	46.9±11.3	85.6±39.7	2.5±1.2
	FL12	28.0±9.4	60.4±34.3	2.5±0.8
	FL4	22.8±3.7	112.1±69.8	1.9±0.6
PND 30	ML12	32.1±8.0	76.2±47.4	1.6 ±0.4
	ML4	61.3±41.4	18.4±14.4	2.0±0.9
	FL12	67.5±29.0	86.5±35.0	4.1±1.3
	FL4	29.2±13.6	78.3±23.8	2.1±0.7
PND 50	ML12	10.6±2.3	0.62	BLD
	ML4	20.9±3.9	0.62	BLD
	FL12	19.3±8.9	17.7±12.7	BLD
	FL4	7.3±2.4	22.1±11.4	BLD
PND 85	ML12	15.6±5.5	5.63±5.01	BLD
	ML4	15.7±6.6	4.50±3.88	BLD
	FL12	6.1±2.1	2.58±1.96	BLD
	FL4	6.7±2.1	0.62	BLD
PND 150	ML12	23.4±9.6	14.5±6.2	BLD
	ML4	35.3±20.0	15.5±3.6	BLD
	FL12	29.3±26.4	16.4±3.7	BLD
	FL4	22.6±8.0	19.1±0.7	BLD

Table 10. Circulating levels of IL-1β, IL-6 and TNFα at 10, 21, 30, 50, 85 and 150 days of age, from small (L4) and control (L12) litters. **IL-1β:** $F_{(23, 147)}= 1.6$, $p<0.05$; **IL-6:** $F_{(23, 148)}= 2.1$, $p<0.005$; **TNFα:** $F_{(3, 31)}= 6.1$, $p<0.003$. [a]different from rats of same age and sex (litter size effect), [b]different from males of same age and litter size, [c]different from preceding age (same sex and litter size), BLD: below limit of detection * non-fasting samples.

1.3.10 Testosterone levels

Circulating testosterone levels in males varied according to age ($F_{(5,50)}$ = 15.1, p<0.0001), showing an increase in levels at PND85 and a further increase at PND150. (Table 11).

		Testosterone (ng/dl)
PND 10*	ML12	0.19±0.05
	ML4	0.76±0.27 [a]
PND 21*	ML12	0.60±0.20
	ML4	0.63±0.21
PND 30	ML12	0.48±0.24
	ML4	0.35±0.13
PND 50	ML12	0.72±0.41
	ML4	0.63±0.15
PND 85	ML12	2.60±0.91
	ML4	2.61±0.87
PND 150	ML12	11.55±3.42
	ML4	18.68±4.46

Table 11. Circulating testosterone levels at 10, 21, 30, 50, 85 and 150 days of age in males from small and control litters. **[a]: Litter size effect.**

1.3.11 17β-Estradiol levels

Circulating 17β-estradiol levels were affected by age ($F_{(5,41)}$ = 6.4, p<0.0001), with an interaction between age and litter size ($F_{(5,41)}$ = 2.7; p<0.04). At PND21, females from small litters tended to have higher levels than control females ($F_{(1,5)}$ = 7.5, p=0.052), as well as at PND30 ($F_{(1,7)}$ = 6.1; p= 0.06). No differences were observed at PND50 or PND85. However, 17β-estradiol levels increased at PND150 in L4 females such that they were higher than in L12 females ($F_{(1, 16)}$ = 7.78; p<0.02; Table 12).

		17β-estradiol (pg/ml)
PND 10*	FL12	10.96±1.51
	FL4	8.51±3.53
PND 21*	FL12	7.06±3.07
	FL4	17.60±2.23
PND 30	FL12	16.69±1.72
	FL4	26.18±3.90
PND 50	FL12	22.86±1.61
	FL4	24.10±1.39
PND 85	FL12	26.61±4.68
	FL4	24.31±1.99
PND 150	FL12	32.45±4.42
	FL4	89.38±18.78[a]

Table 12. Circulating 17β-estradiol levels at 10, 21, 30, 50, 85 and 150 days of age, in females from small and control litters. [a] Litter size effect.

1.3.12 Circulating triglycerides at PND150.

Circulating triglyceride levels at PND150 were affected by litter size ($F_{(1,21)}$ = 5.7, p<0.03), being increased in rats from small litters, but only in males ($F_{(3, 24)}$ = 4.1, p<0.05; Figure 28).

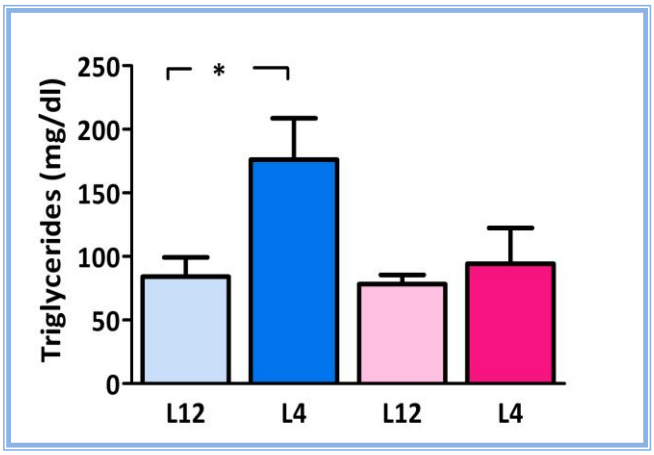


Figure 28. Circulating triglyceride levels at PND150 in male and female rats from L12 and L4 litters. * = p<0.05.

1.3.13 Non-sterified fatty acids (NEFA) at PND150.

Niether litter size nor sex affected circulating NEFA levels at PND150 (ML12: 0.77 ± 0.06, ML4: 1.00 ± 0.08, FL12: 1.03 ± 0.09, FL4: 0.96 ± 0.09 mmol/L).

1.4 Effects of litter size, sex and age on adipokine expression in adipose tissue

Because the amount of adipose tissue changed as a consequence of neonatal overnutrition in a sexually dimorphic manner, as did circulating adipokine levels, we analyzed adipokine expression in adipose tissue at PNDs 10, 21, 85 and 150 to better understand these changes.

1.4.1 Adipokine expression in subcutaneous adipose tissue

As early as 10 days of life, overnutrition ($F_{(1,20)}$ = 12.8, p<0.002) produced an increase in leptin mRNA levels (Figure 29A) in SCAT of both sexes, although this increase was only statistically significant in males. Adiponectin mRNA levels (Figure 29 B) were not significantly modified.

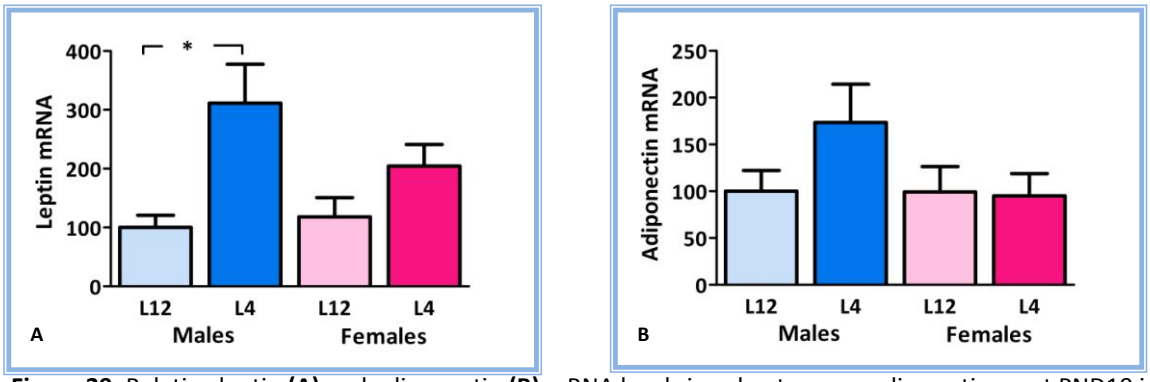


Figure 29. Relative leptin **(A)** and adiponectin **(B)** mRNA levels in subcutaneous adipose tissue at PND10 in male and female rats from litters of 4 (L4) and 12 (L12) pups. Leptin mRNA: ($F_{(3, 19)}$ = 4.9) * = p<0.02.

IL-1β mRNA levels (Table 13) were reduced in L4 compared to L12 rats ($F_{(1,16)}$ = 11.6; p<0.004) with this being significant only in females. IL-6 and TNFα mRNA levels (Table 13) were not affected by either sex or litter size.

At PND21, litter size ($F_{(1,19)}$ = 14.7, p<0.001) continued to affect leptin mRNA levels in SCAT, but with this being significant only in males (Figure 30 A). Adiponectin mRNA levels were modulated by litter size ($F_{(1,19)}$ = 20.7, p<0.0001) with an interaction between litter size and sex ($F_{(1,19)}$ = 5.7, p<0.03) as this affect was only significant in males (Figure 30 B).

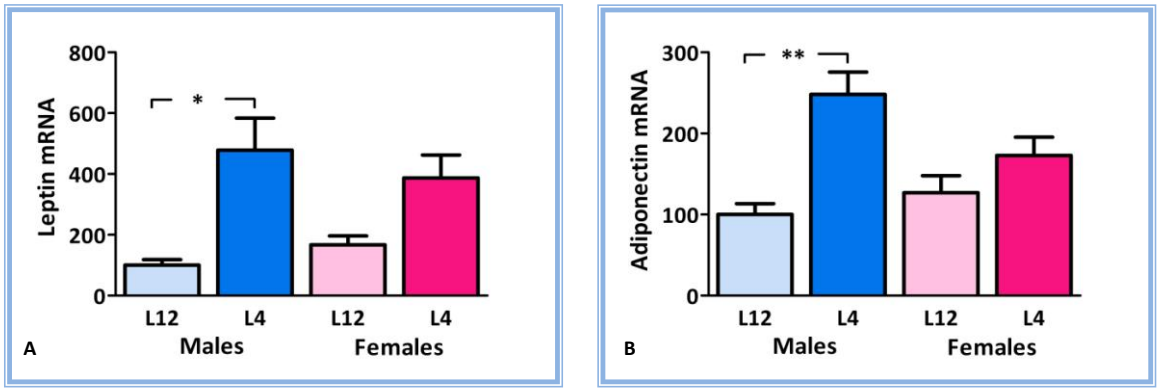


Figure 30. Leptin (**A)** and adiponectin **(B)** mRNA levels in subcutaneous adipose tissue at PND21 in male and female rats from litters of 4 (L4) and 12 (L12) pups. Leptin mRNA: ($F_{(3, 22)}$ = 7.1); Adiponectin mRNA: ($F_{(3, 22)}$ = 8.7). * = p<0.02; **= p<0.001.

In general, the expression of cytokines in SCAT tended to be decreased in over-nourished animals at PND21, although a significant effect of litter size was only observed on IL6 levels ($F_{(1,18)}$ = 6.5, p<0.02), with L12 rats having higher levels than L4 in both sexes. No effect of litter size or sex was found on IL1β or TNFα mRNA levels at PND21.

At PND85 there was an overall effect of sex on leptin mRNA levels ($F_{(1,18)}$ = 5.3, p<0.04, Figure 31A). Likewise, IL1β mRNA levels were affected by sex (ML12: 100 ± 27.7, ML4: 78.2 ± 27.2, FL12: 7.6 ± 2.2, FL4: 8.2 ± 4.1; $F_{(1,18)}$ = 6.9, p<0.02). Adiponectin mRNA levels (Figure 31B) were not statistically different between groups. As to IL6 and TNFα levels most of the results were very late in amplifying and thus not reliable.

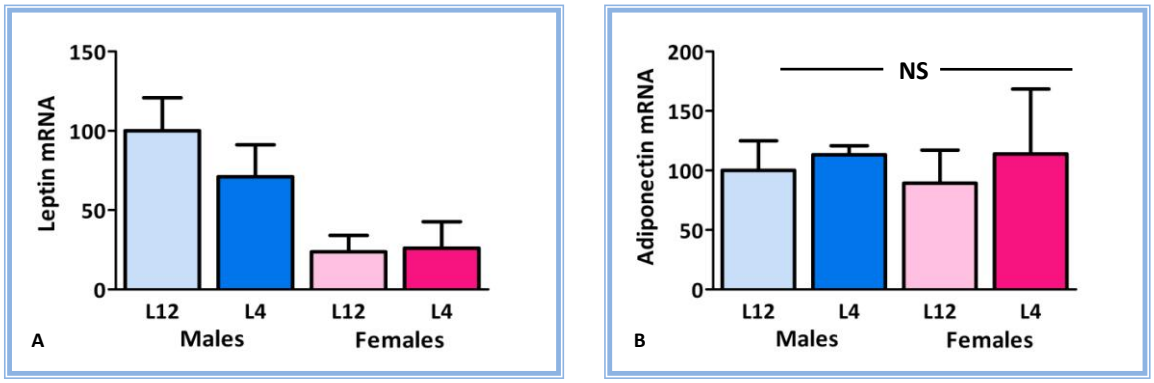


Figure 31. Leptin **(A)** and adiponectin **(B)** mRNA levels in in SCAT at PND85 in male and female rats from litters of 4 (L4) and 12 (L12) pups.

At PND150, leptin mRNA levels (Figure 32A) were different between the sexes ($F_{(1,18)}$ = 62.8, p<0.0001) and there was a litter size effect on adiponectin mRNA levels (Figure 32B) ($F_{(1,19)}$= 7.1, p<0.02). At PND150, expression of IL1β, IL6 and TNFα in SCAT was below the detection limits of the assay.

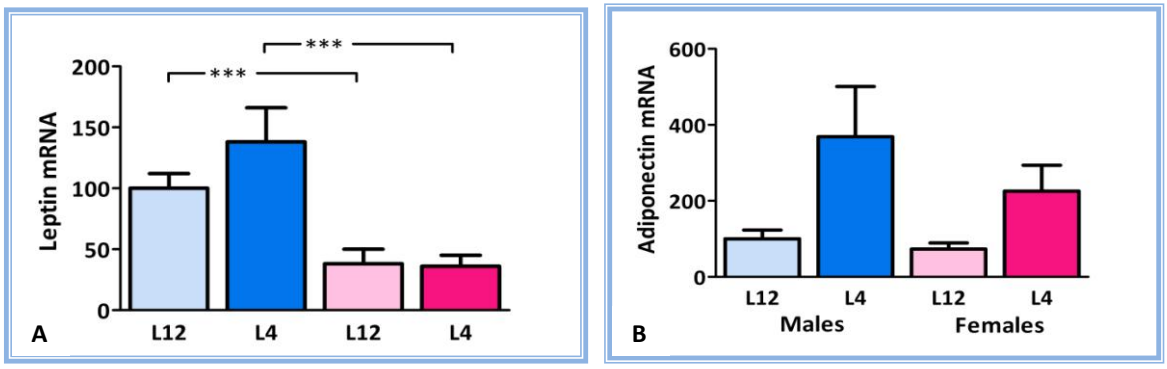


Figure 32. Leptin (A) and adiponectin (B) mRNA levels in subcutaneous adipose tissue at PND 150 in male and female rats from litters of 4 (L4) and 12 (L12) pups.

1.4.2 Adipokine expression in visceral adipose tissue

At PND10 there was insufficient VAT for analysis.

At PND21, L4 rats had higher leptin mRNA levels than L12 rats in both sexes (litter size effect: $F_{(1,21)}$ = 21.7, p<0.0001; Figure 33 A). Adiponectin mRNA levels were not affected by sex or litter size, although males from small litters clearly tended to

have increased expression levels of this adipokine compared to control males and females (Figure 33B).

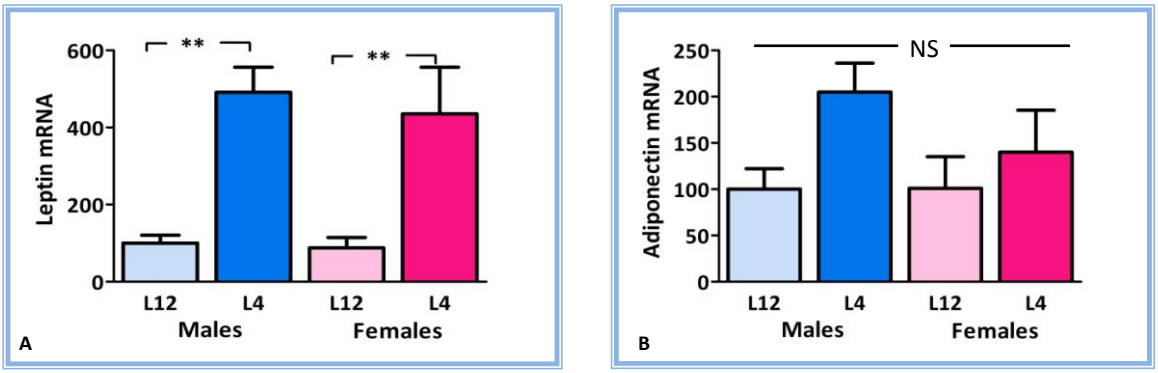


Figure 33. Leptin **(A)** and adiponectin **(B)** mRNA levels in visceral adipose tissue at PND21 in male and female rats from litters of 4 (L4) and 12 (L12) pups. Leptin mRNA levels: $F_{(3,20)} = 7.3$ **= $p<0.003$.

IL6 mRNA levels in VAT were increased in L4 rats of both sexes ($F_{(3,23)} = 6.6$; $p<0.01$). Males had higher levels of the inflammatory cytokine IL1β than females (sex effect: $F_{(1,19)} = 12.7$, $p<0.002$), with this being significant between control males and females ($F_{(3,22)} = 5.2$; $p<0.009$; Table 14). Males also had higher TNFα mRNA levels in VAT compared to females (sex effect: $F_{(1,20)} = 4.9$; $p<0.04$).

At PND85, leptin mRNA levels in VAT were lower in females compared to males (sex effect: $F_{(1,24)} = 18.1$, $p<0.0001$, Figure 34A). There was a similar effect of sex on adiponectin ($F_{(1,24)} = 11.1$, $p<0.003$ Figure 34B), IL1β ($F_{(1,19)} = 8.9$, $p<0.008$), IL6 ($F_{(1,22)} = 23.9$, $p<0.0001$) and TNFα ($F_{(1,23)} = 22.6$, $p<0.0001$) (Table 15), with females having lower levels than males.

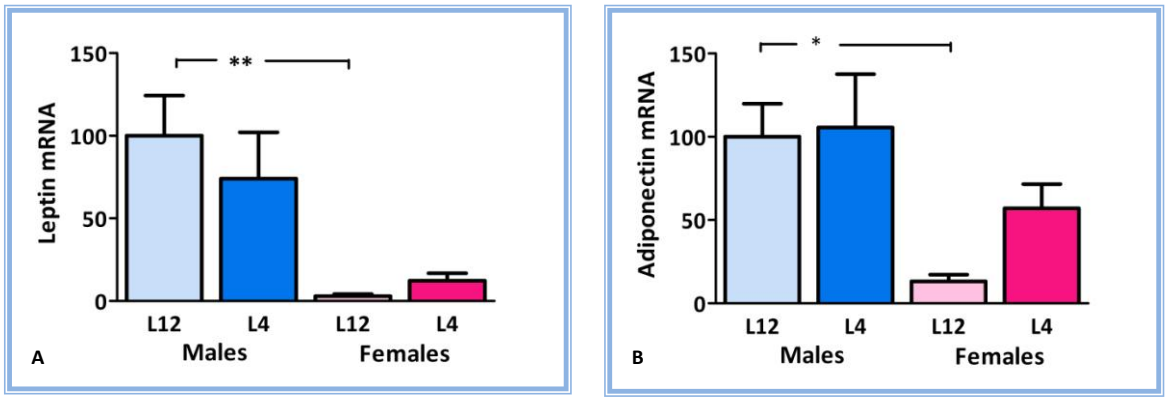


Figure 34. Leptin (A) and adiponectin (B) mRNA levels in visceral adipose tissue at PND85 in male and female rats from litters of 4 (L4) and 12 (L12) pups. Leptin mRNA: ($F_{(3,23)}$ = 6.7); Adiponectin mRNA: ($F_{(3,23)}$ = 4.5) **= p<0.01; *= p<0.03.

At PND150, leptin expression remained higher in males than females ($F_{(1, 22)}$ = 71.2; p<0.0001) regardless of litter size (Figure 35A). Adiponectin mRNA levels were also higher in males than females from both litter sizes ($F_{(1,23)}$ = 63.8; p<0.0001; Figure 35B).

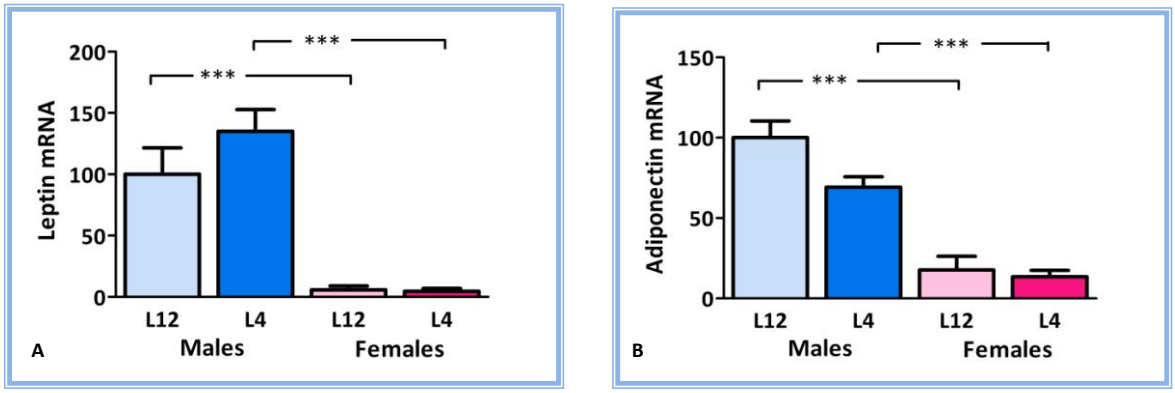


Figure 35 . Leptin (A) and adiponectin (B) mRNA levels in visceral adipose tissue at PND150 in male and female rats from litters of 4 (L4) and 12 (L12) pups. Leptin mRNA: $F_{(3, 21)}$ = 24.4; Adiponectin mRNA: $F_{(3, 22)}$ = 24.5; ***=p<0.0001.

However, IL1β mRNA were higher in females compared to males ($F_{(1,23)}$ = 29.4, p<0.0001) with both L12 and L4 females having higher IL1β mRNA levels than L12 and L4 males ($F_{(3,22)}$ = 9.99; p<0.0001; Table 16). TNFα mRNA levels were affected by litter size ($F_{(1,23)}$ = 9.9, p<0.01), being higher in L4 compared to L12 males ($F_{(3, 22)}$ = 4.22, p<0.03.

IL6 mRNA levels were below the detection limit in many of the samples and therefore not reported.

		SCAT mRNA			VAT mRNA		
		IL1β	**IL6**	**TNFα**	**IL1β**	**IL6**	**TNFα**
PND 10*	**ML12**	100±21.3	100±10.5	100±10.5	TNA	TNA	TNA
	ML4	100.5±29.6	99.6±56.6	88.9±13.2	TNA	TNA	TNA
	FL12	156.4±44.6	94.9±22.4	89.8±12.3	TNA	TNA	TNA
	FL4	32.4±13.3[a]	38.7±12.1	54.3±11.1	TNA	TNA	TNA
PND 21*	**ML12**	100±55.4	100±38.7	100±28.9	100±23.7	100±18.5	100±21.5
	ML4	42.5±9.3	19.3±1.7[a]	95.0±25.7	70.3±21.5	339.8±73.8[a]	161.4±53.6
	FL12	97.8±42.5	112.3±46.6	142.1±32.9	18.4±4.7[b]	72.8±20.1	50.2±13.8
	FL4	51.3±23.6	20.7±8.2[a]	41.7±6.3	36.2±8.1	331.0±79.9[a]	75.4±13.2
PND 85	**ML12**	BLD	BLD	BLD	100±42.1	100±28.9	100.0±28.8
	ML4	BLD	BLD	BLD	70.0±19.3	124.3±30.3	124.3±30.3
	FL12	BLD	BLD	BLD	47.7±29.1	174.8±135.4	31.8±24.7[b]
	FL4	BLD	BLD	BLD	51.0±23.5	22.9±12.4[b]	15.8±90.1[b]
PND 150	**ML12**	BLD	BLD	BLD	100±29.5	BLD	100±39.5
	ML4	BLD	BLD	BLD	80.5±37.0	BLD	450.5±186.6[a]
	FL12	BLD	BLD	BLD	703.9±142.6[b]	BLD	211.0±49.4
	FL4	BLD	BLD	BLD	617.3±145.8[b]	BLD	256.4±65.2

Table 16. Relative mRNA levels of cytokines in subcutaneous and visceral adipose tissue at post-natal days (PND) 10, 21, 85 and 150 in male (M) and female (F) rats raised in litters (L) 4 or 12 pups. [a] different from rats of same age and sex (litter effect),[b] different from males of same age and litter size (sex effect). [@]Litter size effect by 2-ways ANOVA. * Non-fasting samples. TNA: tissue not available. BLD: below limit of detection

1.5 Central effects of early overnutrition throughout development in males and females.

To correlate the metabolic changes observed as a consequence of neonatal overnutrition with possible signs of hypothalamic inflammation, hypothalamic levels of leptin, IL-6, IL-1β, IL-10 and TNF-α were determined at PND10, 50 and 150.

1.5.1 Hypothalamic cytokine levels at PND 10

At PND10 there was an overall litter size effect on hypothalamic leptin levels ($F_{(1,24)}$ = 6.7, p<0.02), with L4 rats tending to have higher levels than L12 rats (Figure 36A).

There was an interaction between sex and litter size ($F_{(1,20)}$ = 5.3, p<0.05) on hypothalamic IL6 levels, with this cytokine tending to increase in L4 males and decrease in L4 females.

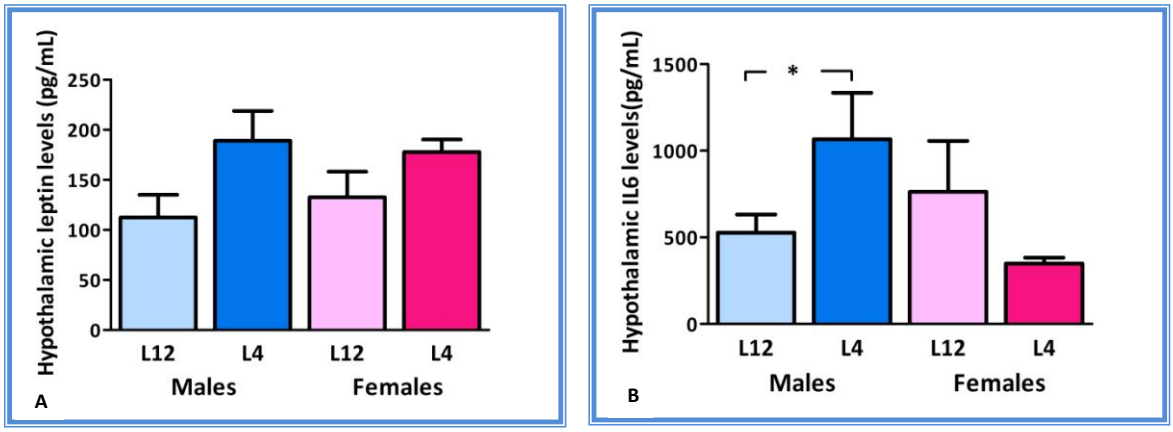


Figure 36. Leptin **(A)** and IL6 **(B)** levels in the hypothalamus at PND 10 in male and female rats from litters of 4 (L4) and 12 (L12) pups. IL6: T-test, *p<0.05.

No significant differences were found in hypothalamic IL-1β, IL-10, or TNF-α levels, although there was a trend for all of these cytokines to increase in L4 males (Table 17).

		Hypothalamic cytokine levels		
		IL10 (pg/mL)	**IL1β (pg/mL)**	**TNFα (pg/mL)**
PND 10*	**ML12**	22.7±5.7	27.1±4.9	1.8±0.1
	ML4	42±11.1	39.6±8.7	4.5±1.6
	FL12	31.7±7.7	34.1±6.3	2.5±1.2
	FL4	27.4±3.9	29.2±3.4	0.8±0.1

Table 17. Hypothalamic levels of IL10, IL1 β and TNFα at PND10 in males and females from L12 and L4.
*: Non- fasting samples.

1.5.2 Hypothalamic cytokine levels at PND50

At PND50, when the litter size-induced changes in body weight or circulating metabolic factors were no longer observed, leptin levels in the hypothalamus did not differ between groups. However, L4 males had higher hypothalamic IL6 levels compared to females from the same litter size (sex effect: $F_{(1,24)} = 4.8$, $p<0.05$; Figure 37).

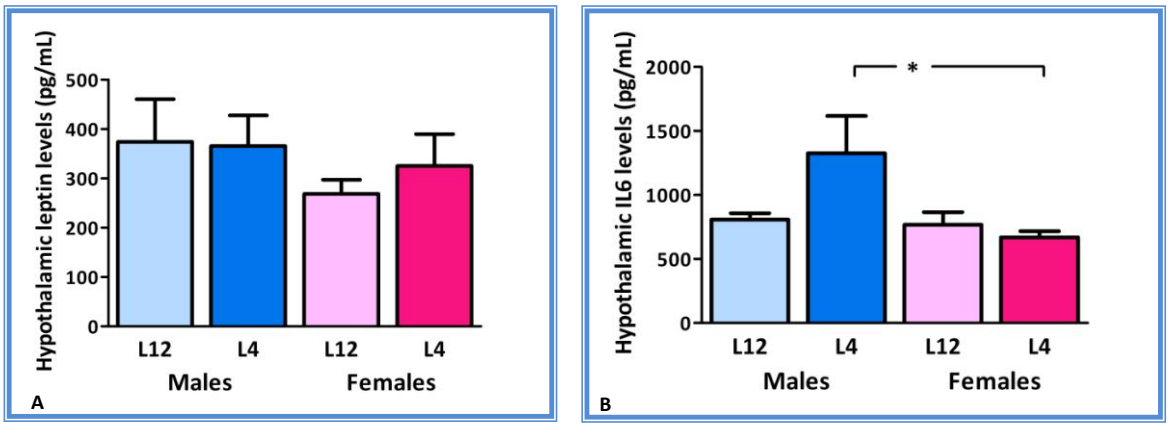


Figure 37. Leptin **(A)** and IL6 **(B)** levels in the hypothalamus at PND 50 in male and female rats from litters of 4 (L4) and 12 (L12) pups. IL6: ANOVA: $F_{(3, 23)} = 3.5$ *= $p<0.05$.

There were no significant differences between groups in hypothalamic levels of IL10 or IL1β. There was an overall sex effect on TNFα levels ($F_{(1, 22)} = 4.5$, $p<0.05$) with levels being generally higher in males.

		Hypothalamic cytokine levels		
		IL10 (pg/mL)	**IL1β (pg/mL)**	**TNFα (pg/mL)**
PND 50	**ML12**	101±17.9	70.9±10	1.6±0.2
	ML4	108±17.5	76.7±9	5.0±1.8
	FL12	85.6±11.4	57.9±4.9	1.2±0.2
	FL4	197±74.8	79.3±14.7	1.2±0.1

Table 18. IL10, IL1 β and TNFα hypothalamic levels at PND50 in males and females from L12 and L4.

1.5.3 Hypothalamic cytokine levels at PND 150

At PND150, leptin levels in the hypothalamus were found to be higher in males than in females (sex effect: $F_{(1,24)} = 6.1$, $p<0.03$, Figure 38).

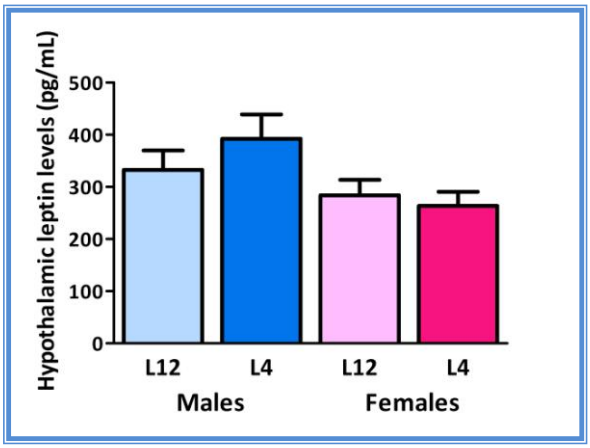


Figure 38. Leptin levels in the hypothalamus at PND 150 in male and female rats from litters of 4 (L4) and 12 (L12) pups.

No significant differences in hypothalamic IL1β, IL6 or IL10 levels were found, although L4 males tended to have higher levels of all these cytokines (Table 19).

		Hypothalamic cytokine levels		
		IL10 (pg/mL)	**IL1β (pg/mL)**	**IL-6 (pg/mL)**
PND 150	**ML12**	50.0±12.0	45.3±7.7	706.5±205.4
	ML4	69.7±14.3	51.9±7.3	1144.1±417.2
	FL12	55.4±8.9	42.3±4.2	614.9±88.6
	FL4	53.1±5.3	40.6±3.8	347.6±55.1

Table 19. Hypothalamic IL10, IL1 β and IL6 levels at PND150 in males and females from L12 and L4.

In contrast, TNFα levels in the hypothalamus were affected by sex ($F_{(1,11)}$ = 42.6, $p<0.0001$) and litter size ($F_{(1,11)}$ = 22.17, $p<0.02$), with an interaction between these two factors ($F_{(1,11)}$ = 14.3, $p<0.01$). Males from small litters had higher hypothalamic TNFα levels than those from control litters and higher levels than females from small litters (Figure 39).

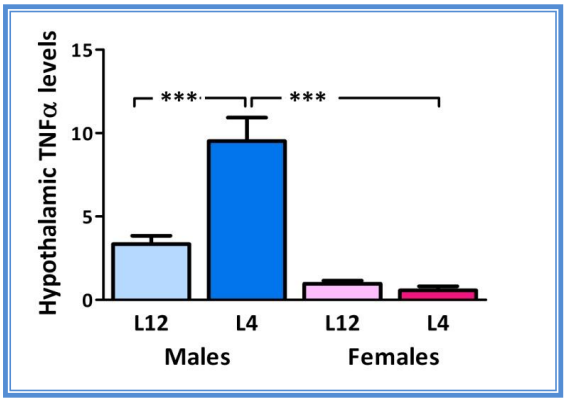


Figure 39. Hypothalamic TNFα levels at PND150 in male and female rats from litters of 4 (L4) and 12 (L12) pups. $F_{(3,10)}$ = 24.2, ***= p<0.0001.

1.5.4 Effects of early overnutrition and sex on hypothalamic glial proteins

Hypothalamic GFAP protein levels were not affected by sex or litter size at PND10, PND21, PND30, PND50 or PND85 (Table 20). At PND 150, hypothalamic GFAP levels were affected by litter size ($F_{(1, 20)}$ = 5.5, p<0.03; Figure 40A), with L4 males having higher levels than L12 males.

At PND150, the microglia marker, Iba 1, was influenced by litter size ($F_{(1, 20)}$ = 5.9, p<0.03; Figure 40B), with its levels being lower in the rats that had been exposed to neonatal overnutrition, regardless of sex.

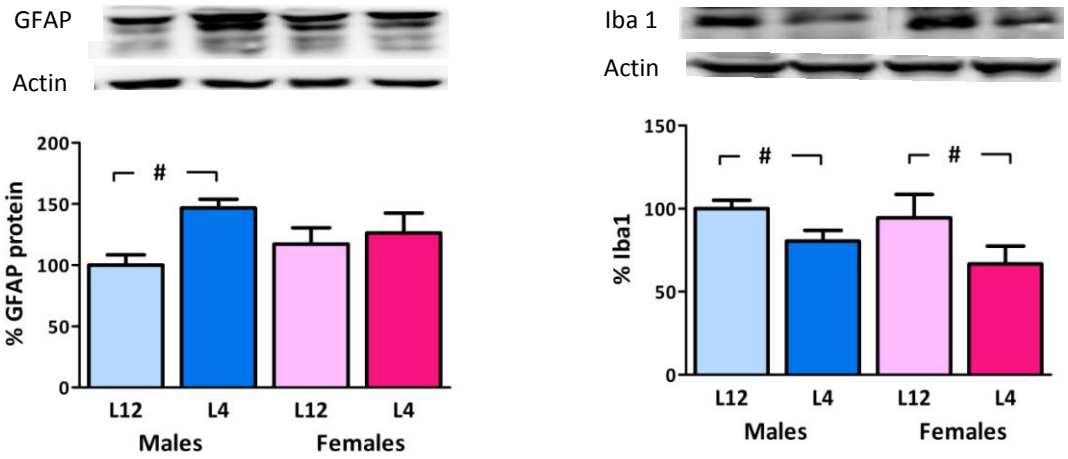


Figure 40. Relative hypothalamic GFAP (A) and Iba1 (B) protein levels in male and female rats from L12 and L4 at PND 150. N=6. #: p<0.05 Litter size effect by 2-ways ANOVA.

		GFAP
PND 10*	**ML12**	100 ± 7.8
	ML4	97.7 ± 12.9
	FL12	93.7 ± 12.9
	FL4	103.1 ± 12.9
PND 21*	**ML12**	100 ± 6.7
	ML4	101 ± 8.4
	FL12	101 ± 6.5
	FL4	107.6 ± 12
PND 30	**ML12**	100 ± 4.2
	ML4	73.9 ± 14.2
	FL12	105.8 ± 5.7
	FL4	95.7 ± 12.2
PND 50	**ML12**	100 ± 12.6
	ML4	106.1 ± 17.4
	FL12	98.8 ± 10
	FL4	81.6 ± 17.2
PND 85	**ML12**	100 ± 10
	ML4	88.4 ± 5
	FL12	117.1 ± 202
	FL4	87.8 ± 5.8

Table 20. Hypothalamic GFAP protein levels at PND10, PND21, PND30, PND50 and PND85, which levels were not significantly different between the experimental groups.

Hypothalamic vimentin protein levels were affected by litter size at PND30 ($F_{(1, 24)} = 6.5$, $p<0.02$; Figure 41), when the animals exposed to early over nutrition had lower levels of this protein. There was no effect at any other ages (results not shown).

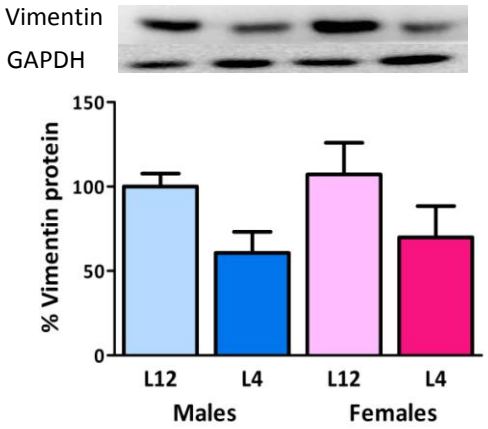


Figure 41. Relative hypothalamic vimentin protein levels in male and female rats from L12 and L4 at PND 30. N=6

To investigate whether neuronal maturation was affected by neonatal overnutrition during the prepubertal stages, nestin levels were analyzed at PND 10, 21 and 30. There was no effect of sex or litter size at PND10 or 21. In contrast, at PND30, nestin protein levels tended to be increased in the animals raised in small litters in both sexes (ML12: 100 ± 28.1, ML4: 191.1 ± 79.0, FL12: 37.3 ± 11.8, FL4:175.5 ± 60.1 %ML12; litter size effect p=0.058).

Given that changes in hypothlamic GFAP levels were found in response to early overnutrition in males, but not females, at PND150 we asked whether this was associated with increased markers of inflammation and/or changes in the activation of intracellular pathways associated with leptin and insulin signaling.

The p-Stat3$^{(Tyr705)}$ protein levels were increased by litter size ($F_{(1,20)}$ = 6.1, p<0.03) in both sexes. However, p-Stat3$^{(Ser\ 634)}$ protein levels were unaffected (data not shown).

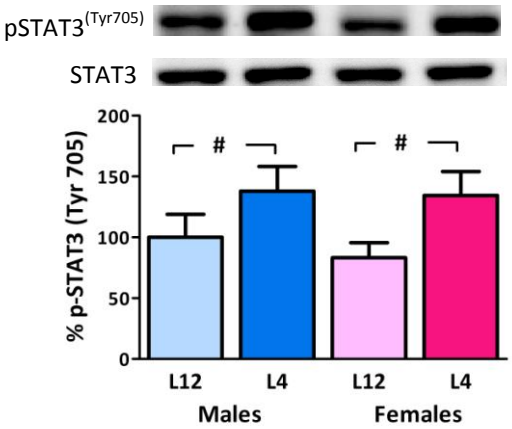


Figure 42. Relative hypothalamic pSTAT3$^{(Tyr705)}$ protein levels in male and female rats from L12 and L4 at PND 30. N=6 #: p<0.03 litter size effect by 2-ways ANOVA.

There was an interaction between litter size and sex on SOCS3 protein levels ($F_{(1,18)}$ = 5.1, p<0.05), with this inhibitor of cytokine signaling tending to decrease in L4 males and increase in L4 females.

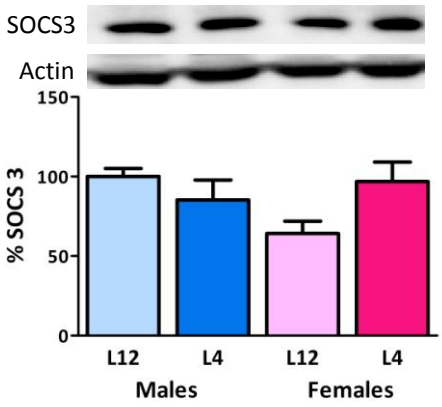


Figure 43. Relative hypothalamic SOCS3 protein levels in male and female rats from L12 and L4 at PND 30. N=6

Hypothalamic protein levels of p-IRS, p-AKT, p-Iκβ and p-PTEN were not different between groups at PND150 (data not shown).

1.5.5 Early overnutrition and sex influence on astrocyte cell number and morphology

Although neonatal overnutrition did not increased GFAP levels in PND150 females, it is possible that the number or morphology of astrocytes could be affected. Hence, we analyzed the number and morphology of astrocytes in the hypothalamus of male and female rats at PND150.

There was an effect of litter size ($F_{(1,19)} = 9.8$, $p<0.005$; Figure 44A), with an increase in the mean number of GFAP cells/area in the arcuate nucleus of the hypothalamus of males that had been exposed to early overnutrition. There was an overall effect of sex ($F_{(1,19)} = 5.4$, $p<0.04$; Figure 44B) on the number of projections per GFAP cell, with females tending to have more primary projections. Primary projections were also measured to determine their length, an effect of litter size was found ($F_{(1,19)} = 19.4$, $p<0.003$; Figure 44C), with ML4 having shorter primary projections than ML12 and no effect on females.

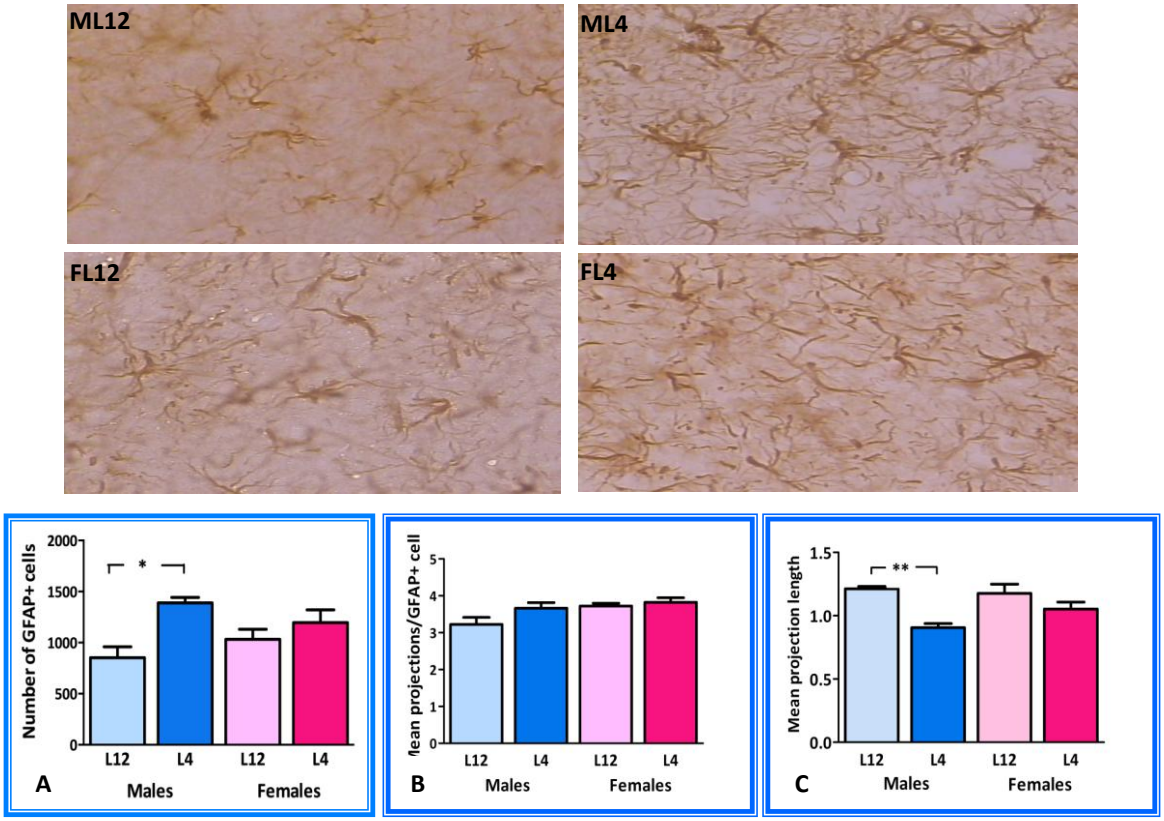


Figure 44. Number of GFAP positive cells/area (A), number of projections per GFAP positive cell (B) and primary projections length (C) in the arcuate nucleus of male and female rats from L12 and L4 at PND 150. Number of cells/area: $F_{(3, 22)}$ = 3.8, *=p<0.0 3; Length of projections: $F_{(3,22)}$= 8.1**=p<0.01

As leptin receptor expression in astrocytes has been reported to increase in hypothalamic astrocytes as a result of HFD-induced obesity **(Hsuchou et al., 2009)**, we analyzed whether the increase in weight in male rats due to neonatal nutrition is associated with changes in LepR levels in astrocyte of the arcuate nucleus. The level of LepR immunoreactivity over GFAP positive somas was increased in rats raised in a small litter (litter effect: $F_{(1,8)}$=24.6, p<0.001; Figure 45), regardless of sex.

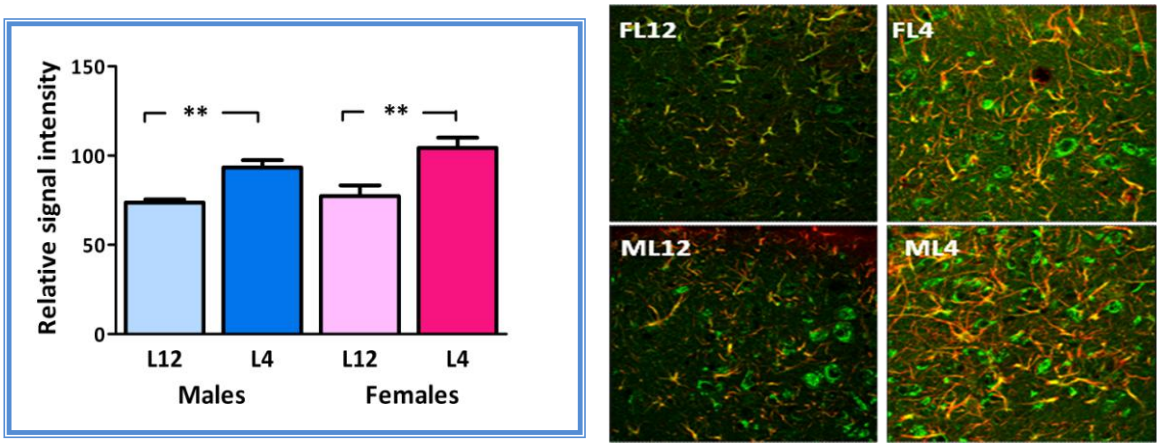


Figure 45. Relative signal intensity of ObR over GFAP cells in male and female rats from L12 and L4 at PND 90.

1.5.6 Early overnutrition and sex effects on microglia cell number

There were no significant differences in the mean number of Iba1+ cells/area in the arcuate nucleus of the hypothalamus of 150 day old rats (Figure 46), although the number of Iba1+ cells tended to decrease in rats with neonatal overnutrition, similar to that observed for Iba1 protein levels in the hypothalamus.

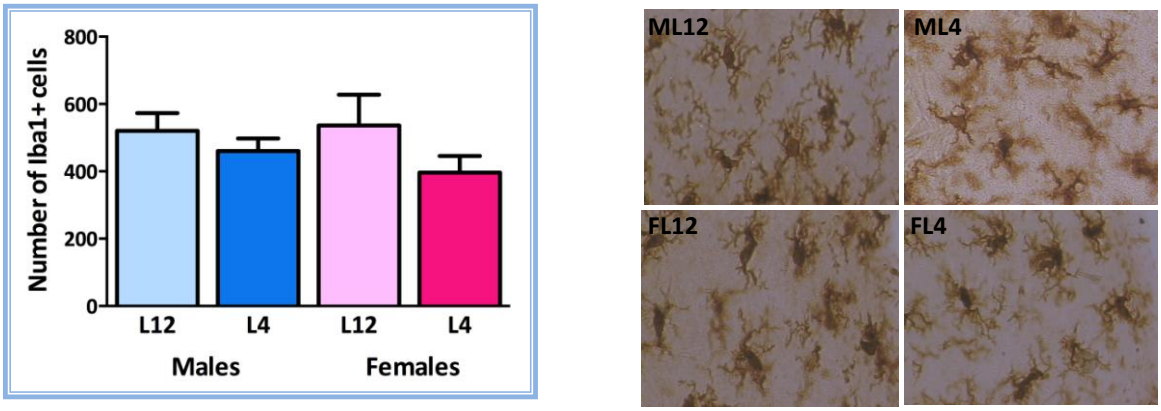


Figure 46. Mean number of Iba1 positive cells/ and immunohistochemitry images in the arcuate nucleus of male and female rats from L12 and L4 at PND 150.

2. Neonatal androgenization

2.1 Metabolic effects of neonatal androgenization

2.1.1 Metabolic effects of neonatal androgenization at PND10

At PND10, testosterone levels remained elevated in females that received testosterone on PND1 (M: 6.6 ± 3.4, F: 1.2 ± 0.4. AF: 7.0 ± 2.9 ng/ml). To determine if neonatal testosterone levels could play a role in the development of the sexually dimorphic effects observed at PND10 in the neonatal overnutrition study, testosterone levels were also measured in females at this age. At PND10 there was an effect of litter size ($F_{(1,18)}$ = 4.4, $p<0.05$) with an interaction between sex and litter size ($F_{(1,18)}$ = 5.7, $p<0.03$), as L12 males had lower levels than L4 males, but with no effect in females (ML12: 0.19 ± 0.05, ML4: 0.76 ± 0.27, FL12PND10: 0.35 ± 0.04, FL4PND10: 0.31 ± 0.02 ng/ml).

Circulating 17β-estradiol levels showed no differences between groups (M: 37.2 ± 4.0, F: 39.7 ± 6.1, AF: 28.4 ± 2.3 pg/ml)

Neonatal androgenization did not alter body weight (M: 17.7 ± 0.4, F: 17.8 ± 0.4, AF: 18.3 ± 0.4 g) or body length (M: 7.4 ± 0.1, F: 7.5 ± 0.1, AF: 7.7 ± 0.1 cm) at PND10.

The amount of SCAT was higher in females than in males and even greater in androgenized females (Figure 47).

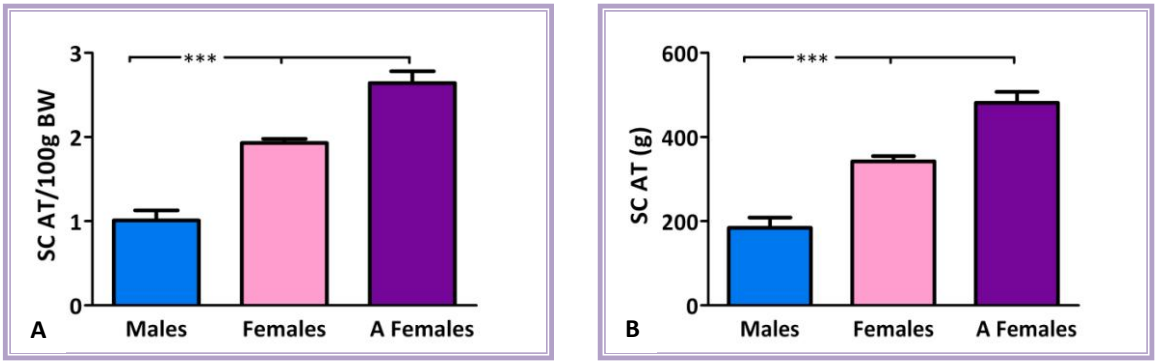


Figure 47. Percentage **(A)** and absolute **(B)** amount of subcutaneous fat (SCAT) at PND10 in males, females and androgenized females SCAT/100g BW: ($F_{(2,28)}$= 53.9); SCAT (g): ($F_{(2,28)}$= 47.8) ***= $p<0.0001$.

At PND10, glycemia (M: 112.3 ± 3.1, F: 104.1 ± 4.0, AF: 102.1 ± 3.7 mg/dl) and insulin levels (M: 0.69 ± 0.24, F: 0.72 ± 0.19, AF: 0.49 ± 0.07 ng/ml) were not different between groups.

Serum leptin levels were higher in females compared to males, but androgenized females were not different from either males or females ($F_{(1,18)}$ = 10.3; p<0.01; Figure 48). There was a similar effect on serum adiponectin levels, with higher levels of this adipokine in females compared to males ($F_{(1,25)}$ = 11.3; p<0.01) with androgenization reducing adiponectin levels so that they were no longer different from males.

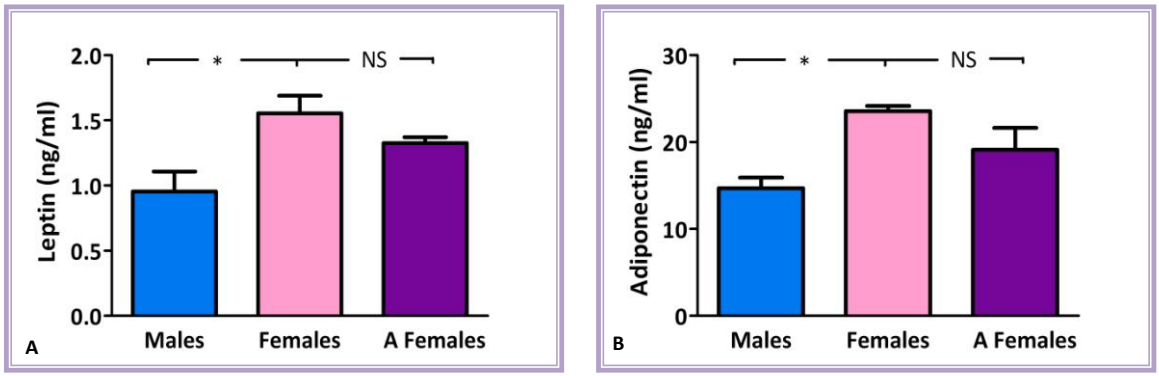


Figure 48. Serum leptin **(A)** and adiponectin **(B)** levels at PND10 in males, females and androgenized females. Leptin: ($F_{(2,17)}$ = 6.3, p<0.02; Adiponectin: ($F_{(2,22)}$ = 5.6; p<0.01) *= p<0.05.

Circulating levels of the inflammatory cytokines IL1β (M: 41.8 ± 24.7, F: 39.2 ± 9.2, AF: 27.1 ± 14.9 pg/ml) and TNFα (M: 1.7 ± 0.3, F: 1.1 ± 0.4, AF: 1.0 ± 0.4 pg/ml) were not affected by neonatal androgenization**.** In contrast, circulating IL6 levels were lower in females than in males, with androgenization of females ablating this difference ($F_{(2,16)}$ = 4.0, p<0.05; (Figure 49).

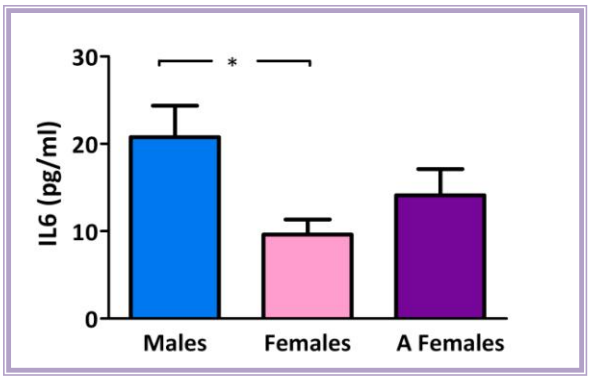


Figure 49. Serum IL6 levels at PND10 in males, females and androgenized females. ($F_{(2,16)}$ = 4.0, *= p<0.05

2.1.2 Metabolic effects of neonatal androgenization at PND90

At PND90 circulating testosterone levels were not affected by neonatal testosterone injection, with males having significantly higher levels than both control and androgenized females ($F_{(1,29)}$ = 20.0, p<0.0001; Figure 50). No significant differences were found in circulating 17β-estradiol levels (M: 49.1 ± 2.1, F: 47.6 ± 2.8, AF: 42.1 ± 2.2 pg/ml).

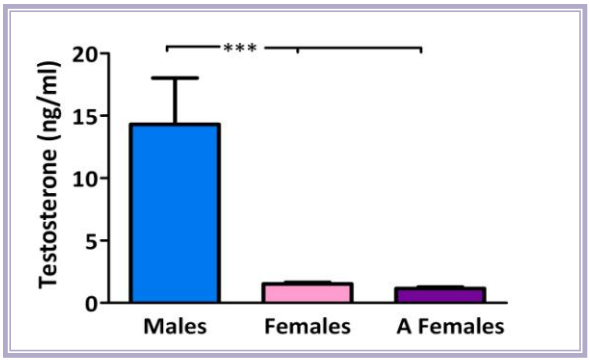


Figure 50. Circulating testosterone levels at PND90 in males, females and androgenized females. Testosterone: $F_{(2, 28)}$ = 13.5, ***= p<0.0001.

At PND90, males were heavier than both females and androgenized females ($F_{(1,31)}$ = 231.3, p<0.0001) and the latter weighed more than control females (Figure 51). No differences in length were observed at this age.

B

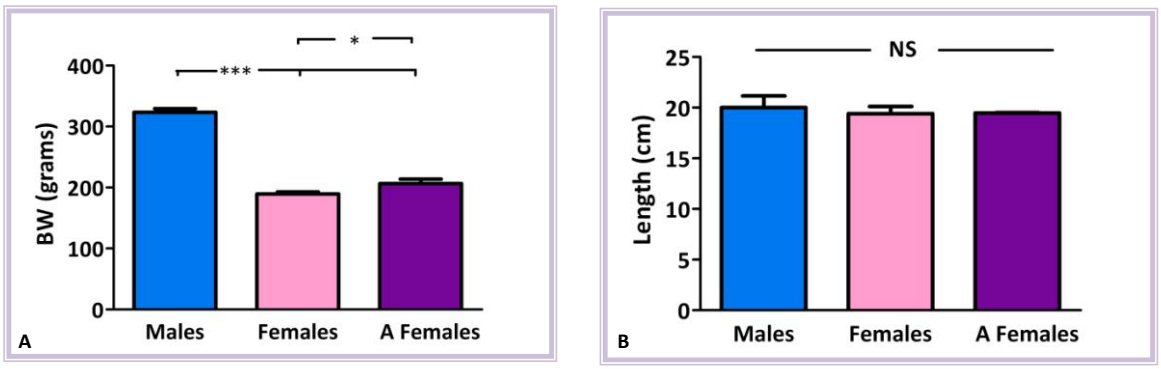


Figure 51. Representation of BW **(A)** and length **(B)** at PND90 in males, females and androgenized females. BW: $F_{(2,30)}$ = 128.6 ***=p<0.001; T-student: F *vs* AF *= p<0.05 .

The percentage of both SCAT ($F_{(1,22)}$ = 84.7, p<0.0001) and VAT ($F_{(1,21)}$ = 15, p<0.001) was higher in males compared to both female groups, with no effect of neonatal testosterone treatment, Figure 52.

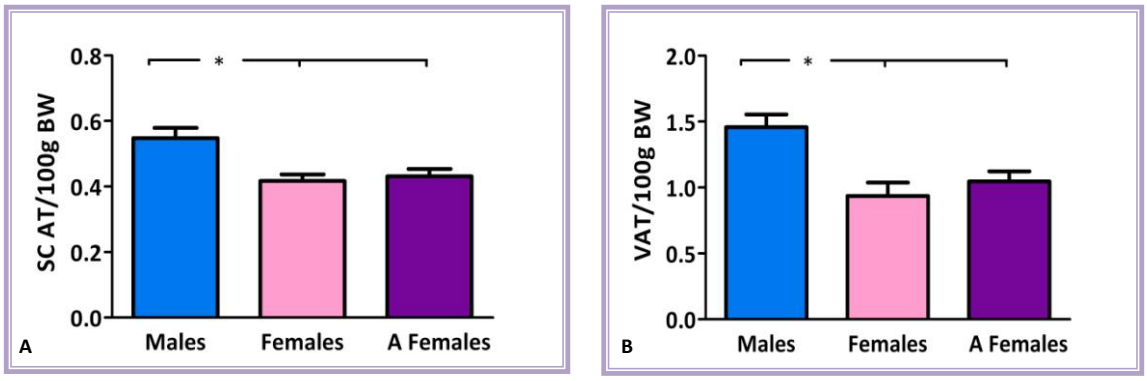


Figure 52. Percentage of subcutaneous adipose tissue (SCAT; **A**) and visceral adipose tissue (VAT; **B**) at PND90 in males, females and androgenized females. SCAT/100 g BW: $F_{(2,21)}$ = 7.9; VAT/100g BW: $F_{(2,20)}$ = 7.9 *= p<0.01.

At PND90, glycemia was higher in males compared to females (sex effect: $F_{(1,26)}$ = 5.1; p<0.05, Figure 53A) and neonatal androgenization of females increased glucose levels such that they were no longer different from males. Insulin levels were lower in both females groups (sex effect: $F_{(1,13)}$ = 6.8, p<0.01; Figure 53B).

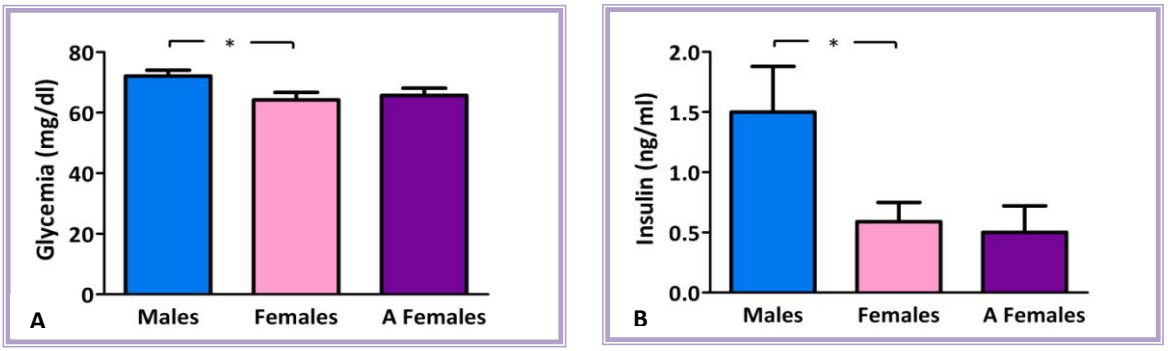


Figure 53. Serum glucose **(A)** and insulin **(B)** levels at PND 90 in males, females and androgenized females**.** Glycemia: $F_{(2,28)}$ = 3.7 *= p<0.05 ; Insulin: $F_{(2,15)}$ = 5.1 **= p<0.01

Serum leptin levels were lower in females compared to males and neonatal testosterone treatment resulted in leptin levels being higher than in control females ($F_{(1,18)}$ = 10.3, p<0.01; Figure 54A) and not different from males. At PND90, circulating adiponectin levels (Figure 54B) did not vary between the experimental groups.

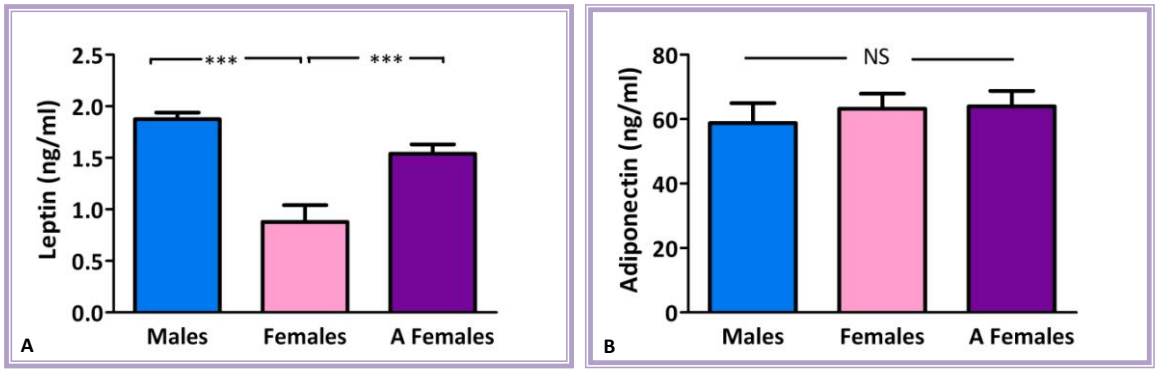


Figure 54. Serum leptin **(A)** and adiponectin **(B)** levels at PND90 in males, females and androgenized females. Leptin: $F_{(2,17)}$ = 22.3; ***= p<0.0001.

Circulating levels of IL1β were lower in females compared to males ($F_{(1,17)}$ = 15.1, p<0.003) and neonatal androgenization increased the levels of this cytokine in adult female rats ($F_{(1,17)}$ = 8.8, p<0.03; Fig. 55). Serum TNFα levels were below the limit of detection of the assay employed.

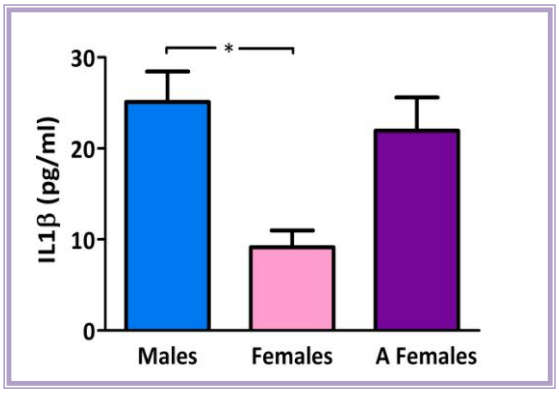


Figure 55. Serum IL1β levels at PND90 in males, females and androgenized females. ANOVA: $F_{(2,16)} = 5.9$ *= p<0.03.

2.2 Cytokine profile in adipose tissue

2.2.1 Expression of cytokines in subcutaneous adipose tissue at PND10

The injection of testosterone to neonatal females had no significant effect on the mRNA levels of leptin, adiponectin, IL1β, IL6 or TNFα in SCAT (Table 21) at PND10.

	Leptin mRNA	Adiponectin mRNA	IL1β mRNA	IL6 mRNA	TNFα mRNA
MALES	100 ± 26.3	100 ± 21.0	100 ± 22.8	100± 20.8	100±34.1
FEMALES	138.8± 21.7	130.4 ± 9.5	57.8± 14.8	74.1±16.3	115.1±13.3
ANDROGENIZED FEMALES	142.9± 27.2	103.7 ± 15.3	80.3± 14.4	94.1±17.6	132.5 ± 13

Table 21. Expression of leptin, adiponectin, IL1β, IL6 and TNFα mRNA levels in subcutaneous adipose tissue at PND10 in males, females and androgenized females. All data are normalized to the expression level of males.

2.2.2 Expression of adipokines and cytokines in subcutaneous adipose tissue at PND90

The expression of leptin, adiponectin and TNFα did not differ between groups in SCAT, while IL1β and IL6 were not reliably detectable in most samples (Table 22) at PND90.

	Leptin mRNA	Adiponectin mRNA	IL1β mRNA	IL6 mRNA	TNFα mRNA
MALES	100 ± 67.8	100 ± 50.2	BLD	BLD	100 ± 64.8
FEMALES	151.2 ± 77.6	188.1 ± 27.1	BLD	BLD	156.6 ± 43.9
ANDROGENIZED FEMALES	170.2 ± 74.3	293.8 ± 90.3	BLD	BLD	129.5 ± 19.8

Table 22. Expression of leptin, adiponectin, IL1β, IL6 and TNFα mRNA levels in subcutaneous adipose tissue at PND90 in males, females and androgenized females. BLD= below limits of detection. Data are normalized to the levels in males.

2.2.3 Expression of adipokines and cytokines in visceral adipose tissue at PND90

At PND90, leptin mRNA levels in VAT were higher in males compared to females of both groups, but androgenization of females increased leptin expression so that it was higher than in normal females ($F_{(2,14)}$ = 18.5, $p<0.0002$; Fig. 56). Adiponectin ($F_{(2,14)}$ = 6.5, $p<0.01$; Fig. 57A), IL6 ($F_{(2,15)}$ = 9.7, $p<0.003$; Fig 57B) and TNFα ($F_{(2,15)}$ = 15.1, $p<0.0001$; Fig. 57C) were lower in both groups of females compared to males. IL1β mRNA levels were not significantly different between groups (M: 100 ± 41.4, F: 30.6 ± 23.7, AF: 282.9 ± 154.9 % M).

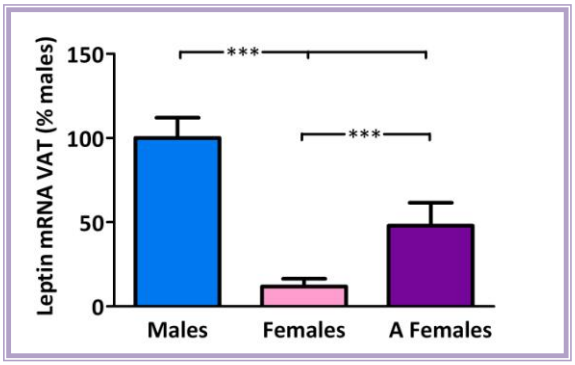


Figure 56. Relative leptin mRNA levels in visceral adipose tissue at PND90 in males, females and androgenized females. ***= p<0.0001.

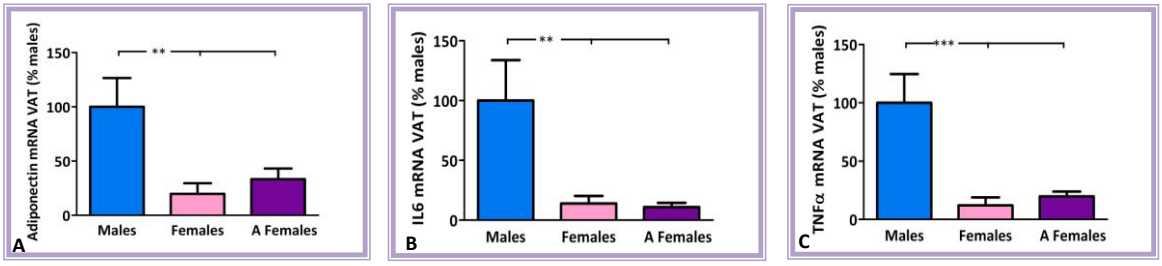


Figure 57. Relative adiponectin **(A)**, IL6 (A) and TNFα **(B)** mRNA levels in visceral adipose tissue at PND 90 in males, females and androgenized females. **= p<0.001, ***=p<0.0001.

2.3 Histological analysis of visceral adipose tissue at PND90

The histological study of VAT at PND90, indicated that VAT adipocytes were larger in males than females and andogenized females ($F_{(1,7)}$ = 21.9, p<0.003; Figure 58), with no differences between females and androgenized females.

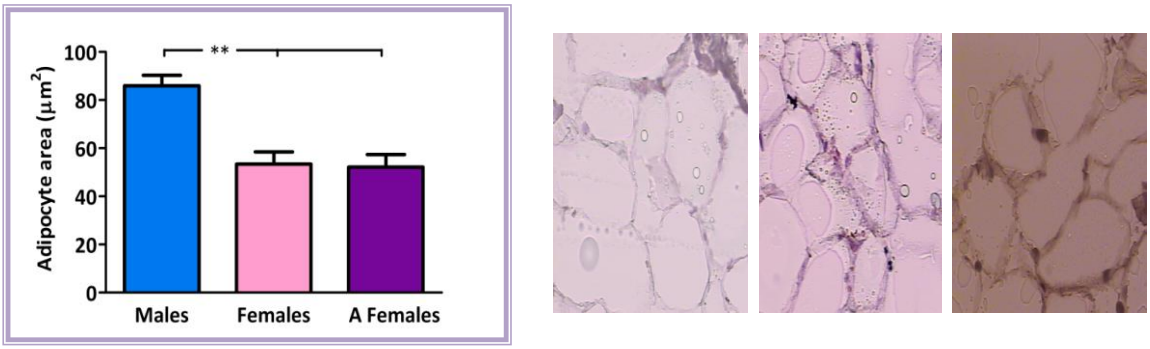


Figure 58. Relative adipocyte area in visceral adipose tissue at PND 90 in males, females and androgenized females. $F_{(2,9)}$ = 13.9, **= p<0.005.

2.4 Central effects of neonatal androgenization of female rats

2.4.1 Effects of neonatal androgenization on glial proteins in the hypothalamus at postnatal days 10 and 90

At PND10 neonatal androgenization had no effect on GFAP (M: 100 ± 24.4; F: 91.5 ± 22.3; AF: 100.2 ± 18.6 % M) or vimentin (M: 100 ± 42.7; F: 71.1 ± 17.9; AF: 82.9 ± 7.8 %M) protein levels in the hypothalamus. However, at PND90 females tended to have lower hypothalamic GFAP levels than males, while neonatal androgenization increased GFAP levels such that they were significantly higher than in the control females ($F_{(1,14)}$ = 8.4, p<0.03; Figure 59A). Vimentin protein levels in the hypothalamus tended to be higher in females than in males, but this was not statistically significant due to high variability within the groups (Figure 59B).

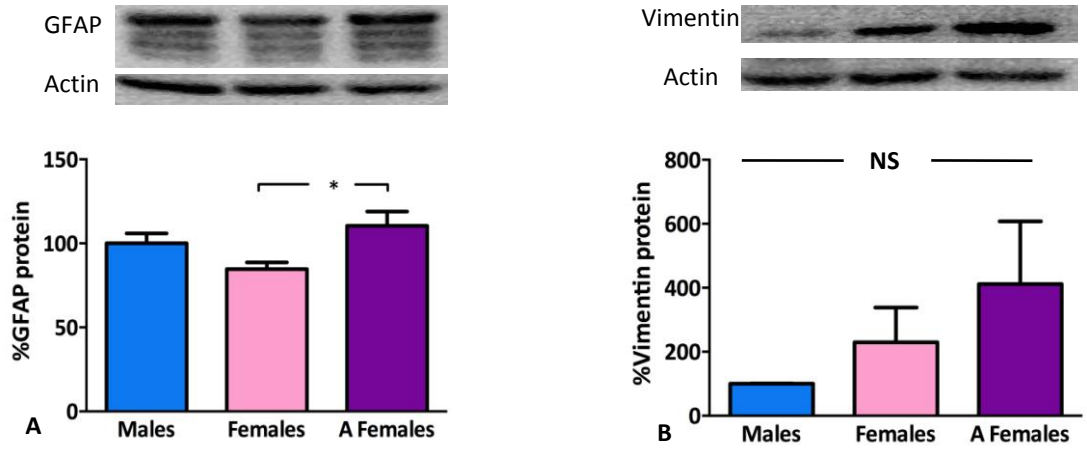


Figure 59. Relative GFAP (A) and vimentin (B) protein levels in the hypothalamus at PND90 in males, females and androgenized females. GFAP: ($F_{(2,16)}$ = 4.2) *= p<0.05. NS = not significant.

3. Primary astrocyte cultures

Neonatal overnutrition resulted in changes in GFAP expression in the hypothalamus of male, but not female, rats. Indeed, the number of GFAP positive cells was higher in males from small litters compared to control males at PND150. Moreover, the effects seen on hypothalamic inflammatory markers were different between males and females. Therefore, we analyzed whether male and female hypothalamic astrocytes *in vitro* respond differently to hormonal signals and fatty acids (FAs).

3.1 Dose response curve of male and female hypothalamic astrocytes to palmitic acid

We performed a dose response curve to palmitic acid in primary astrocyte cultures of both sexes. Astrocytes were exposed to increasing concentrations of palmitic acid: 0.05, 0.1, 0.25 and 0.5mM during 24 hours as concentrations between 0.05 and 0.6 mM have been shown to induce cytokine release and are within a physiological range in serum, as NEFA levels in rat range from 100–200 µM in control, non-fasting conditions to 500–600 µM in fasted animals **(Rustan et al. 1992)**, **(Xu et al. 2010)**. Samples were processed to determine if the expression of diverse proteins was affected as a consequence of FAs exposure.

3.1.1 Change in cell number in response to palmitic acid treatment

We first determined if PA affects the number of astrocytes *in vitro*. No significant change in the number of cells was found in response to this FA (Figure 60).

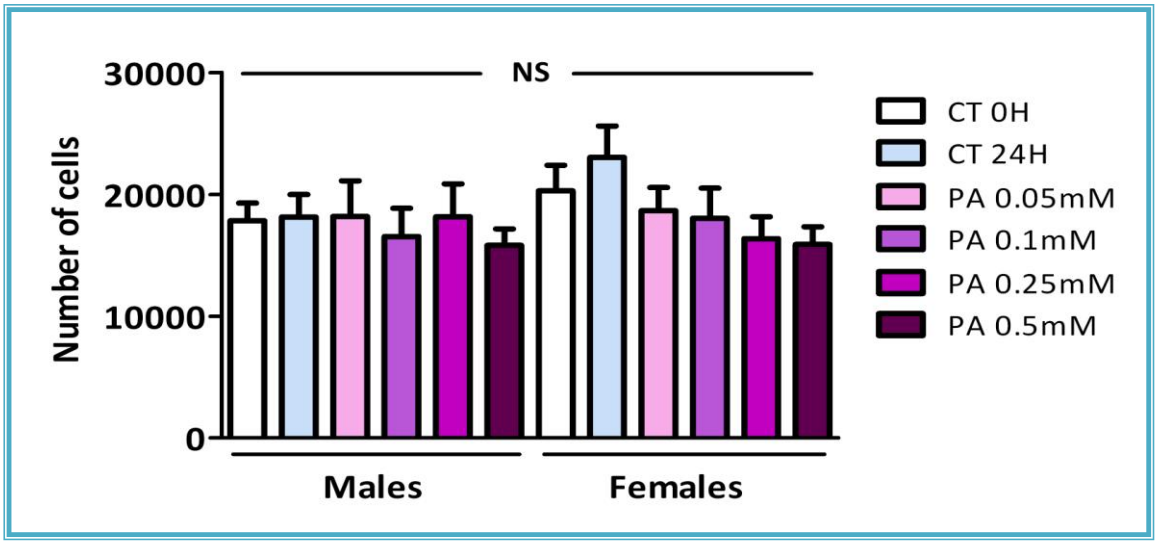


Figure 60. Number of cells in primary astrocyte cultures from 2 day old male and female Wistar rats after treatment with increasing levels of palmitic acid (PA) for 24 hours. N=4 cultures with each experimental group being performed in triplicate in each culture.

3.1.2 Changes in the mRNA levels of GFAP, IL6, IL1β, TNFα, CHOP and IGF-1 in response to increasing concentrations of palmitic acid

GFAP mRNA levels were progressively decreased in response to increasing concentrations of PA ($F_{(4,30)}$ = 21.34; p<0.0001; Figure 61).

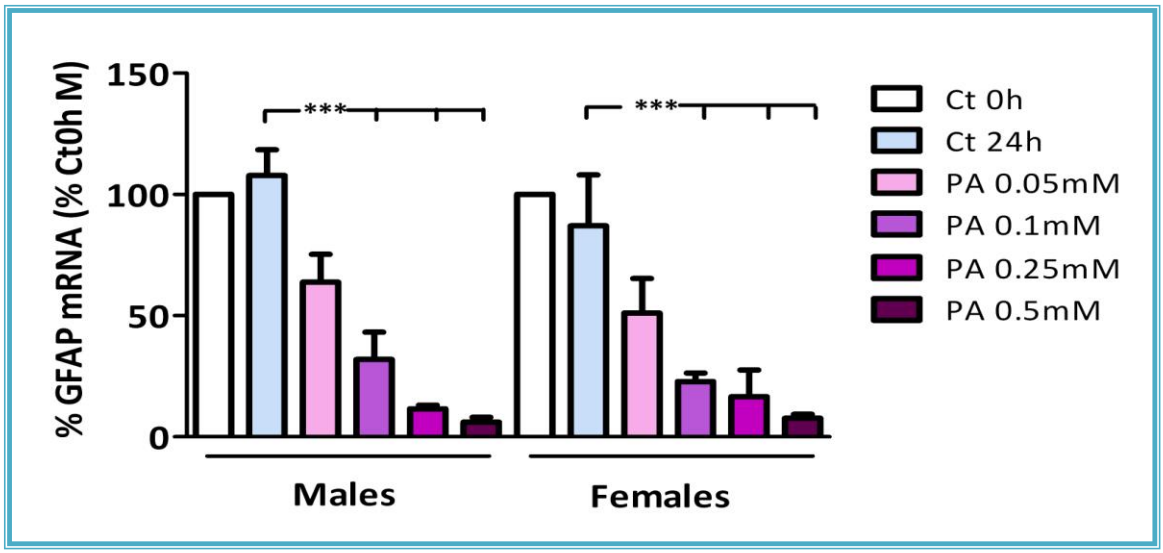


Figure 61. Relative GFAP mRNA levels in primary hypothalamic astrocyte cultures from 2 day old male and female Wistar rats. ($F_{(9,39)}$ = 9.9; p<0.0001). Ct: control; PA: Palmitic acid. N=4 cultures with at least 3 repetitions of each group in experiment.

Carnitine palmytoil transferase 1a increased in astrocytes from both sexes in response to PA ($F_{(4,31)}$ = 6; p<0.002; Figure 62), although specific differences between the groups were not found in the posthoc analysis.

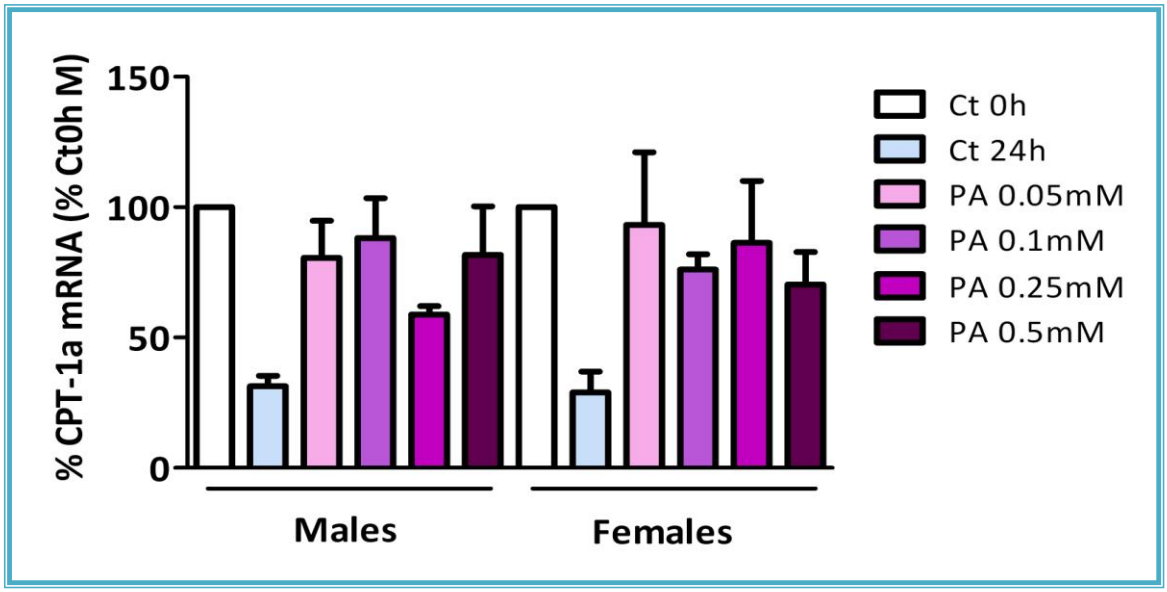


Figure 62. Relative CPT-1a mRNA levels in primary hypothalamic astrocyte cultures from 2 day old male and female Wistar rats. ($F_{(9,40)}$ = 2.9; p<0.02). Ct: control; PA: Palmitic acid. N=4 cultures with at least 3 repetitions of each group in experiment.

The expression of IL6 increased with increasing levels of palmitic acid in both sexes ($F_{(4, 29)}$ = 5.4, p<0.003; Figure 63).

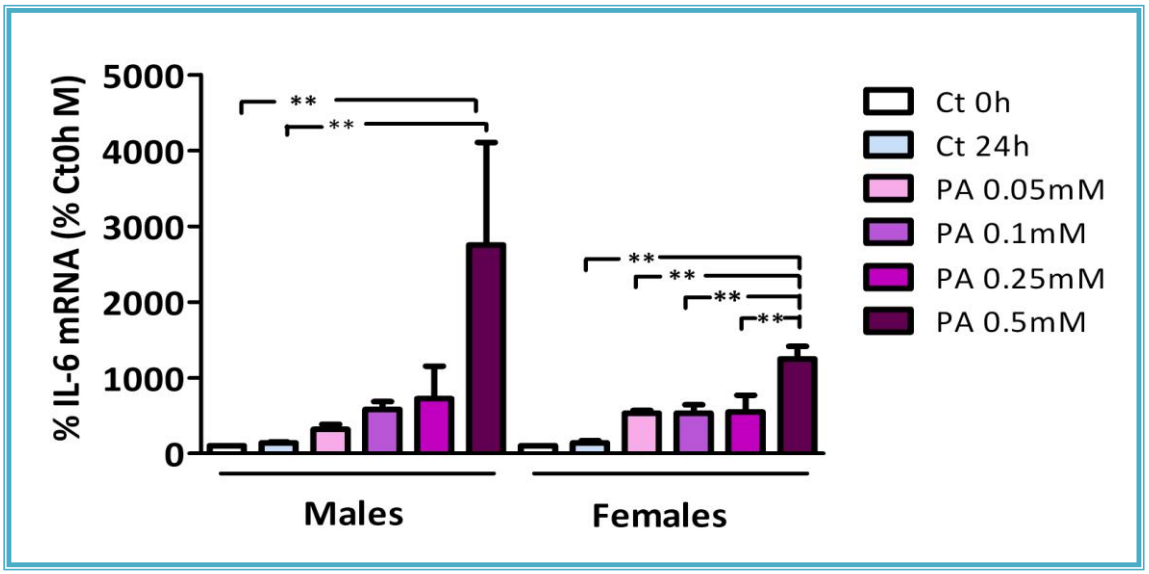


Figure 63. Relative IL6 mRNA levels in primary hypothalamic astrocyte cultures from 2 day old male and female Wistar rats. Ct: control; PA: Palmitic acid. ANOVA: ($F_{(9,38)}$ = 3, p<0.03). N=4 cultures with at least 3 repetitions of each group in each experiment.

No significant effects were found on IL1β mRNA levels (Males: Ct0: 100, Ct24h: 49.9 ± 15.7, PA 0.05 mM: 61.2 ± 18.6, PA 0.1 mM: 91.2 ± 34.2, PA 0.25 mM: 96.0 ± 50.9, PA 0.5mM**: 159** ± 115.7; Females: Ct0h: 104.3 ± 35.8, Ct24h: 54.9 ± 21, PA 0.05 mM: 116.9 ± 31.6, PA 0.1 mM: 83.4 ± 28.9, PA 0.25 mM: 34.4 ± 8.8, PA 0.5 mM: 144.1 ± 66.6 %Ct0 Males).

There was an effect of sex on TNFα expression, with females expressing lower levels than males ($F_{(1,29)}$ = 4.7, p<0.05; Figure 64). No effect of PA was found.

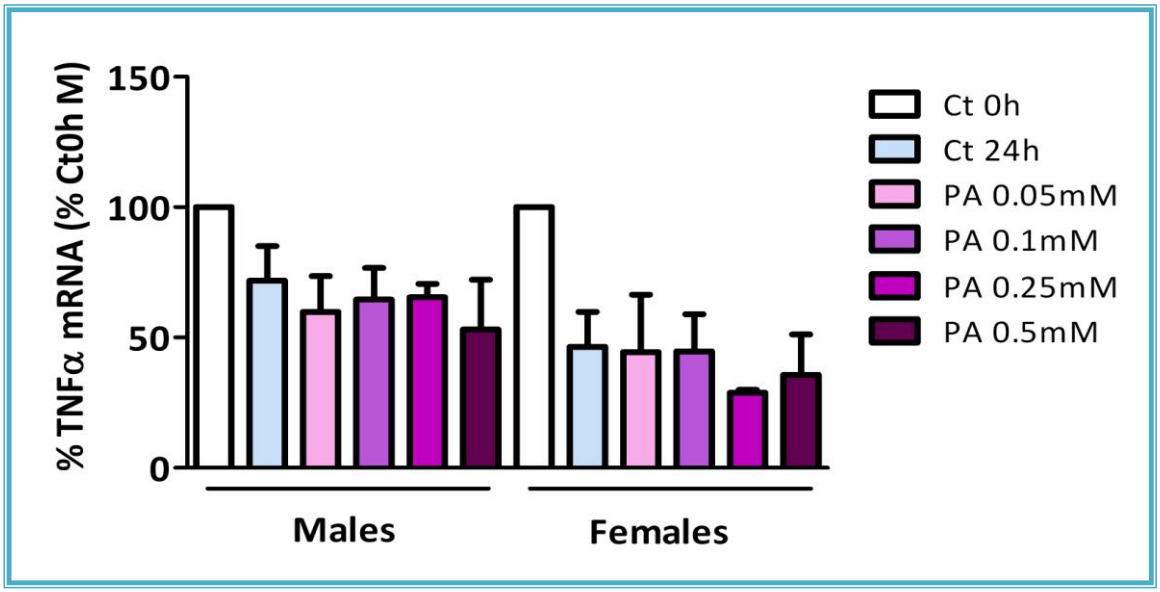


Figure 64. Relative TNFα mRNA levels in primary hypothalamic astrocyte cultures from 2 day old male and female Wistar rats. Ct: control; PA: Palmitic acid. N=4 cultures with at least 3 repetitions of each group in each experiment.

Levels of CHOP mRNA were increased after PA exposure ($F_{(4,31)}$ = 8.6, p<0.0001; Figure 65). This difference reached significance at the highest dose in males.

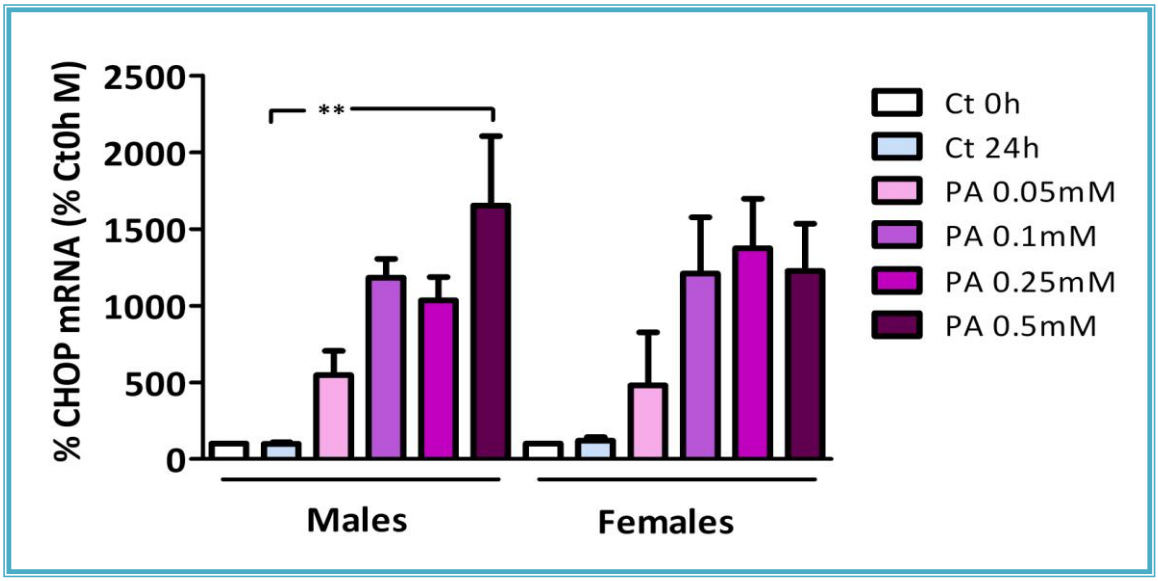


Figure 65. Relative CHOP mRNA levels in primary hypothalamic astrocyte cultures from 2 day old male and female Wistar rats. Ct: control; PA: Palmitic acid. ANOVA: ($F_{(9,40)}$ = 4, p<0.003). N=4 cultures with at least 3 repetitions of each group in each experiment.

On the contrary, the mRNA levels of the trophic factor IGF1 were reduced when exposed to PA ($F_{(4,31)}$ = 6.3, p<0.003; Figure 66), with this effect being more pronounced in females.

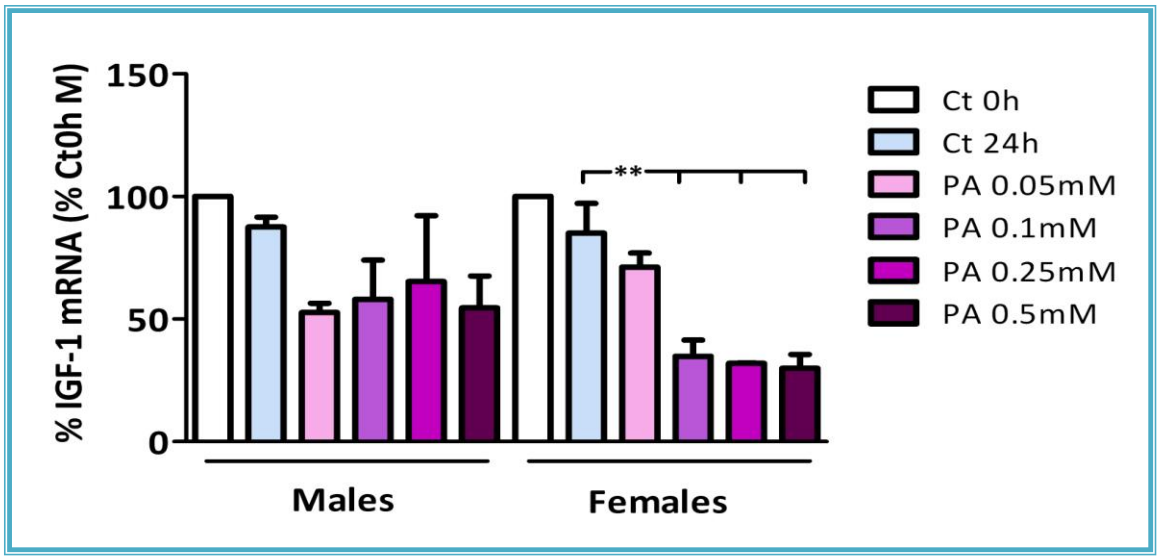


Figure 66. IGF-1 mRNA levels in primary hypothalamic astrocyte cultures from 2 day old male and female Wistar rats. Ct: control; PA: Palmitic acid. ANOVA: ($F_{(9,40)}$ = 3.8, p<0.003) **p<0.003. N=4 cultures with at least, 3 repetitions of each group in each experiment.

3.1.3 Nitrites and nitrates released in response to PA

Palmitic acid stimulated the release of NO_2 and NO_3 to the culture media in a dose dependant manner in astrocytes from both sexes ($F_{(4,28)}$ = 4.4, p<0.01; Figure 67).

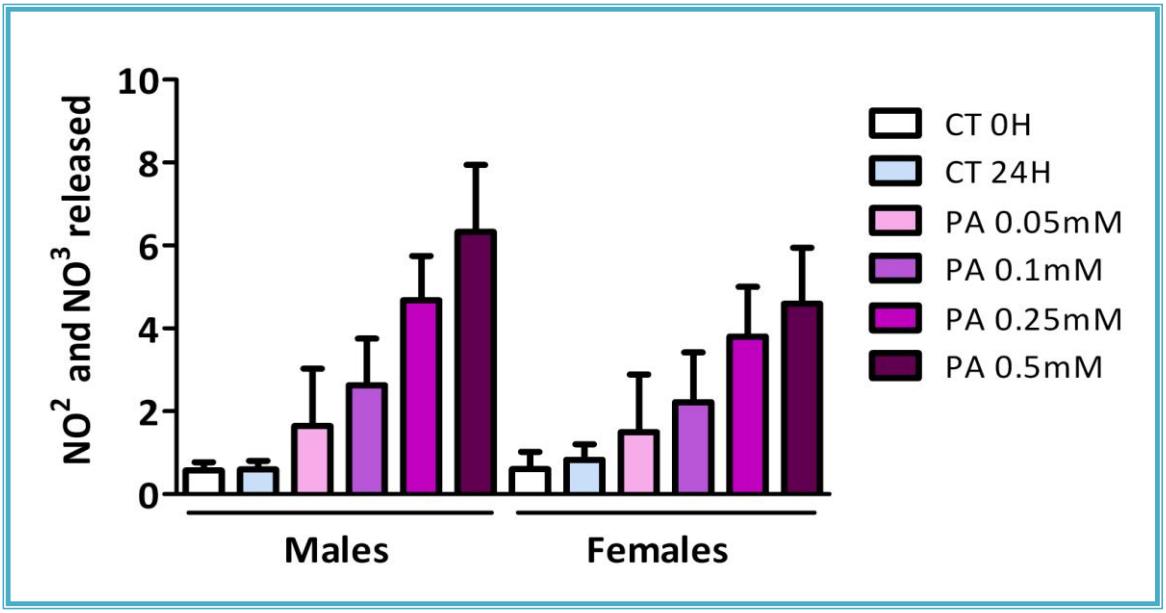


Figure 67. Concentration of NO_2 and NO_3 in the culture medium of hypothalamic astrocytes after 24 hours of palmitic acid (PA) exposure. Ct: control; ANOVA: ($F_{(9,37)}$ = 2.2), p=0.057. N=4 cultures with at least 3 repetitions of each experimental group in each culture.

3.2 Response of male and female astrocytes to saturated and unsaturated fatty acids.

The hypothalamic inflammatory response to FAs has been shown to depend on the type of FFAs, therefore we analyzed if male and female astrocytes were differentially affected by different classes of FFAs. Astrocytes were exposed to oleic (monounsaturated) and palmitic (saturated) acids for 24 hours, as these are common FFAs found in our diet. In addition, as these FFAs are not normally consumed individually, the effect of the combination of OA and PA on astrocytes was also analyzed.

3.2.1 Changes in the mRNA levels of GFAP, CPT-1a IL-6, IL-1β, TNFα, CHOP and IGF-1

Astrocyte cultures were treated with 0.5 mM of each FA, as this is reported to be at the high end of physiological concentrations **(Thaler and Schwartz, 2010; Gupta et al., 2012; Yuan et al., 2013)**.

Palmitic acid alone drastically reduced the mRNA levels of the astroglial marker GFAP ($F_{(1,24)}$ = 47.7, p<0.0001). There was no effect of OA on GFAP mRNA levels; However, there was an interaction between PA and OA ($F_{(1,24)}$ = 9, p<0.01), with OA partially blocking the effect of PA. In addition, GFAP mRNA levels were affected by sex ($F_{(1,24)}$ = 4.3, p<0.05; Figure68).

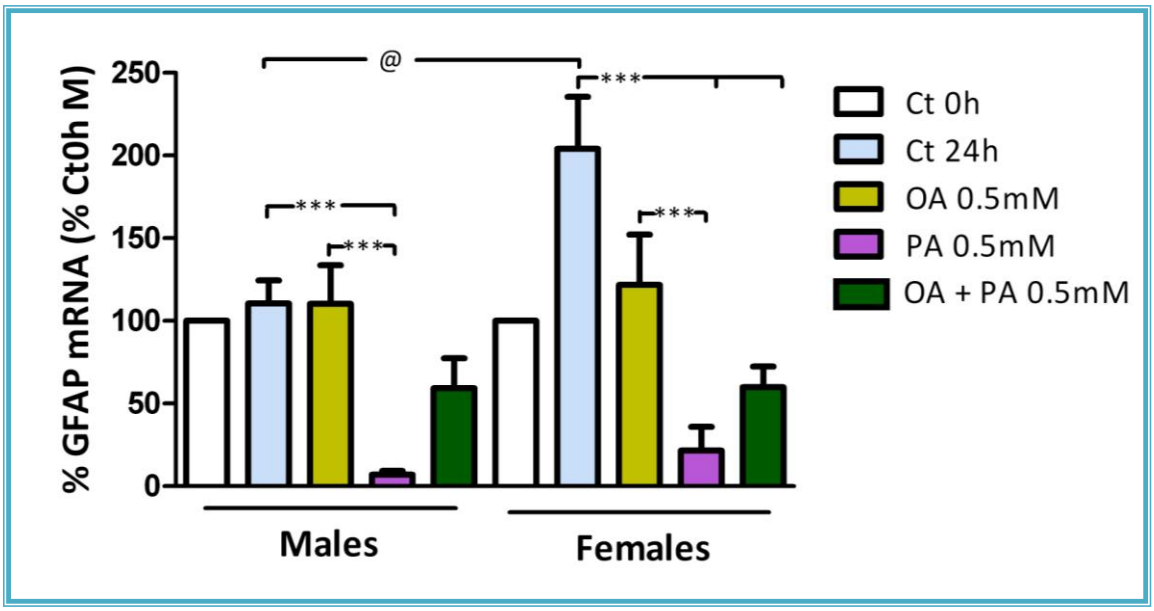


Figure 68. Relative GFAP mRNA levels in primary hypothalamic astrocyte cultures from 2 day old male and female Wistar rats. Ct: control; $F_{(7, 31)}$ = 10; ***: p<0.0001, @: p<0.05 by T-test. N=4 cultures with at least 3 repetitions of each experimental group in each one.

CPT-1a mRNA levels were modulated by OA ($F_{(1, 17)}$ = 24.3 p<0.0001), with this FA increasing CPT-1a mRNA levels regardless of the presence or not of PA (Figure69).

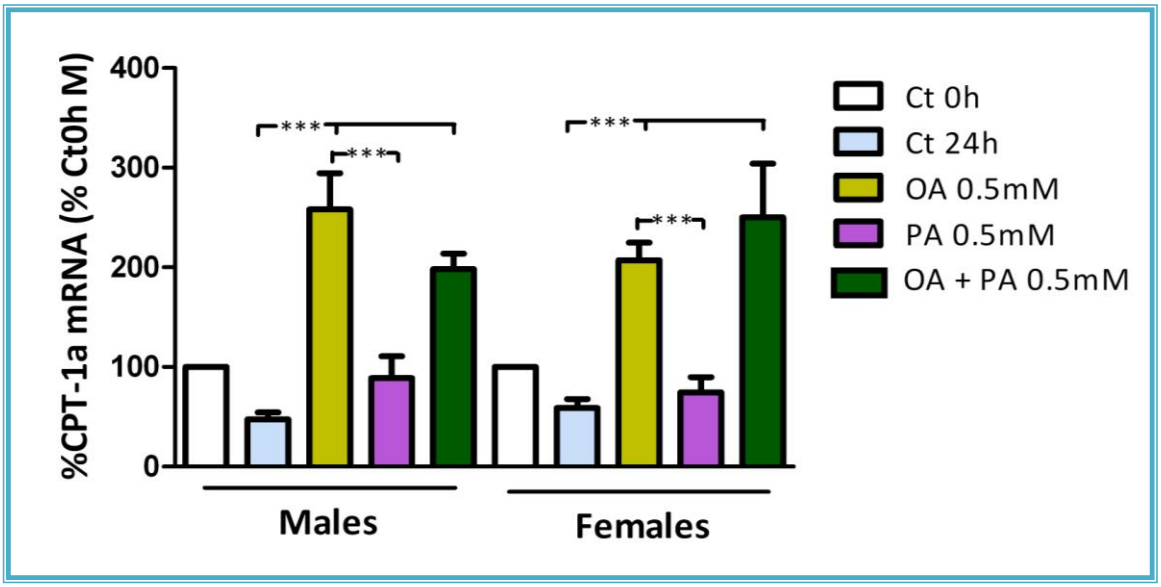


Figure 69. Relative CPT-1a mRNA levels in primary hypothalamic astrocyte cultures from 2 day old male and female Wistar rats. Ct: control; $F_{(7, 31)}$ = 11.2; ***: p<0.0001. N=4 cultures with at least 3 repetitions of each experimental group in each one.

Palmitic acid induced the expression of IL6 mRNA levels in both sexes ($F_{(1, 17)}$ = 24.3 p<0.0001;Figure 70). Oleic acid had no effect alone, but reduced the effect of PA ($F_{(1, 17)}$ = 24.3 p<0.0001).

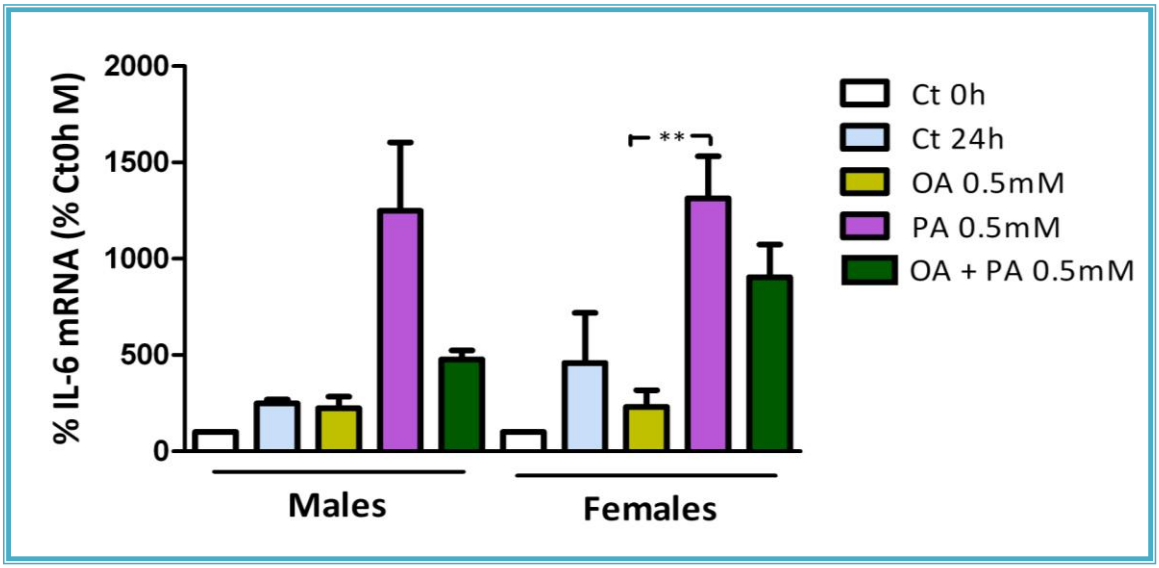


Figure 70. Relative IL6 mRNA levels in primary astrocyte cultures from 2 day old male and female Wistar rats. Ct: control; ($F_{(7,24)}$ = 5.1); **:p<0.005. N=4 cultures with at least, 3 repetitions of each experimental groups in each one.

IL1β mRNA levels were unaffected (MCt-0h: 100; MCt-24h: 140.1 ± 37.1; M-OA: 138.2 ± 35.2; M-PA: 168.5 ± 148.8; M-OA+PA: 73.7 ± 25.7; F-Ct24h: 417.2 ± 134.1; F-OA: 212.1 ± 89.4; F-PA: 181.7 ± 97.5; F-OA+PA: 89.6 ± 16 % MCt0h).

TNFα mRNA levels were affected by PA ($F_{(1,20)}$ = 36.4 p<0.0001) and OA ($F_{(1, 20)}$ = 14.4 p<0.003), with an interaction between PA and sex ($F_{(1, 20)}$ = 4.2 p=0.054) and PA and OA ($F_{(1, 20)}$ = 5.4 p<0.05; Figure 71). Both OA and PA reduced TNFα mRNA levels, with an additive effect of these FAs.

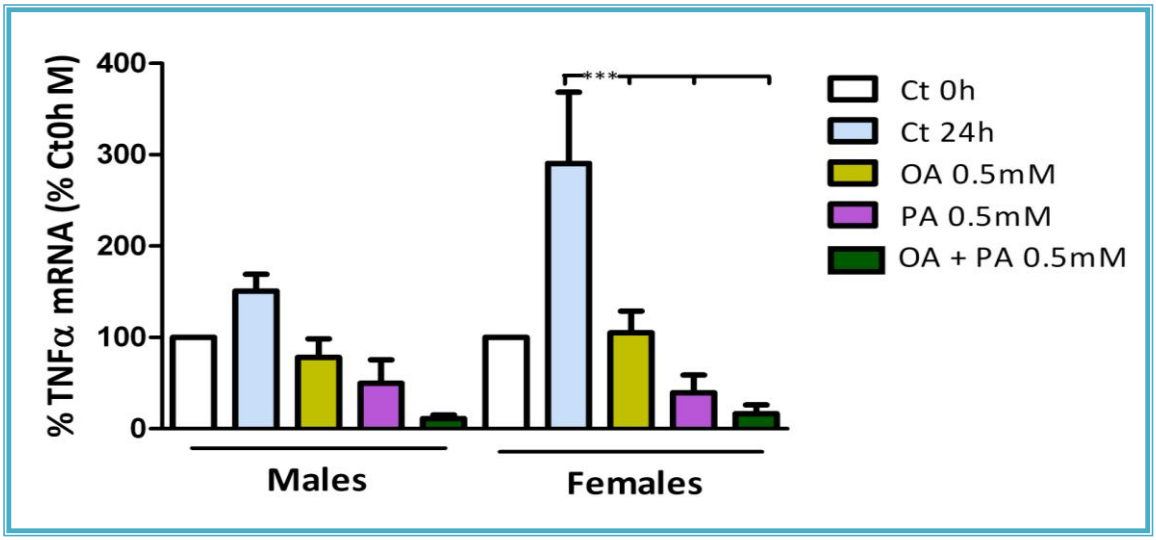


Figure 71. TNFα mRNA levels in astrocyte primary cultures from 2 days old male and female Wistar rats. Ct: control; h: hours. ANOVA: ($F_{(7,27)}$ = 8.9, p< 0.0001). N=3 cultures with at least 3 repetitions of each experimental group in each one.

We analyzed CHOP mRNA levels, which is activated by ER stress and promotes apoptosis. We found expression of this gene to be affected by OA ($F_{(1,16)}$ = 5.3, p<0.05) and PA ($F_{(1,16)}$ = 17.3, p<0.002; Figure 72), with PA inducing CHOP and OA inhibiting the effect of PA.

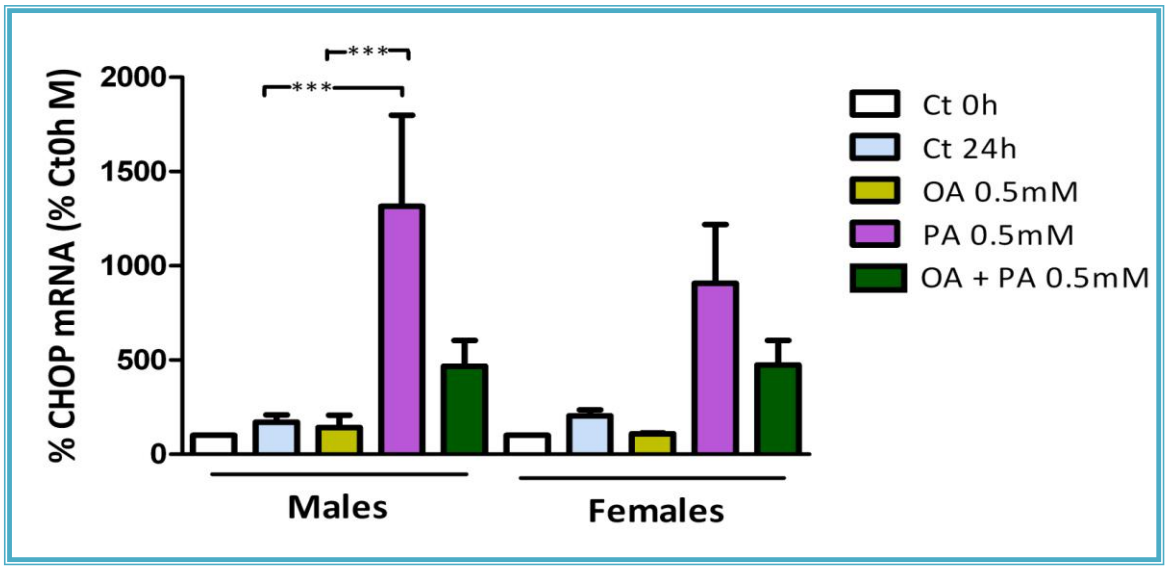


Figure 72. CHOP mRNA levels in astrocyte primary cultures from 2 days old male and female Wistar rats. Ct: control; h: hours. ANOVA: ($F_{(7, 23)}$ = 4, p< 0.02). **: p<0.02. N=4 cultures with at least 3 repetitions of each experimental group in each one.

As IGF-1 production by astrocytes is involved neuronal protection, we analyzed the expression of this trophic factor. IGF-1 mRNA levels (Figure 73) were decreased by PA ($F_{(1,24)}$ = 16.5, p<0.0001). On the contrary, OA stimulated IGF-1 expression ($F_{(1, 24)}$ = 11.8, p<0.03), with this effect being significant in male astrocyte cultures. The addition of both FAs simultaneously resulted in no change in IGF-1 mRNA levels, suggesting that PA affects the ability of these cells to produce this neuronal protector factor in response to OA.

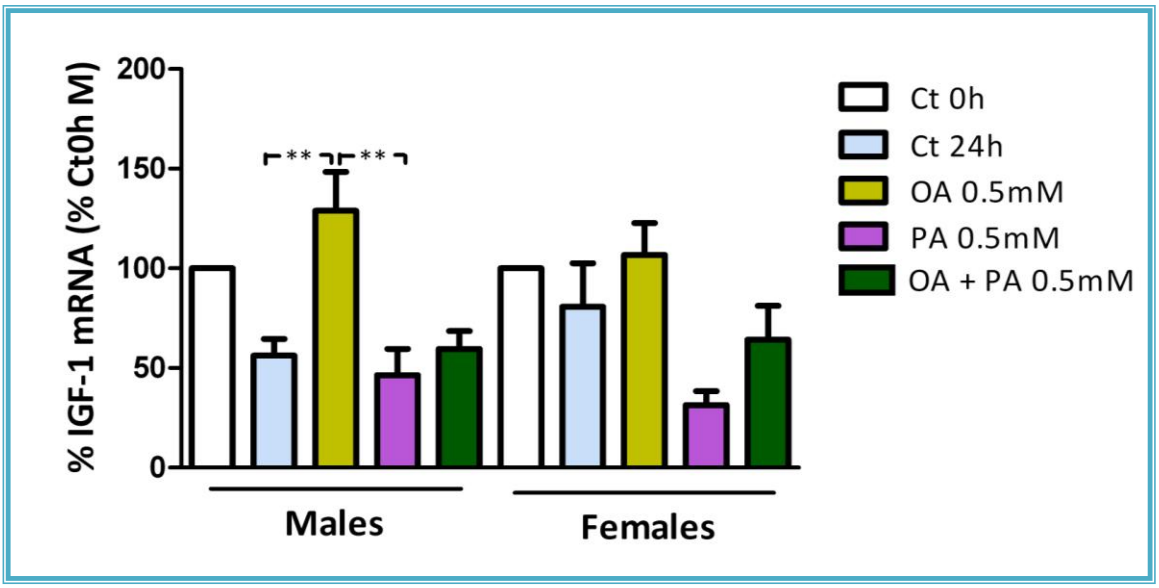


Figure 73. Relative IGF-1 mRNA levels in primary astrocyte cultures from 2 day old male and female Wistar rats. Ct: control; ($F_{(7,31)}$ = 4.7, p< 0.003). **: p<0.02. N=4 cultures with at least 3 repetitions of each experimental group in each one.

3.2.2 Nitrites and nitrates released to the culture media after FAs treatment

As astrocytes are activated and can respond to FAs by releasing inflammatory signals, we asked if they also released neurotoxic free radicals such as nitric oxide (NO) in response to FAs.

The amount of NO_2 and NO_3 released to the culture media by astrocytes was affected by PA exposure ($F_{(1,17)}$ = 32.6, p<0.0001), with an interaction between OA and PA ($F_{(1,17)}$ = 8.1 p<0.02; Figure 74). Although OA alone had no significant effect, in combination with PA the release of NO_2 and NO_3 was significantly increased.

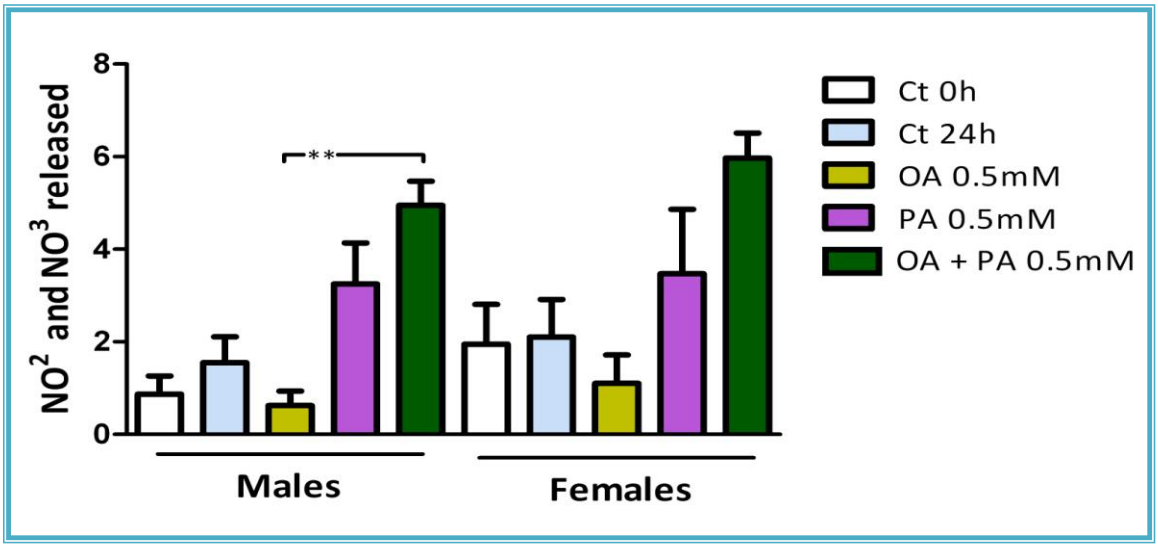


Figure 74. Concentration of NO_2 and NO_3 in the culture media of hypothalamic astrocytes after 24 hours of exposure to palmitic acid (PA), oleic acid (OA) or a combination of these two fatty acids. Ct: control; ($F_{(7,24)}$ = 6.1, p< 0.002). N=4 cultures with at least 3 repetitions of each experimental group in each one.

3.3 Palmitic acid and 17β -estradiol

To determine if estrogens can modify the astrocytic response to PA, 17β–estradiol (E2) was added to the astrocyte cultures 3 hours before PA (0.1 mM) exposure. There was no effect of sex on the mRNA levels of either ERα or ERβ. ERα mRNA levels (Figure 75) were decreased by PA ($F_{(1,17)}$ = 15.4, p<0.002) and E2 increased the levels of this receptor ($F_{(1,17)}$ = 16.7, p<0.002). Besides, E2 pretreatment followed by PA impede the decrease in ERα in females but not in males.

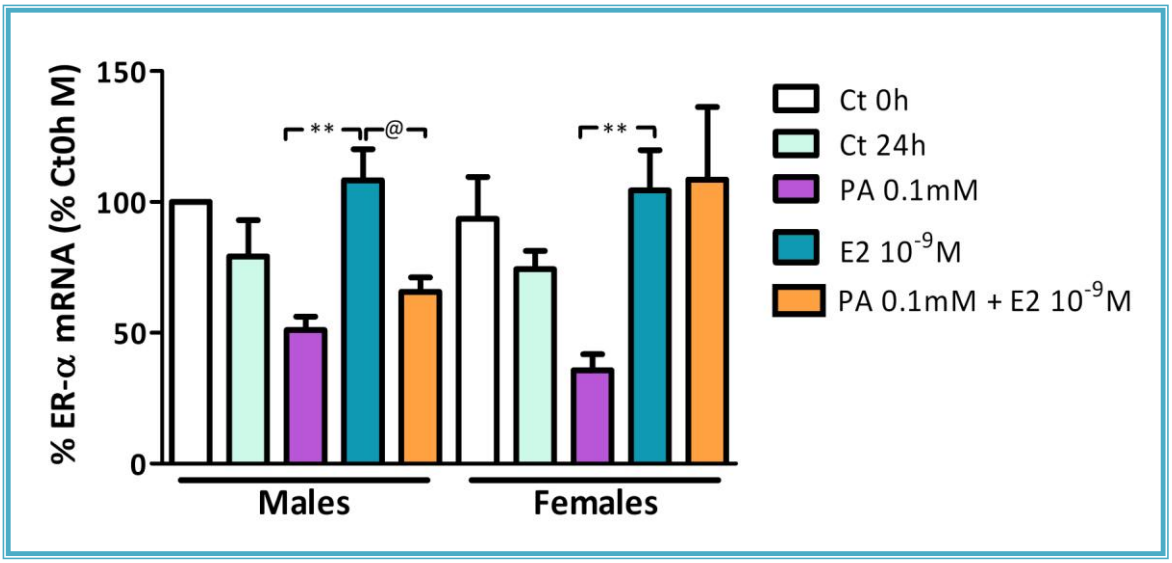


Figure 75. Relative ERα mRNA levels in primary astrocyte cultures from 2 day old male and female Wistar rats. Ct: control; PA: Palmitic acid; E2: 17β-estradiol. ANOVA ($F_{(7,24)}$ = 3.5, p<0.03).
N=4 cultures with at least, 3 repetitions of each experimental groups in each one.

As found in the previous experiments, GFAP expression was reduced by PA, but this decrease did not reach significance in this experiment (Figure 76).

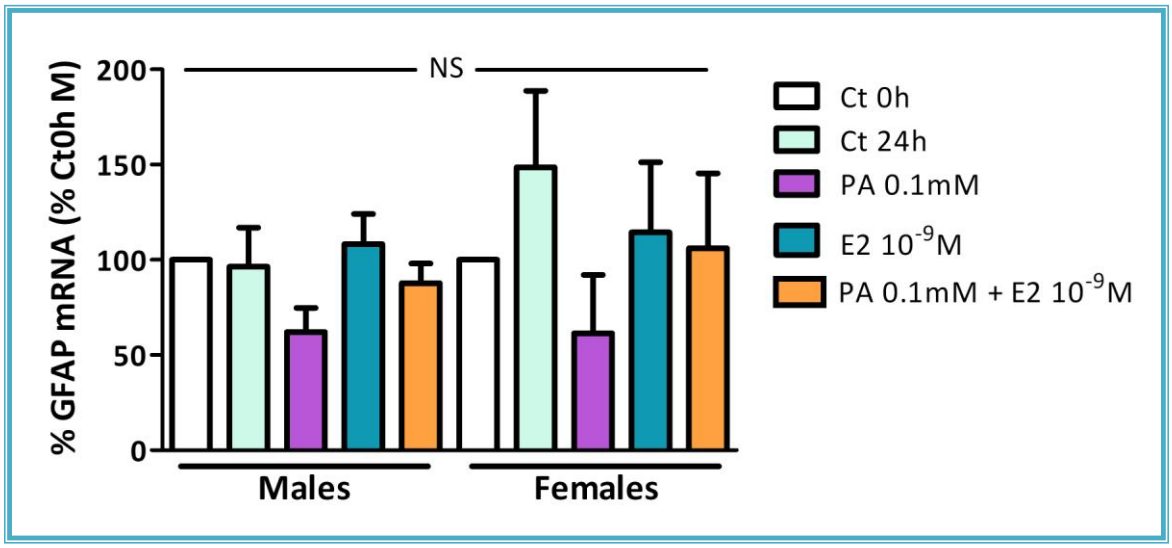


Figure 76. Relative GFAP mRNA levels in primary astrocyte cultures from 2 day old male and female Wistar rats. Ct: control; PA: Palmitic acid; E2: 17β-estradiol. N=4 cultures with at least 3 repetitions of each experimental group in each one.

IL6 mRNA levels (Figure 77) were affected by PA ($F_{(1, 20)} = 48$, $p<0.0001$), E2 ($F_{(1, 20)} = 15.4$, $p<0.002$) and the interaction between these two factors ($F_{(1, 34)} = 13.4$, $p<0.03$). Palmitic acid increased IL6 mRNA levels, and the previous addition of E2 reduced this effect.

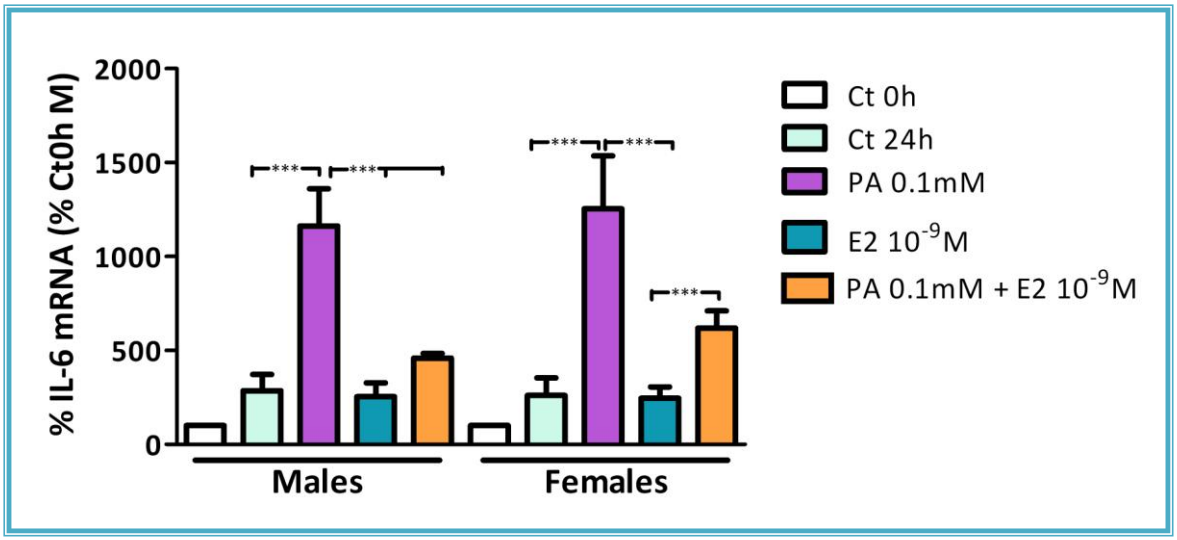


Figure 77. Relative IL6 mRNA levels in primary hypothalamic astrocyte cultures from 2 day old male and female Wistar rats. Ct: control; PA: Palmitic acid; E2: 17β-estradiol. ANOVA ($F_{(7, 27)}= 10.6$, $p<0.0001$). N=4 cultures with at least 3 repetitions of each experimental group in each one.

The expression of IL1β (Figure 78) was also affected by PA ($F_{(1, 19)} = 7.9$, $p<0.02$) and E2 ($F_{(1, 19)} = 4.7$, $p<0.05$). Although PA increased IL-1β levels and this was reduced by E2, no significance differences were found by one-way ANOVA probably due to the increased variability.

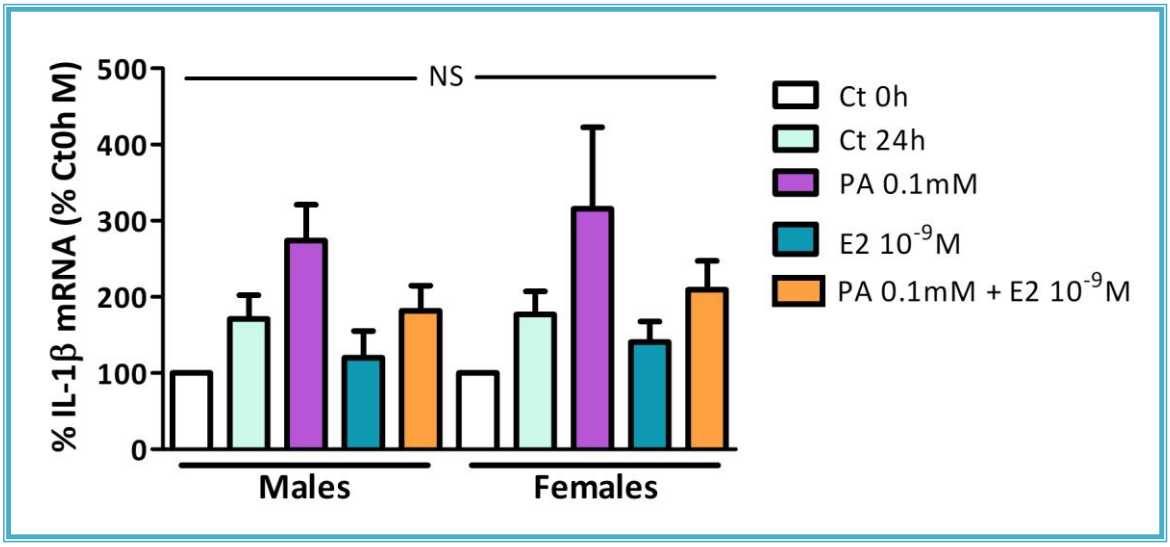


Figure 78. Relative IL1β mRNA levels in primary astrocyte cultures from 2 day old male and female Wistar rats. Ct: control; PA: Palmitic acid; E2: 17β-estradiol. N=4 cultures with at least 3 repetitions of each experimental group in each one.

Similar results were found with TNFα mRNA levels, where PA tended to increase the levels of this cytokine and E2 impeded this increase, although these changes were not significant (MCt-24h: 147 ± 4.4; M-PA: 215.9 ± 86.9; M-E2: 124.4 ± 32.1; M-PA+E2: 132.3 ± 28.3; F-Ct24h: 169.9 ± 66.2; F-PA: 204.3 ± 82.3; F-E2: 164.2 ± 31.9; F-PA+E2: 196.6 ± 109.8), again most likely due to the high variability.

IL10 mRNA levels decreased in astrocytes in response to PA ($F_{(1,19)}$ = 13.4, $p<0.003$; Figure 79), but with individual differences not being found in the posthoc analysis.

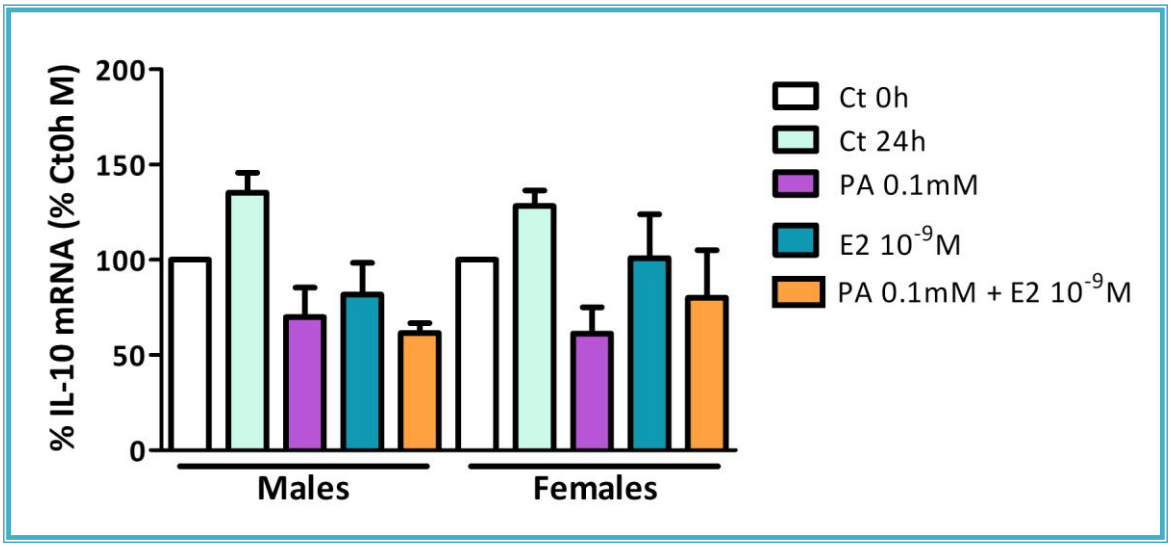
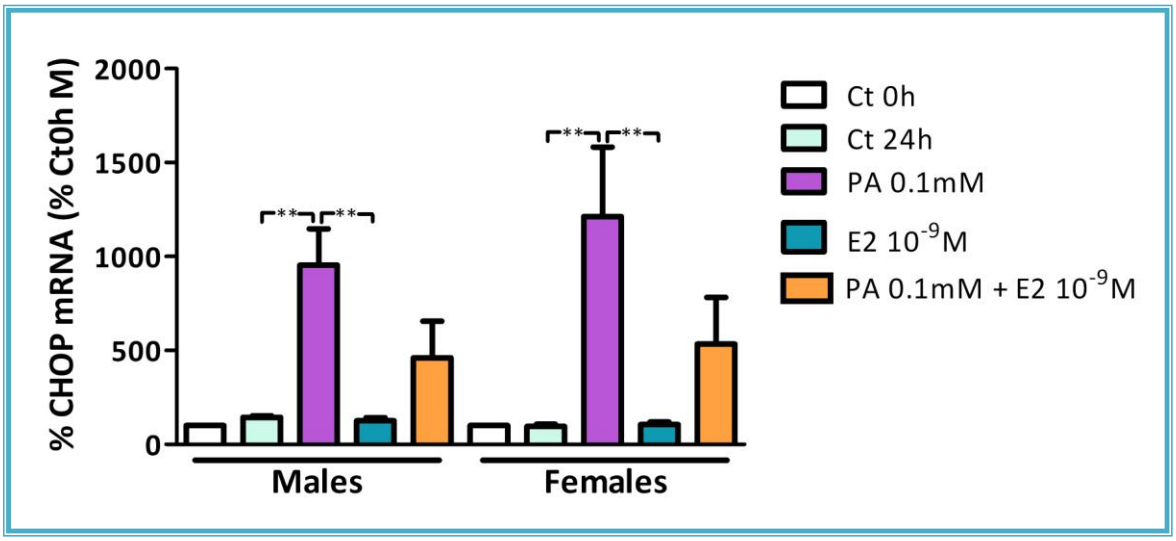


Figure 79. Relative IL10 mRNA levels in primary astrocyte cultures from 2 day old male and female Wistar rats. Ct: control; PA: Palmitic acid; E2: 17β-estradiol. ANOVA ($F_{(7, 26)}$ = 2.9, p<0.03). N=4 cultures with at least 3 repetitions of each experimental group in each one.

The mRNA levels of IGF-1 tended to decrease with PA, but these effects did not reach significance in this experiment (MCt0h: 100; MCt-24h: 92.7 ± 18.2; M-PA: 65.2 ± 6.8; M-E2: 89 ± 25; M-PA+E2: 62.4 ± 7.4; F-Ct24h: 68.7 ± 12.9; F-PA: 50 ± 18.3; F-E2: 72.8 ± 12.2; F-PA+E2: 68.3 ± 11.8).

The mRNA levels of CHOP (Figure 80) were affected after PA exposure ($F_{(1,17)}$ = 29, p<0.0001), E2 ($F_{(1,17)}$ = 5.6, p<0.05) and the interaction between these two factors ($F_{(1,19)}$ = 5.4, p<0.05). There was an increase in response to PA that was reduced by pre-incubation with E2.

Figure 80. Relative CHOP mRNA levels in primary astrocyte cultures from 2 day old male and female



Wistar rats. Ct: control; PA: Palmitic acid; E2: 17β-estradiol. ANOVA ($F_{(7, 24)} = 5.8$, $p<0.002$). N=4 cultures with at least 3 repetitions of each experimental group in each one.

3.4 Palmitic acid and the Toll receptor 4 antagonist RS-LPS

It has been proposed that FAs can activate glial cells through activation of TLR4 (**Milanski et al., 2009**). Hence, we analyzed whether the observed astroglial responses to palmitic acid could be inhibited with a TLR4 antagonist. Palmitic acid at 0.1mM was combined with LPS-RS to determine if the expression of inflammatory cytokines induced by PA was via Toll receptor 4 in astrocytes. The mRNA levels of (IL) 1β, 6, TNFα, CHOP, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (NFKBIA) and inhibitor of kappa light polypeptide gene enhancer in B-cells kinase beta (IKBKB) were determined.

IL1β mRNA levels (MCt-24h: 60 ± 9.3; M-RS-LPS: 685.5 ± 2031; M-PA: 105.5 ± 8.9; M-PA+RS-LPS: 1330.5 ± 684.8; F-Ct24h: 39.9 ± 2.9; F-RS-LPS: 420 ± 246.4; F-PA: 46.4 ± 9.6; F-PA+RS-LPS: 843.8 ± 574) were increased by RS-LPS ($F_{(1,16)} = 10.2$, $p<0.01$) and females tended to have lower levels than males, although no specific differences were found in the posthoc analysis.

IL6 mRNA levels (Figure 81) were increased by both PA ($F_{(1,16)}$ = 8.9, p<0.01) and RS-LPS ($F_{(1,16)}$ = 10.9, p<0.01), with this increase being greatest with the combination of these two substances.

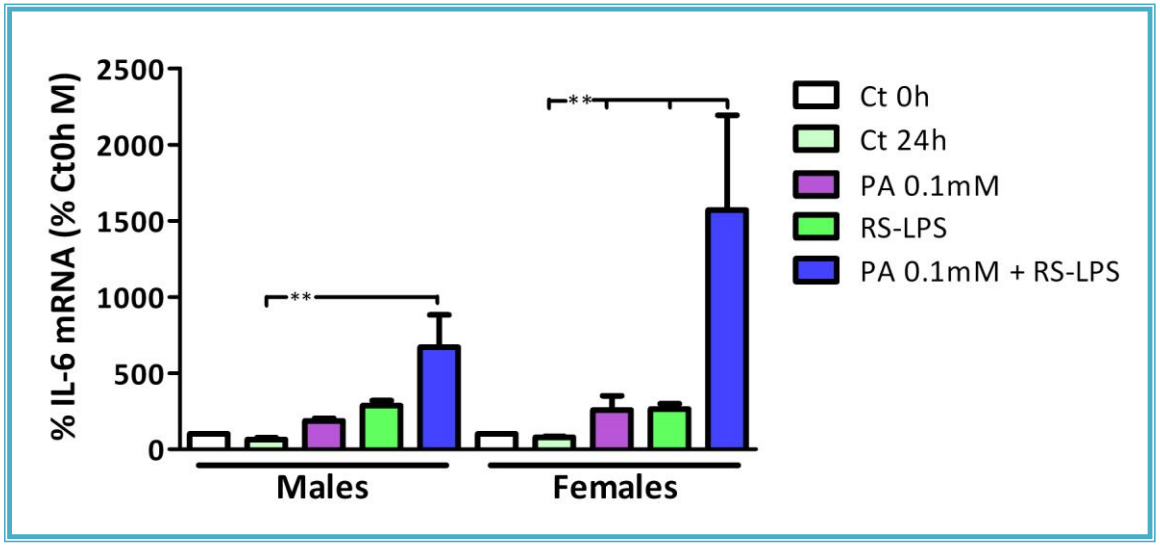


Figure 81. Relative IL6 mRNA levels in primary astrocyte cultures from 2 day old male and female Wistar rats. Ct: control; PA: Palmitic acid; LPS-RS: Lipopolysaccharide from the photosynthetic bacterium Rhodobacter sphaeroides. ANOVA ($F_{(7,23)}$ = 4.5, p<0.01). N=4 cultures with at least 3 repetitions of all experimental groups in each one.

TNFα mRNA levels (Figure 82) were increased by RS-LPS ($F_{(1,16)}$ = 17.8, p<0.01).

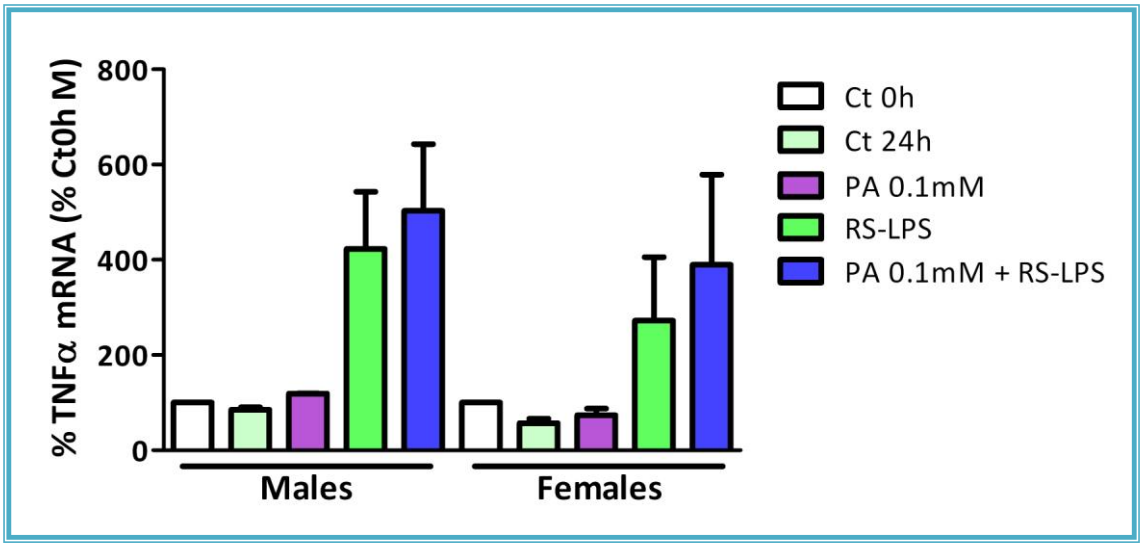


Figure 82. TNFα mRNA levels in astrocyte primary cultures from 2 days old male and female Wistar rats. Ct: control; h: hours; PA: Palmitic acid; LPS-RS: Lipopolysaccharide from the photosynthetic bacterium Rhodobacter sphaeroides. mM: mili Molar. ANOVA ($F_{(7, 23)}$ = 2.9, p<0.05). N=4 cultures with at least, 3 experimental groups in each one

CHOP mRNA levels (Figure 83) were affected by PA ($F_{(1,16)}$ = 9.6, p<0.01) and RS-LPS ($F_{(1, 16)}$ = 5.5, p<0.05).

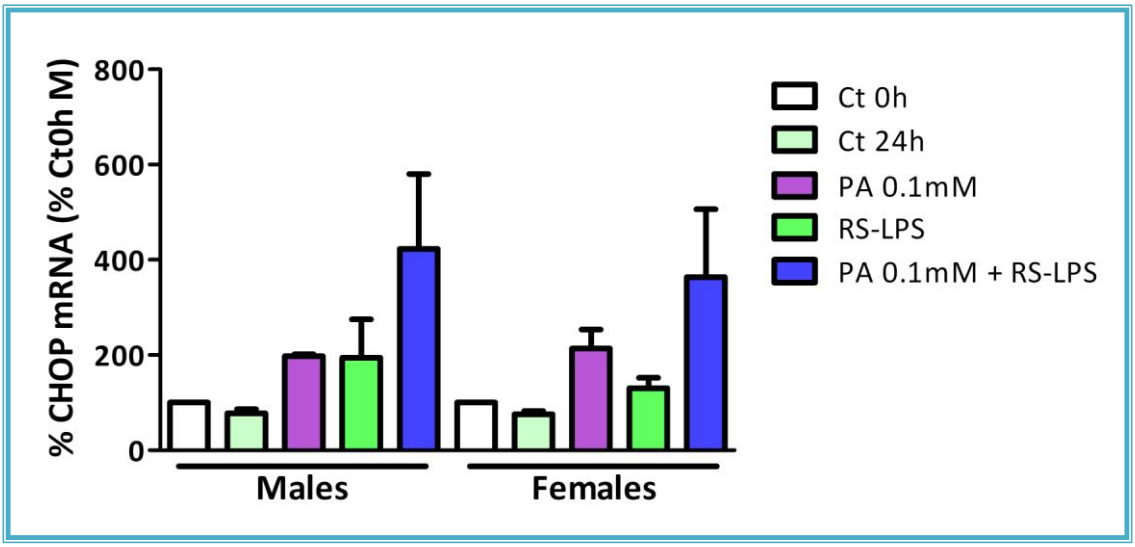


Figure 83. CHOP mRNA levels in astrocyte primary cultures from 2 days old male and female Wistar rats. Ct: control; h: hours; PA: Palmitic acid; LPS-RS: Lipopolysaccharide from the photosynthetic bacterium Rhodobacter sphaeroides. mM: mili Molar. N=4 cultures with at least, 3 experimental groups in each one.

NFκB inhibitor α (NFKBIA) ($F_{(1, 16)}$ = 6.5, p<0.03; Figure 84) and IKKβ (IKBKB) ($F_{(1, 16)}$ = 11, p<0.01; Figure 85) mRNA levels tended to increase by RS-LPS and the combination with PA although these differences did not reach significance due to variability.

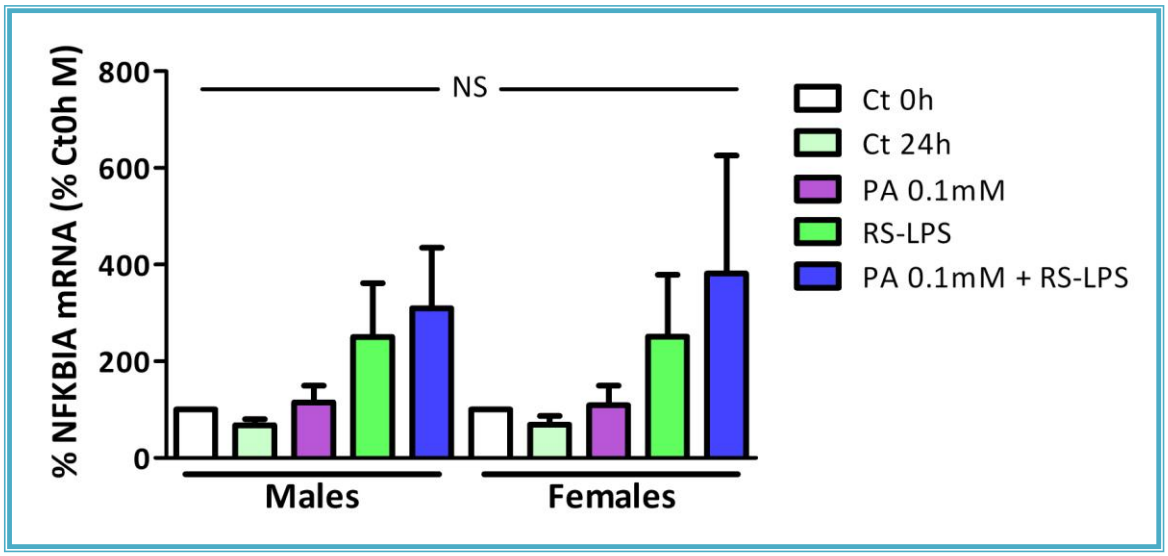


Figure 84. NFKBIA mRNA levels in astrocyte primary cultures from 2 days old male and female Wistar rats. Ct: control; h: hours; PA: Palmitic acid; LPS-RS: Lipopolysaccharide from the photosynthetic bacterium Rhodobacter sphaeroides. mM: mili Molar. N=4 cultures with at least, 3 experimental groups in each one.

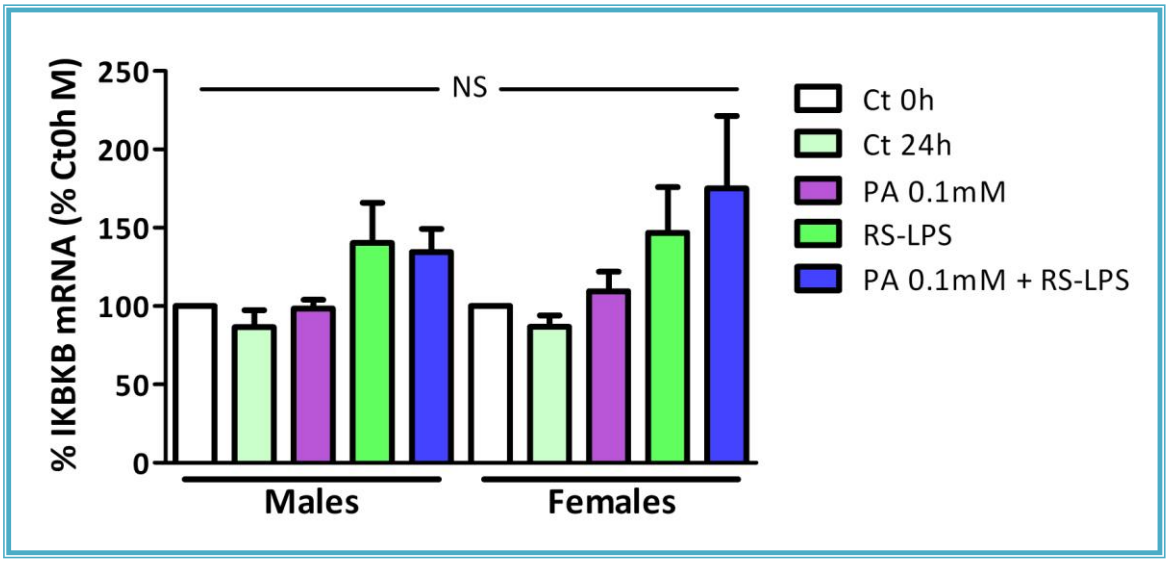


Figure 85. IKBKB mRNA levels in astrocyte primary cultures from 2 days old male and female Wistar rats. Ct: control; h: hours; PA: Palmitic acid; LPS-RS: Lipopolysaccharide from the photosynthetic bacterium Rhodobacter sphaeroides. mM: mili Molar. ANOVA ($F_{(9, 29)} = 2.5$, $p<0.05$). N=4 cultures with at least, 3 experimental groups in each one.

3.5 Intracellular signaling in astrocytes in response to PA

Astrocyte lysates were processed after PA 0.1mM exposure to detect possible changes in the following phosphoproteins: cAMP response element-binding protein (CREB), c-Jun N-terminal kinase (JNK), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), P38 mitrogen activated protein kinase (p38), extracellular signaling-regulated kinases 1 (ERK1) and 2 (ERK2), protein kinase B (Akt), the 70kDa ribosomal S6 kinase ($p70^{S6K}$), STAT3 and signal transducer and activator of transcription 5 (STAT5).

Table 22 encompasses the mean fluorescent intensity of the cited phosphoproteins corrected by their corresponding total proteins.

	Males		Females	
	Ct 15 min	**PA 15 min**	**Ct 15 min**	**PA 15 min**
p-CREB	100 ± 0	66.6 ± 23.9	79.7 ± 24.6	43.1 ± 17
p-JNK	100 ± 0	109.2 ± 50.6	82.5 ± 29.1	113.5 ± 68.6
p-NF-κB	100 ± 0	138.8 ± 41.2	166 ± 105.9	351.3 ± 236.9
p-P38	100 ± 0	88.7 ± 24.9	99.6 ± 49	158.7 ± 115.7
p-AKT	100 ± 0	91.3 ± 21.4	99.4 ± 24.7	123.2 ± 50.6
p-ERK	100 ± 0	99.1 ± 3.1	96.2 ± 0.9	97.7 ± 2.1
p-P70s6k	100 ± 0	111.6 ± 43	83.4 ± 26	146.6 ± 44.1
p-STAT3	100 ± 0	105.3 ± 42.5	88.4 ± 38.3	132.1 ± 64.3
p-STAT5	100 ± 0	145.5 ± 15.4[a]	125.9 ± 31.6	159.4 ± 71.2

Table 22. Mean fluorescent intensity of CREB, JNK, (NF-κB), p38, ERK2, Akt, $p70^{S6K}$, STAT3 STAT5 in primary astrocyte cultures after 15 minutes with PA incubation. [a] different from control group of the same sex by T-test, $p<0.05$. Ct: control group; PA: Palmitic acid group. N= 3.

VI. DISCUSSION

Obesity and its associated comorbidities continue to be one of the major public health concerns in developing countries, without signs of a decrease in this epidemic. We now know that becoming overweight or obese is not only the result of poor nutrition and/or the lack of physical activity, but also that early nutritional perturbations can have long-term consequences on metabolism. The experimental model employed here, neonatal overnutrition, is known to cause abnormal metabolic responses and obesity in adulthood **(Godfrey and Barker, 2000; Stettler et al., 2002; Viner and Cole, 2006; Barker, 2007; Gluckman et al., 2007; Freedman et al., 2009; Reynolds et al., 2015a)**. In addition, although not thoroughly studied, males and females have been previously reported to respond differently to this and other early nutritional alterations **(Fuente-Martin et al., 2012c; Reynolds et al., 2015a)**. The studies reported here indicate that early postnatal weight gain as a consequence of being raised in a small litter not only causes alterations in diverse metabolic parameters, but that many of these alterations are both age and sex dependent.

In rodents, the long-term disruption of energy homeostasis induced by overfeeding during critical periods of development has been shown to be at least partially due to effects on metabolic neurocircuit formation **(Plagemann et al., 1999b; Fuente-Martin et al., 2012c; Vogt et al., 2014; Collden et al., 2015).** Here we show that being raised in a small litter, which results in increased food intake during this early age due to increased maternal milk availability **(Fiorotto et al., 1991; Fink et al., 2001)**, resulted in increased body weight, body length and fat mass, as well as elevated serum glucose, insulin, leptin and adiponectin levels during the period of nursing in rats of both sexes. Some of the changes could be involved in the modifications in neurocircuit formation. Importantly, increased weight gain during this critical period was not only associated with systemic metabolic alterations, but also with hypothalamic changes, with both metabolic and central changes being different between males and females.

1. Effects of neonatal overnutrition on food intake, body weight and body length throughout development

In rodents, the neonatal period is the most sensitive developmental stage with regards to hormonal programming of the brain, with hormonal fluctuations during this period having an impact in the adult brain **(Sun et al., 2012; Vogt et al., 2014)**. The increased availability and intake of milk **(Fiorotto et al., 1991; Cunha et al., 2009)** is most likely a direct cause of the increased body weight gain of pups raised in small litters, but it is the resulting hormonal and metabolic changes that induce the long-term effects.

We found that pups raised in small litters showed increased body weight, length, SCAT and glycemia, as well as hyperinsulinemia and hyperleptinemia, as early as PND10. These early modifications in L4 pups are most likely due to the increased energy intake during nursing as described above, but could also be influenced by changes in maternal nurturing **(Connor et al., 2012; Reynolds et al., 2014; Segovia et al., 2014)**. Even after weaning, when all rats had *ad libitum* access to a chow diet, L4 rats of both sexes continued to eat more than L12 rats, but only until approximately PND50, suggesting that at this point in the study their higher energy intake is not due to more food availability, but probably to having an increased appetite. An increase in food intake in adult rats previously exposed to early overnutrition has been reported by some authors **(Rodrigues et al., 2011; Fuente-Martin et al., 2012b; Collden et al., 2015)**, while other studies report no effect **(Stefanidis and Spencer, 2012; Bei et al., 2015).** These discrepancies could be due to differences in the age at which the animals were studied, as here we found that this effect changed throughout postnatal development. In addition, even before pubertal onset, males ate more than females, with L4 males already being heavier than L4 females, indicating that even before the influence of post-pubertal gonadal steroids there are differences between the sexes. These early difference could be at least partially due to the neonatal peak of testosterone in males that could affect the neuronal circuits involved in food intake regulation.

The effect of litter size on food intake was present until approximately PND50 and then waned until later adulthood, when L4 males again began to eat more than control males. In association with this, during adolescence and early adulthood, the effects of litter size on body weight and metabolic hormone levels also disappeared. This observation is in accordance with several studies showing that some of the changes produced in the early ages, are normalized when the animal reaches young adulthood **(Vickers et al., 2005; Glavas et al., 2010; Stefanidis and Spencer, 2012).** As many hormonal and metabolic changes occur during puberty, it is possible that the early neonatal effects are masked or modified during this period.

Despite the attenuation of some of these outcomes in young adult rats, the effect of early overnutrition on body weight reappeared in later adulthood, but in a sexually dimorphic manner, as it was only present in males. Litter size effects on body weight and leptin levels have been previously shown to vary with age in male rats, being present early in development and disappearing at 11 weeks of age **(Velkoska et al., 2005)**, while Wiedmer and colleagues **(Wiedmer et al., 2002)** reported that male rats from small litters weighed more than those from large litters from 2 to 5 weeks of age and then again from 10 to 12 weeks of age. Metabolism is known to change with age **(Aasum et al., 2003)**, with an animal or individual becoming more susceptible to body weight gain and fat accumulation as they become older **(Han et al., 2013).** This can occur although energy intake is not increased, as there is an age dependent decrease in energy expenditure **(Han et al., 2011)**. Thus, it is possible that neonatal overnutrition advances the physiological aging of metabolism, with males being more susceptible to this process. Moreover, some of the intracellular mechanisms involved in aging are identical to those activated in obesity associated complications **(Tang et al., 2015)**. For instance, low-grade hypothalamic inflammation can be triggered by both obesity **(De Souza et al., 2005; Zhang et al., 2008; Purkayastha et al., 2011; Cai, 2013)** and aging **(Tang and Cai, 2013; Zhang et al., 2013)**. Several studies have reported that inflammation in the hypothalamus induces the development of systemic aging **(Purkayastha and Cai, 2013; Tang and Cai, 2013; Zhang et al., 2013).** Likewise, ER stress is also linked to both overnutrition **(Zhang et al., 2008; Ozcan et al.,**

2009) and aging **(Greenamyre and Hastings, 2004; Katayama et al., 2004; Wootz et al., 2004).** This inflammation in the hypothalamus also involves glial cells, including astrocytes that can produce cytokines, as well as diverse factors such as transforming growth factor beta (TGF-β) in response to obesity or aging conditions. This process can lead to loss of function of IκBα and consequently, activation of NF-κB **(Yan et al., 2014)**. Here not only did L4 males begin to gain more weight as adults, but they also demonstrated signs of hypothalamic inflammation as TNFα levels were increased. Neither of these processes were observed in females at PND150, but this does not negate the fact that they might occur at an older age.

At PND90, when an increase in body weight was again found in L4 males, there was no difference in food intake. This suggests that the metabolic efficiency of the neonatally over-nourished rats may begin to be affected at this time, at least in males. This is in agreement with a report by Stefanidis and Spencer indicating that there is metabolic compensation by these rats in attempt to restore a normal body weight, but this compensation is different between the sexes **(Stefanidis and Spencer, 2012).** Around PND119, food intake again began to be higher in males from small litters compared to their controls. During the following weeks there are variations in food intake in the neonatally over-nourished rats compared to their controls and this could indicate that these animals are continuously trying to readjust their body weight/food intake. In addition to increased food intake, a decrease in energy expenditure, especially as they get older, could also contribute to body weight gain, but this was not analyzed here.

2. Effects of neonatal overnutrition on circulating parameters throughout development: hormones and cytokines

The long-term effects of being raised in a small litter could be due to modification of the postnatal leptin surge as a result of neonatal overfeeding. Leptin is a key signal in determining the development of hypothalamic neuronal circuits involved in feeding behavior **(Bouret et al., 2004a; Bouret and Simerly, 2007; Bouret, 2013).** Although in the rodent most neurons are born prenatally, the outgrowth of their

projections occurs after birth **(Ishii and Bouret, 2012)**. Hence, changes in leptin levels in perinatal life can impact on the development of hypothalamic "wiring" and result in lifelong metabolic deregulation **(Bouret et al., 2008)**. Some long-term effects of changes in neonatal leptin levels have been shown to become manifest only later in adulthood **(Granado et al., 2011; Granado et al., 2014);** here we found long-term effects until approximately PND50, which disappear to reappear again in later adulthood. In the studies by Granado et al., leptin levels were directly modified; here other factors such as insulin levels in addition to leptin were indirectly modified. This, as well as the temporal differences in these hormonal changes, could underlie the differential outcomes of our studies. Variations in neonatal leptin concentrations can also modify leptin receptors expression in the hypothalamus, possibly modifying feedback mechanisms of this hormone. *Rodrigues et al* reported higher leptin levels at PND21 as a consequence of neonatal overnutrition; but they show no change in leptin receptor protein levels at PND21 or PND180 **(Rodrigues et al., 2009).** However, *Toste et al* showed a decrease in leptin receptor levels in the adult hypothalamus after leptin treatment during lactation **(Toste et al., 2006)**, We observed that neonatally overfed male and female adults presented higher intensity of leptin receptor immunoreactivity specifically over GFAP-positive cells, this is in accordance with **(Hsuchou et al., 2009)**, who showed that diet-induced obesity increases astrocytic leptin receptor expression.

A change in neonatal leptin levels could also have direct effects on adipose tissue development and adipocyte functions, which could modify their ability to produce metabolic cytokines **(Harris et al., 1998).** Leptin expression in adipose tissue was increased at PND10 and PND21 in L4 rats of both sexes, a similar effect has been previously observed in lambs whose mothers where overfed in late gestation **(Muhlhausler et al., 2007)**. Thus, the higher circulating leptin levels at this age are most likely due to the rise in fat mass, as well as increased relative leptin production per gram of adipose tissue. These results suggest that leptin could indeed play a role in the observed long-term effects on metabolism by inducing permanent changes in

the hypothalamic feeding circuits during this sensitive period of development and possibly in adipose tissue development, although this remains to be demonstrated.

Perinatal insulin levels are also implicated in hypothalamic maturation, as this hormone affects the development of projections of the main metabolic neuronal populations **(Plagemann et al., 1999a; Konner et al., 2009; Vogt et al., 2014)**, with aberrant development due to changes in insulin levels disrupting glucose homeostasis and possibly leading to obesity and metabolic disorders such as T2D **(Vogt et al., 2014)**. Here, glycemia and circulating insulin levels were higher in prepubertal L4 rats compared to L12 rats of both sexes. These results are in accordance with several studies that have reported elevated insulin levels in neonatally over-nourished rodents **(Boullu-Ciocca et al., 2005; Pereira et al., 2006)**. Therefore, this perinatal hyperinsulinemia could also participate in the development of metabolic alterations seen in the adult. Moreover, at PND 10 insulin levels in L4 males were higher than in L4 females, which could possibly contribute to the sex differences observed in the long-term outcomes of this experimental manipulation.

At PND 10, adiponectin levels were elevated in L4 male and female rats, with this elevation remaining at weaning in females. The effect of adiponectin on hypothalamic metabolic circuit development remains unknown; however, this adipokine is positively correlated with leptin levels and adiposity in cord blood and is suggested to play a role in early growth **(Kotani et al., 2004; Tsai et al., 2004; Ballesteros et al., 2011)**. Moreover, adiponectin receptors are expressed in the embryonic mouse brain **(Zhou et al., 2005)**. Although adiponectin is implicated in glucose metabolism, with high adiponectin levels acting to increase insulin sensitivity and, on the contrary, decreased adiponectin levels associated with a higher risk of developing insulin resistance **(Yamauchi et al., 2001),** there is little evidence linking adiponectin with insulin sensitivity in neonates **(Kotani et al., 2004; Meral et al., 2011).** Thus, it is possible that during early life, adiponectin has a different physiological role; indeed, PND10 and PND21 pups from L4 litters had higher adiponectin levels despite having higher insulin and HOMA index than their control litter mates. Supporting these observations, neonates are reported to have higher adiponectin levels at birth and in

contrast to adults, adiponectin levels in neonates are positively correlated with birth weight **(Kotani et al., 2004; El-Mazary et al., 2015)**. Hence, in neonatal overnutrition, insulin and adiponectin, two metabolic hormones involved in the control of glucose homeostasis in adulthood, are elevated in early ages and this could participate in development of the observed metabolic effects. Moreover, these hormones are increased in a sexually dimorphic manner and this could possibly contribute to the sex differences in the long-term metabolic outcomes.

Obesity is reported to course with systemic inflammation **(Gregor and Hotamisligil, 2011)**, but the increased weight gain due to neonatal overnutrition did not result in a rise in circulating cytokines at any age. Indeed, in adult animals levels of these cytokines were either very low or undetectable. This of course could be due to the fact that the increase in weight gain was not severe, at least in adults. However, during the neonatal and peripubertal periods, although there was approximately a 40% increase in body weight at PND10 and a 50% increase at PND21 in both sexes, the cytokines measured either decreased or did not change. At PND10, TNF-α and IL-1β levels were actually lower in rats raised in small litters, with no significant change in IL-6 levels. One possibility is that the developing animal responds differently to this increase in weight gain, as discussed below.

The decrease in circulating TNF-α and IL-1β were associated with a significant decline in the mRNA levels of IL-1β in SCAT of females at PND10, but not in males. It is possible that at this age circulating levels of these cytokines do not reflect their production by SCAT. On the contrary, there was no effect of neonatal overnutrition on circulating cytokine levels at PND21, but the mRNA levels of IL6 were increased in VAT and decreased in SCAT of both sexes. These opposite changes in the expression of IL-6 could result in a net result of no change in circulating levels. This interleukin has a dual role, as it can be either anti-inflammatory or pro-inflammatory depending on the basal situation, the type and/or duration of the stimuli and the tissue where it is exerting its actions, with the correlation between IL-6 and obesity and insulin resistance being controversial **(Wallenius et al., 2002; Di Gregorio et al., 2004; Vida et al., 2015)**. In this line, while IL-6 is reported to induce insulin resistance in adipocytes

(Rotter et al., 2003), IL-6 treatment improves insulin-stimulated glucose metabolism in humans and enhances glucose uptake and fatty acid oxidation in myotubes, in addition to exerting anti-inflammatory actions **(Starkie et al., 2003; Carey et al., 2006).** Although there was no effect of litter size on IL-6 levels at these early ages, there was an effect of sex, with males having higher IL-6 levels than females, which could also contribute to the metabolic differences observed between the sexes.

At PND10, testosterone levels were higher in L4 males compared to L12 males, with 17β-estradiol levels being unaffected by neonatal overnutrition. Therefore, the neonatal testosterone surge that occurs in males **(Raisman and Field, 1973)** appears to be modified by early overnutrition and this could be involved in the sexually dimorphic long-term responses. Indeed, the early sex steroid environment is important for the correct development of the hypothalamus **(Lenz and McCarthy, 2010)**, and modifications in androgen levels during early development have been shown to modify later metabolism **(Nohara et al., 2013a; Nohara et al., 2013b; Ongaro et al., 2015).** Hence, during neonatal overnutrition there are early changes in the hormonal environment, including leptin, insulin, adiponectin and testosterone that could influence the normal development of hypothalamic feeding circuits, as well as adipose tissue development and, thereby condition the metabolic status later in life.

During the adolescent and young adult stages (PND30, 50 and 85), few effects of neonatal overnutrition were found. However, as mentioned above, in older adults (PND150) males were again found to be affected, although females were not. Not only was weight increased, but these rats were also hyperleptinemic, as reported in other studies **(Fuente-Martin et al., 2012b; Stefanidis and Spencer, 2012; Ye et al., 2012)**. Circulating adiponectin levels were unaffected, which is in accordance with other studies analyzing neonatally overnourished male rats of 120 **(Velkoska et al., 2005)** and 180 days of age **(Rodrigues et al., 2011).** In contrast, *Boullu-Ciocca et al.* reported hypoadiponectinemia in over-nourished male rats at PND150 **(Boullu-Ciocca et al., 2008).** However, there was an interaction between sex and litter size on adiponectin levels, which tended to be higher in L4 males compared to their controls, with the inverse occurring in females. Adult females had higher adiponectin levels than males

and this could be associated with the lower insulin levels in females, as adiponectin acts as an anti-diabetic hormone.

Triglyceride levels were also elevated in PND150 male rats, in association with the increased body weight. This is consistent with a study by Cai and coworkers in which higher levels of triglycerides were found in males from small litters, in addition to being more elevated than in females **(Cai et al., 2014)**. In contrast, no significant change or sex differences were found in circulating NEFA levels. Together these results suggest that male rats are more susceptible than females to the long-term metabolic effects of neonatal overnutrition.

3. Effects of neonatal overnutrition on adipose tissue throughout development: amount, distribution and cytokine gene expression

Many studies have established that WAT distribution in adult humans and animals is different between the sexes **(Power and Schulkin, 2008; Fuente-Martin et al., 2013a).** Men are prone to accumulate central visceral fat, whereas women store peripheral or SCAT **(Kotani et al., 1994; Demerath et al., 2007)**. However, less is known regarding fat distribution and its possible implications in the prepubertal period. In our experimental model, there was a very rapid increase in SCAT as a consequence of increased food intake during the neonatal period. Moreover, this increase in SCAT was sexually dimorphic even before puberty, with overnourished females having a greater increase in SCAT than males from the same litter size. The same pattern was observed at weaning, but at this age, both female groups had a greater percentage of SCAT than both male groups. These differences in SCAT continued at PND30 with litter size continuing to increase the amount of SCAT at PNDs 50 and 85. However, at PNDs 85 and 150, the sex differences are inverted, with males having more SCAT than females, which suggest a possible influence of post-pubertal gonadal steroids.

Visceral adipose tissue was also increased by neonatal overnutrition at PND21, but in this case L4 males accumulated more VAT than L4 females. Although the effect of litter size did not persist at PND30, males continued to accumulate more VAT than females. SCAT and VAT differences between over-nourished and

control rats dissipated in the young adult rat, being coincident with the loss of effect of neonatal overnutrition on food intake, body weight and body length. At the end of the study, males tended to have higher amounts of both SCAT and VAT than females without reaching significant differences, most likely due to high variability among the experimental groups and the method used. A more refined method, such as MRI, would be more suitable to detect these changes **(Johnson et al., 2008).**

Therefore, even before the pubertal rise in gonadal steroids, clear sex differences exist regarding adipose tissue distribution and accumulation in response to overnutrition. Excess VAT accumulation is a source of proinflammatory cytokines, which contribute to the development of insulin resistance, type 2 diabetes and cardiovascular diseases **(Carey et al., 1997; Weiss, 2007b; Macotela et al., 2009; Shulman, 2014)**. In contrast, SCAT has been associated with beneficial metabolic effects, such as lower cardiovascular and diabetes mortality and protecting against impaired glucose metabolism **(Van Pelt et al., 2002; Tran et al., 2008; Manolopoulos et al., 2010),** with the greater accumulation of SCAT possibly protecting females from some of the negative consequences of obesity. Hence, as the amount and distribution of adipose tissue differs significantly between males and females during the early postnatal period, this could contribute to the earlier development of metabolic complications in males. This is consistent with the fact that at PND10 and PND21 males had a greater increase in circulating insulin levels, which could be associated to the increased amount of VAT and cytokine production by VAT (discussed below), which is related to insulin resistance **(Hotamisligil et al., 1993; Uysal et al., 1997; Bastard et al., 2002; Jager et al., 2007; Nov et al., 2010).** Therefore, even before sex steroids rise, there is a sexual dimorphic response to overnutrition in adipose tissue distribution.

The early increase in fat mass in L4 pups was also directly related with elevated leptin mRNA levels in adipose tissue and circulating leptin concentrations. At PND21, L4 males had higher leptin expression in SCAT than L12 males, an effect that was not significant in females. At this age, VAT expression of leptin was higher in L4 than L12 pups of both sexes. In adulthood (PND85 and 150) males, expressed more leptin mRNA levels than females in SCAT and VAT, regardless of litter size as

previously shown by others in humans and rodents **(Montague et al., 1997; Shen et al., 2009b)**. These increases in leptin expression in adipose tissue correlated with circulating leptin levels.

In normal adults, adiponectin expression in adipose tissue is inversely correlated with the amount of fat mass **(Kern et al., 2003)**. In contrast, during the neonatal period when fat mass was greater in L4 rats, we found circulating adiponectin levels to also be higher. This was coincident with significantly increased expression of adiponectin in adipose tissue in L4 males, with the same trend occurring in females. In adult rats, although there was no effect of neonatal overnutrition on circulating adiponectin levels, there was an overall increase in the expression levels in SCAT at PND150 and in VAT at PND85 and PND150 in males, whereas circulating adiponectin levels were higher in females. How this change in cytokine production affects overall metabolism cannot be determined from the studies presented here, but it is of interest that apparent rise in production is found when effects on bodyweight have begun to reappear in males and males had higher adiponectin mRNA levels than females in both adipose depots. These differences could be attributed to the greater affectation of metabolic control in males, with the increased expression of adiponectin in males being a protective response.

Obesity and the accumulation of white adipose tissue particularly, VAT, are often accompanied by a chronic low-grade inflammatory state **(Gregor and Hotamisligil, 2011)**. At PND10, although there was a significant increase in SCAT in the over-nourished rats, cytokine mRNA levels were not increased. One possibility is that the organism must be exposed to the inflammatory-inducing factors associated with increased weight gain for a more extended period of time. In addition, adipose tissue is still developing in these animals and the characteristics of developing adipose tissue differ from mature adipose tissue **(Rosen and Spiegelman, 2014)**. At this young age, the increase in fat mass could be due to hyperplasia (formation of new adipocytes) more than to hypertrophy (increase in adipocyte size) of the existing adipocytes. The two mechanisms of adipose tissue growth, hyperplasia and hypertrophy, have different outcomes; whereas hyperplasia is characterized by an

increase in adipocyte number by infiltration and proliferation of new and small preadipocytes, which have a higher fat storage capacity and are more sensitive to insulin; hypertrophy of adipose tissue implies that the volume of the adipocyte increases, which is associated with higher metabolic risk **(Weisberg et al., 2003; Lundgren et al., 2007).** Therefore, the observed decrease in expression of IL-1β and IL-6 in SCAT at PND21, as well as in IL-1β at PND10 in females SCAT, could possibly be associated with adipose tissue expansion due to proliferation more than hypertrophy of the existing adipocytes, although this observation requires further investigation. Indeed, as adipocytes differentiate, their expression of cytokines changes, with for example, only mature adipocytes producing leptin **(Rosen and Spiegelman, 2014)**. Hence, the early changes in adipokine expression could be representative of modifications in adipocyte proliferation and maturation in response to early over-nutrition.

In contrast, at PND21, over-nourished rats had higher expression of IL-6 in VAT. This observation does not necessarily indicate inflammation, as it is possible that the initial response to nutrient and energy excess in VAT is to produce this cytokine as an acute anti-inflammatory response in an attempt to fight against the excessive nutritional insult. IL-6 has been shown to have inhibitory actions on TNF-α and IL-1β through activation of interleukin-1 receptor antagonist (IL-1ra) **(Tilg et al., 1994)**. Although this anti-inflammatory effect has been more thoroughly studied in response to the IL6 increase after exercise **(Starkie et al., 2003)**, it is possible that this mechanism could participate in other situations such as early overnutrition. However, if weight gain becomes chronic, this cytokine could contribute to the pathophysiological inflammatory process in obesity **(Rotter et al., 2003)**.

In adults, cytokine expression in SCAT was undetectable, indicating the lack of inflammatory processes in this tissue. The only indication of inflammation in VAT was the increased expression of TNF-α in L4 males, which is coincident with the increase in bodyweight in these animals. Males raised in small litters are reported to have increased content of IL-6 mRNA in mesenteric adipose tissue at 150 days of age **(Boullu-Ciocca et al., 2008),** although the expression of this cytokine was undetectable

in the two adipose depots studied here**.** As different functions and implications on health are associated with the different types of adipose tissue, this cytokine may also have different roles in each type of adipose tissue. In this line, it has been reported that omental adipose tissue release 2-3 times more IL6 than subcutaneous abdominal adipose tissue **(Fried et al., 1998)**. Visceral adipose tissue is directly related with secondary complications of obesity and this may be the first site that responds to the excess of calories, secreting IL-6 at first as an anti-inflammatory signal, as explained above. It is also possible that this rise in IL6 represents a signal that is already associated with the induction of insulin resistance, as previously reported **(Rotter et al., 2003).** These changes did not occur in SCAT, which is not as tightly associated with obesity secondary complications.

Increased TNF-α is reported to be directly related to metabolic syndrome and impairment of insulin signaling **(Hotamisligil et al., 1993).** However, although L4 males had increased expression of TNF-α in VAT at PND150, no change in their HOMA index suggests that systemic insulin resistance is not present. Circulating levels of this cytokine were below the level of detection of the assay employed, thus other tissues might not be affected. Moreover, it is suggested that TNF-α does not induce insulin resistance when IL6 is down regulated in adipose tissue **(Sultan et al., 2009),** and here IL-6 expression was undetectable in VAT. High circulating leptin levels also contribute to maintain the chronic inflammatory state **(Paz-Filho et al., 2012)** and at the same time, pro-inflammatory cytokines induce the increase in circulating leptin levels and its expression in adipose tissue **(Grunfeld et al., 1996; Faggioni et al., 1998).** Thus, together these results could indicate that these male rats are beginning to exhibit signs of further metabolic impairment. Moreover, leptin has differential effects on male and female adipocytes **(Guo et al., 2009)** and this could also contribute to the sex differences observed.

4. The neonatal sex steroid environment influences the long-term responses to neonatal overnutrition

Adult males and females are known to respond differently to some metabolic challenges, including being raised in a small or large litter **(Fuente-Martin et al., 2012c; Sanchez-Garrido et al., 2014; Sanchez-Garrido et al., 2015)**,and this is at least partially due to post-pubertal differences in sex steroids **(Castellano et al., 2011; Sanchez-Garrido et al., 2013)**. As males and females responded differently to neonatal overnutrition even prepubertally, we hypothesized that these differences could be due, at least in part, to sex differences in the neonatal hormonal milieu. It is possible that the neonatal testosterone surge that occurs in males **(Miyachi et al., 1973)** could be involved, not only in the differential response to overnutrition during the neonatal period, but also in the sex differences in long-term metabolic outcomes. Administration of testosterone to neonatal females has been classically used as an experimental model to study the mechanisms underlying sex differences in the brain **(Raisman and Field, 1973).** This model has also been employed to analyze the long-term effects of increased neonatal androgen levels on the female reproductive axis and the development of polycystic ovary syndrome **(Dunlap et al., 1972; Gellert et al., 1977; Goomer et al., 1977; Ongaro et al., 2015)**, and more recently on metabolic abnormalities **(Nohara et al., 2013c; Mauvais-Jarvis, 2014; Ongaro et al., 2015)**.

Testosterone levels remained elevated at PND10 in females that had received an injection of this sex steroid on PND1, and this was associated with the loss of some early sex differences. For example, the higher levels of adiponectin seen at PND10 in females compared to males in the neonatal overnutrition experiment were reduced by androgenization of females, such that they were no longer different from males. This could be due to the direct inhibitory effect of testosterone on adiponectin synthesis **(Xu et al., 2005; Capllonch-Amer et al., 2014)**. Likewise, the lower levels of IL-6 in PND10 females were increased in androgenized females and were no longer different from males. This could be due to androgen effects on adipocyte differentiation, which can affect their expression of cytokines **(Chazenbalk et al., 2013; O'Reilly et al., 2014)**. However, neonatal androgenization exacerbated the sex

difference in subcutaneous fat mass observed at PND10. This observation is of interest as studies have indicated that the number of X chromosomes determines adiposity **(Chen et al., 2012)** and the results obtained here suggest that this might also affect the response to early sex steroid levels. Indeed, hyperandrogenism in polycystic ovarian syndrome (PCOS) is associated with increased abdominal fat in women **(Dunaif, 1997)**. Thus, some of the sex differences seen at PND10 could be at least partially due to sex differences in testosterone levels at this early age. Furthermore, testosterone levels were increased in over-nourished PND10 males compared to their controls. This rise in sex steroid levels could play a role in the development of long-term metabolic effects in over-nourished males and contribute to the sexually dimorphic outcomes of this experimental manipulation.

Adult levels of testosterone and estradiol were unaffected in females that had received testosterone neonatal treatment. However, there were long-term changes in metabolic parameters in response to neonatal androgenization, as previously reported in other studies **(Perello et al., 2003; Ongaro et al., 2013)**. Although body weight and glycemia were slightly increased at PND90 in androgenized females, the most drastic effects were observed on circulating leptin and IL1β levels, which rose above normal female values. The rise in serum leptin correlated with increased expression of this adipokine in VAT, while the sexual dimorphism in visceral adipose expression of IL6 and TNFα was not affected by neonatal testosterone. Thus, the neonatal rise in testosterone in males could play a role in the development of sexual dimorphisms in adipose tissue function. However, the fact that the production of some adipokines was actually increased above the levels found in both males and females suggests that female rats exposed to increased levels of testosterone, at least at this period of development, are prone to develop metabolic pathologies. Indeed, neonatal androgenization of females is associated with metabolic dysfunctions and PCOS development in the adult **(Roland et al., 2010; Nohara et al., 2013c; Mauvais-Jarvis, 2014)**.

The factors involved in the sex differences observed in the long-term responses to neonatal overnutrition could thus include differential changes in

testosterone and some metabolic factors such as insulin. These changes could affect the development of central metabolic circuits, as previously shown **(Mauvais-Jarvis, 2014; Vogt et al., 2014)**, as well as that of other metabolic tissues such as adipose tissue. These developmental modifications would then also interact with the differential hormonal environment found in post-pubertal animals. For example, higher estrogen levels in post-pubertal females would have protective effects through inhibition of food intake and increasing energy expenditure **(Palmer and Gray, 1986; Musatov et al., 2007; Xu et al., 2011)** and it is possible that this could reduce or delay the long-term effects of rapid neonatal weight gain. Estrogens also modulate adipose tissue deposition and function, favoring lipid accumulation in the subcutaneous depot in women and visceral fat deposition in men. Indeed, direct administration of estrogens to the CNS was shown to drastically reduce visceral adiposity **(Adler et al., 2012)** and increase thermogenesis **(Martínez de Morentin et al., 2014)**. Thus, another factor involved in the sexual dimorphic responses could be the differences found with regards to the amount, distribution and function of adipose tissue between the sexes from very early ages, as previously discussed, and the later interaction of this adipose tissue with the adult sex steroid environment.

5. Central response to neonatal overnutrition in males and females

Neonatal overnutrition has been previously reported to modify the levels of hypothalamic neuropeptides in the adult **(Davidowa et al., 2003; Velkoska et al., 2005; Bouret et al., 2007; Chen et al., 2009)**. Here we analyzed the association between the rapid early weight gain due to neonatal overnutrition and the hypothalamic changes regarding inflammatory markers specifically, in glial cells.

Astrocytes in neuroendocrine control

Astrocytes participate in the regulation of the neuroendocrine functions including reproduction, osmotic control and energy homeostasis **(Tweedle and Hatton, 1977; Garcia-Segura et al., 1996b; Garcia-Caceres et al., 2012)**. Although astrocytes have also been shown to participate in the neuroendocrine control of metabolism

(Cheunsuang and Morris, 2005; Horvath et al., 2010; Garcia-Caceres et al., 2011; Yi et al., 2011; Fuente-Martin et al., 2012a), much is yet to be learned , including whether astrocytes participate in the long-term metabolic effects of neonatal nutritional or hormonal changes.

Hypothalamic development in rodents takes place mainly during the postnatal period **(Markakis, 2002; Bouret, 2013; Alvarez-Bolado et al., 2015)**, with astrogenesis occurring primarily during the second and third postnatal weeks **(Bandeira et al., 2009; Freeman, 2010).** Therefore, changes in the hormonal environment, such as in leptin, glucocorticoids, insulin, sex steroids or in other factors known to influence the development of this brain area **(Bouret, 2010b; 2013)**, occurring during this period could affect not only neuronal, but also astrocyte development. Moreover, astrocytes are essential for neuronal proliferation, maturation, synaptic connectivity and homeostasis maintenance in the extracellular space **(Chowen et al., 1996; Fields and Stevens-Graham, 2002),** which indicates that any modification in these glial cells could impact on neuronal development and function, including the regulation of energy homeostasis and overall metabolism.

5.1 Effects of neonaltal overnutrition on astrocytes

In the hypothalamus of adult male rats that had been exposed to neonatal overnutrition, GFAP levels are increased and this is associated with an increase in the number of GFAP-positive astrocytes and structural changes in these glial cells **(Garcia-Caceres et al., 2011; Fuente-Martin et al., 2012a).** One possibility is that this increase in the number of hypothalamic astrocytes is due to developmental influences. The neonatal leptin surge that normally occurs between postnatal days 5 and 13 promotes not only promotes neuronal outgrowth and maturation, but also astrogenesis **(Ahima et al., 1998; Bandeira et al., 2009; Rottkamp et al., 2015)** and astrocyte development **(Udagawa et al., 2006; Fisette and Alquier, 2015).** Moreover, *Wang et al* recently showed that leptin receptor ablation in GFAP-positive astrocytes of embryonic mice reduces leptin-induced STAT3 activation in the hypothalamus and make these mice more vulnerable to diet-induced obesity **(Wang et al., 2015),**

indicating a key role of leptin signaling in astrocytes in the organization of hypothalamic neurocircuits. Thus, it is coherent to hypothesize that the rise in circulating leptin levels induced at PND10 by neonatal overnutrition could modulate astrocyte development. However, at this early age, GFAP protein levels in the hypothalamus were not modified in L4 pups despite having increased leptin levels. This does not necessarily indicate that there is no change in astrocyte development or even the number of astrocytes at this age. We have to take into consideration that GFAP protein levels were analyzed in the whole hypothalamus and not in specific hypothalamic areas directly involved in food intake regulation. Of note, immature astrocytes and radial glia mainly express vimentin early in development; whereas GFAP is more highely expressed in differentiated astroglial cells**(Bignami and Dahl, 1995; Gomes et al., 1999)**. Moreover, during CNS development, GFAP can label radial glial, which give rise to neurons, astrocytes and oligodendrocytes. Thus, it is possible that at this early age, GFAP levels also reflect other cell types. Under normal circumstances GFAP levels increase with age **(Nichols et al., 1993)** and it is possible that at this early age, when the astrocyte population is being formed, a high percentage of astrocytes are GFAP negative **(Sofroniew, 2009)**. Althgouh vimentin is expressed in immature astrocytes **(Pixley and de Vellis, 1984; Bignami and Dahl, 1995)**, at PND10 hypothalamic vimentin did not vary in L4 pups from control levels in males or females. However, at PND30 vimentin levels were lower in L4 pups of both sexes, suggesting overnutrition might accelerate astrocyte maturation.

Further studies are necessary to determine if astrocyte proliferation/development is affected by this manipulation. However, it is of interest to note that the level of expression of the leptin receptor in hypothalamic astrocytes appears to be increased, as indicated by the double immunohistochemistry analysis reported here. These changes were found in the hypothalamus of both adult male and female rats that had experienced neonatal overnutrition. In contrast, although the number of GFAP positive cells in the hypothalamus of adult males that had experienced neonatal overnutrition was increased as previously reported **(Fuente-Martin et al., 2012a),** this effect was not observed in females. Based on these results,

one might hypothesize that both the number and phenotype of the hypothalamic astrocytes are modulated by the early nutritional environment.

Neonatal overnutrition did not affect hypothalamic levels of GFAP at any timpoint throughout development until PND150, when neonatally overnourished males had higher levels than control males. The changes in GFAP levels did not correlate with changes in bodyweight, as L4 males began to weigh more than their controls around PND90. Moreover, as stated above over-nourished animals of both sexes weighed more than their controls before weaning and GFAP levels were unaffected. Although it has been reported that activation of glial cells is not directly related to changes in body weight and that signals such as leptin are most likely involved **(Gao et al., 2014)**, here changes in GFAP levels did not correlate with modifications in either body weight or serum leptin levels, at least in neonatal animals. Previously we have found hypothalamic GFAP levels to be modified in neonatally overnourished males at PND85-90, **(Fuente-Martin et al., 2012a)**; however, in contrast to what we observed here the animals in the previous study were already hyperleptinemic at this earlier age. It is unclear why there was a delay in the development of hyperleptinema in the rats in the studies reported here, even though the rats had begun to weigh significantly more than their controls. However, it does appear that there is an influence of hyperleptinemia on the increase in hypothalamic GFAP levels, although other factors are most likely involved.

Hypothalamic GFAP levels have been shown to increase in other models of obesity, such as in HFD-induced obese rats **(Hsuchou et al., 2009).** These obese animals are hyperleptinemic and leptin increases GFAP levels both in vivo and *in vitro* **(Garcia-Caceres et al., 2011)**, whereas low or null leptin levels are associated with decreased GFAP in spite of the animals presenting obesity **(Ahima et al., 1999)**. Hyperleptinemia is reported to be associated with increased hypothalamic leptin levels **(Hsuchou et al., 2009).** In our study, at PND150 neonatally overnourished male rats were hyperleptinemic, but the levels of leptin in the hypothalamus were not significantly increased, although there was a tendancy to do so. This could indicate a possible impairment of the leptin transport system in L4 males, which is suggested to occur

into become saturated in obese animals **(Banks et al., 1999; Burguera et al., 2000)**. It is of interest to note that males had higher hypothalamic leptin levels than females, reflecting what occurs in serum.

Circulating leptin increases in obese individuals and in response to fat rich diets **(Argente et al., 1997; Hoffler et al., 2009),** and obese subjects often develop leptin resistance which is thought to participate in further weight gain and secondary complications **(El-Haschimi et al., 2000; Scarpace and Zhang, 2009).** The increased resistance is reported to be partially due to decreased leptin transport into the brain **(Caro et al., 1996; Schwartz et al., 1996)** and/or decreased signaling in response to leptin **(Van Heek et al., 1997).** At PND150, hypothalamic p-Stat3$^{(Tyr705)}$ levels were increased by neonatal overnutrition in both sexes with no changes in SOCS3 levels, an inhibitor of cytokine signaling including of the leptin signaling pathway; thus, we found no signs of central leptin resistance. This observation is in contrast to a study by *Rodrigues et al* where they found decreased pSTAT3 and an increase in SOCS3 expression in the hypothalamus of neonatally overnourished rats **(Rodrigues et al., 2011).** In the study by Rodrigues the rats also desplayed persistent hyperphagia, which also indicates central leptin resistance; however, the detected no hyperleptinemia in these 180 day old animals. The explanation for this is unclear. . At PND150, in our study the animals were also eating more, so it is possible that a failure in leptin signaling is beginning to appear although not detected here. Indeed, the age of the rats could be an important factor, since the animals in which central leptin resistance was found were 180 days old and it is possible that our animals would continue to develop further metabolic complications, including central leptin resistance.

As mentioned above, we found an increase in the intensity of leptin receptor immunoreactivity over GFAP positive cells in hypothalamic astrocytes of male and female adult rats that were neonatally overfed. This increase in astrocytic leptin receptor expression was correlated with increased weight in both sexes, as the neonatally overnourished rats of both sexes weighed more at the timpoint used in this study. This increased leptin receptor expression in adulthood could be the result

of developmental influences, or of signals related to the weight gain. The increase in leptin concentrations due to early overnutrition have been previously reported to modify the expression of the leptin receptor in the hypothalamus **(Toste et al., 2006; Manuel-Apolinar et al., 2010).** Thus, the observed hyperleptinemia at PND10 and 21 in both sexes could be responsible for the increase in leptin receptors in astrocytes in adulthood.

The expression of leptin receptors in astrocytes increases in response to diet induced-obesity, at least in males **(Hsuchou et al., 2009)**. The physiological role of leptin signaling in astrocytes was established by *Kim et al* **(Kim et al., 2014a)**. In this study that the lack of the leptin receptor in adult GFAP-positive cells, was shown to blunt the anorectic effect of leptin and increase the response to ghrelin as well as the response to fasting when the animals were refeeding. In addition, leptin receptor ablation in GFAP cells modified astrocyte morphology, reduced glial coverage and altered synaptic inputs to AgRP and POMC neurons of the ARC.

Together these data indicate that astrocytes are respond to early nutritional disturbances, possibly in a sexually dimorphic pattern, and that they could be involved in the long-term metabolic consequences. However, further investigation is required todetermine the different responses to metabolic challenges in male and female astrocytes.

5.2 Hypothalamic inflammatory signals in response to neonatal overnutrition

The hypothalamus is now known to be an important site of cytokine bioactivity within the CNS, as they appear to modulate appetite and energy homeostasis **(Schobitz et al., 1993; Wong and Pinkney, 2004).** Hypothalamic inflammation was first reported in obese rodents by *De Souza* and coworkers approximately 10 years ago, when mice maintained on HFD were found to have an increase in the production of inflammatory cytokines and insulin resistance in the hypothalamus **(De Souza et al., 2005)**. Inflammation within the hypothalamus in association with obesity

appears to occur as a consequence of a combination of factors, including the type of diet and the resulting changes in circulating nutrients and hormones. These factors can to lead to an inflammatory response disrupting metabolic, glucose and cardiovascular homeostasis **(Cai and Liu, 2012)**. The inflammatory response of the hypothalamus to HFD-induced obesity can thus lead to decreased sensitivity to leptin and/or insulin and therefore have negative consequences on the regulation of food intake, triggering the development of secondary complications, such as type 2 diabetes **(Carvalheira et al., 2003).** However, there is little information regarding the changes in cytokine levels in obesity that is not due to HFD, such as increased weight gain as a result of early overnutrition, or in young animals.

At PND10, hypothalamic IL-6 levels were increased by overnutrition in males, but not in females. This could indicate an increase in central production of this cytokine, or increased transport of IL-6 across the BBB **(Banks et al., 1995),** although no increase in serum levels was found. The fact that this rise was only observed in males in response to overnutrition is of interest, as both sexes had increased weight gain and increased leptin levels. Androgenization of females resultsed in a rise in circulating IL6, and it is possible that the central differences in this cytokine are due to the sex steroid environment at this time. The increase of IL-6 at this early age could be related to the regulation of food intake **(Wallenius et al., 2002; Flores et al., 2006; Senaris et al., 2011)**, and not necessarily inflammatory processes.

Hypothalamic inflammation during development in response to early overfeeding was studied in pups by *Ziko et al*., where they found a reduction in IL-1β expression in overfed pups at PND14 and suppression of TNFα in overfed pups at PND7 **(Ziko et al., 2014),** with no change in IL-6 expression at any of these early ages. This is similar to our observations, where we found no change in protein levels of IL-1β or TNFα, and supports the hypothesis that hypothalamic inflammation does not occur in these young overweight animals.

Peripheral and central IL-6 levels have been previously reported to be increased in adult male rats (8 weeks old) in response to neonatal overfeeding

(Stofkova et al., 2009; Tapia-González et al., 2011; Ziko et al., 2014) and we found an increase in the hypothalamus of males at PND50, although it did not reach signficance**.** The higher levels of central IL-6 were not associated with a change in body weight at this age and IL-6 could act as an anorectic signal to prevent overfeeding (**Ropelle et al., 2010**). Indeed, in older animals that had increased food intake and were overweight, IL-6 levels in the hypothalamus were not increased.

No differences in hypothalamic IL-1β or TNF-α levels were found at PND50, which was in accordance with *Ziko et al.* where no changes in these proinflammatory cytokines were found in the hypothalamus of neonatally overfed male adults at PND70 **(Ziko et al., 2014).** Here similar results are also shown in females. Levels of the proinflammatory cytokine IL-1β in the hippocampus are reported to be higher in males than in females **(Bilbo and Tsang, 2010),** but we found no differences between the sexes in the levels of hypothalamic IL-1β, IL6, or IL10. In contrast, TNFα levels were higher in males than in females, regardless of littersize.

Hypothalamic inflammation has been tightly associated with increased TNF-α levels and signaling **(Thaler et al., 2012; Wang et al., 2012; Valdearcos et al., 2015)**. At PND150, hypothalamic TNF-α levels were only found to be increased in neonatally overnourished males, which was associated to increased body weight. This observation could indicate that these animals could develop hypothalamic insulin and/or leptin resistance, as observed in other studies **(De Souza et al., 2005; Milanski et al., 2009; Posey et al., 2009; Thaler et al., 2013),** although hypothalamic levels of p-Stat3$^{(Tyr705)}$ were increased with no change in SOCS3. However, the techniques employed would not detect changes if only select cell populations become leptin or insulin resistant**.** The elevated hypothalamic TNFα levels could be related to the increase in GFAP protein levels and GFAP+ cell number in L4 males. Increased hypothalamic TNF-α levels in response to saturated fatty acids has been associated with activated microglia **(Valdearcos et al., 2014);** however, here we did not detect changes in Iba1 protein levels or immunostaining as a consequence of neonatal overnutrition. Microglial activation does not always correlate with weight gain **(Fuente-Martin et al., 2013b; Gao et al., 2014; Garcia-Caceres et al., 2014)**, indicating that

other factors are involved, such as the diet associated with the weight gain. At PND150, hypothalamic TNF-α levels were higher in males than females, regardless of litter size. This could also indicate that overfed males are starting to show vulnerability to the early overnutrition as they get older, which does not occur in females, at least at this age. Thus TNFα could be one factor participating in the sexual dimorphism in response to aging.

6. Sex differences in the responses to neonatal overnutrition

According to the results presented, it is clear that modification of the nutritional status during the sensitive period of lactation, produces a hormonal imbalance during the initial postnatal weeks in L4 pups and triggers short and long-term effects not only on peripheral, but also central systems that regulate energy balance in a sexually dimorphic manner. The most striking differences that we found between the sexes were the long-term effects of neonatal overnutrition reflected in an increased body weight in males, but not females, as well as a change in GFAP immunopositive cells in males but not females. This is consistent with previous studies in which neonatal overnutrition alters body weight in adulthood **(Glavas et al., 2010; Hou et al., 2011; Fuente-Martin et al., 2012b; Bei et al., 2015; Collden et al., 2015)** and is associated with an increase in the number of GFAP-positive cells in the hypothalamus **(Garcia-Caceres et al., 2011; Fuente-Martin et al., 2012a),** although all of these studies employed only male animals. In addition, androgenized females had increased hypothalamic GFAP compared to control females and this was associated with higher body weight and circulating leptin levels, which is consistent with previous studies that show that perinatal androgens cause sexual differences in GFAP expression and morphology in different brain areas **(Garcia-Segura et al., 1988; Chowen et al., 1995; Mong et al., 1999; Amateau and McCarthy, 2002; Conejo et al., 2005).** Whether glial changes due to early weight gain as a consequence of neonatal overnutrition affect synaptic inputs to metabolic neurons and if this is sexually dimorphic requires further investigation.

Thus, taking together body weight, GFAP protein levels and the number of GFAP positive cells, these results suggest that males and females have a differential long-term response to neonatal overnutrition. There are scarce data showing the long-term effects in females. However, as seen here some studies indicate that long-term effects are influenced by sex, with males suggested to be more susceptible than females to early nutritional disturbances **(Bassett and Craig, 1988; Boubred et al., 2009; Fuente-Martin et al., 2012c; Stefanidis and Spencer, 2012).** As previously discussed, neonatal testosterone among other factors could be involved in the sexually dimorphic long-term effects of early nutritional alterations.

6.1 Response of male and female hypothalamic astrocytes *in vitro*

Astrocytes can participate in hypothalamic inflammation and HFD-induced obesity results in hypothalamic astrogliosis **(Horvath et al., 2010; Thaler et al., 2012; Buckman et al., 2013).** Along with microglia, astrocytes participate in the central inflammatory response **(Rivest, 2009; Thaler et al., 2012; Buckman et al., 2014; Morselli et al., 2014; Valdearcos et al., 2014),** producing pro- and anti-inflammatory cytokines, chemokines or other factors in response to a stimulus; therefore, these glial cells could be the source of the increases in central cytokine levels. Indeed, astrocytes are directly exposed to nutrients, including FAs, and have essential roles in relaying information concerning hormonal and nutritional signals to neurons; therefore, FAs could produce modifications in astrocytes that could indirectly affect neuronal regulation of energy balance. Specifically, the saturated FA, palmitic acid, is reported to induce pro-inflammatory cytokine production and central leptin sensitivity, but this leptin resistance is not observed in response to the unsaturated oleic acid **(Karaskov et al., 2006; Milanski et al., 2009; Gupta et al., 2012; Morselli et al., 2014).** The initial responses of glial cells are thought to be mainly protective; however, a prolonged inflammatory response and glial activation can be damaging and contribute to further metabolic complications.

Studies indicate that the inflammatory response of astrocytes differs between the sexes, with a more exaggerated inflammatory response from male compared to

female astrocytes. **(Liu et al., 2007; Santos-Galindo et al., 2011; Morselli et al., 2015)**, Likewise, we found sex differences in the long-term glial and inflammatory response to neonatal overnutrition, including changes in GFAP and hypothalamic cytokines, with males being more affected than females. Estrogens could be involved in the protective effects in females **(Wang et al., 2014a; Zhang et al., 2014)**, but sex differences in astrocytes could also be due to differences in the sex chromosomes or from sex steroid exposure during development **(Arnold, 2009).** Here we analyzed whether the response of these glial cells to fatty acids was sex dependent and if estrogens affected this response.

7. The effect of fatty acids on hypothalamic primary astrocytes from male and female rats

7.1 Palmitic acid

Studies show that excesive ingestion of dietary fats is associated with increased GFAP expression and astrocyte reactivity in the brain **(Hsuchou et al., 2009; Horvath et al., 2010).** Palmitic acid is the most abundant saturated FA in the diet and in plasma. In addition, plasma PA levels increase in obesity **(Opie and Walfish, 1963; Reaven et al., 1988)** and this FA has been suggested to directly induce hypothalamic inflammation **(Milanski et al., 2009; Cheng et al., 2015)**. Importantly, obese patients with metabolic syndrome have a higher FA uptake into the brain than healthy patients, with HFD intake increasing this uptake **(Wang et al., 1994; Karmi et al., 2010).** However, in addition to FAs, other metabolic signals are modified in obesity and reach the brain and therefore, could be also responsible for the observed changes in glia and inflammation. Indeed, HFD ingestion alters circulating levels of insulin, glucocorticoids, estrogens and cytokines **(Tannenbaum et al., 1997; Kubota et al., 1999; Cohen, 2000; Hoffler et al., 2009),** which can modify astrocytes **(Toran-Allerand et al., 1991; Garcia-Segura et al., 1996b; Garcia-Caceres et al., 2011)** and these factors could be involved in astrocyte activation independently of FAs. Indeed, *ob/ob* mice in which leptin levels are nule, GFAP levels are decreased **(Ahima et al., 1999)**. Astrocytes from mixed male and female cerebral cortices have been shown to respond to FAs *in vitro*

(Gupta et al., 2012); hence, we aimed to further understand the response of these glial cells, but specifically in the hypothalamus, to increased levels of FAs in both sexes.

Hypothalamic astrocytes cultured *in vitro* responded to palmitic by decreasing GFAP mRNA levels and this response was similar in both sexes. The decrease in GFAP expression was not associated with a decrease in cell number. These results are in contrast to the study by Morselli and colleagues **(Morselli et al., 2015)** where GFAP expression was increased after exposure to palmitic acid. In the study by Morselli et al., the time of exposure to this fatty acid was only 8 hours compared to 24 hours used here. As the protein levels of GFAP in the hypothalamus and in hypothalamic astrocyte cultures change in a biophasic manner in response to leptin **(Garcia-Caceres et al., 2011)**, it is possible that a similar phenomenon occurs in response to FAs. Another possibility is that palmitic acid is inhibiting growth, as shown by *Beeharry et al* **(Beeharry et al., 2003)**, and this occurs after longer exposition**.** Moreover, the astrocyte cultures of *Morselli et al* were derived from mice and thus the response could be different in rat astrocytes. In support of this, we have found that the GFAP response to ghrelin differs between astrocyte cultures from male mice and male rats **(Fuente-Martin et al., in press).** Reactive astrocyes are structurally more complex and present higher GFAP level **(Gomes et al., 1999)**, therefore a decrease in GFAP levels could indicate decreased astrocyte differentiation and/or proliferation. Moreover, astrocyte morphology could also be affected and the decrease in GFAP due to fewer or shorter projections as a result of palmitc acid. Further studies are necessary to determine if the change in mRNA levels is associated with similar changes in protein levels and in astrocyte morphology, as well as to determine if the changes in GFAP mRNA levels in response to palmitic acid depend on the time of exposure to this FA.

Free fatty acids, especially saturated FAs, can activate an inflammatory response in different cell types **(Boden and Shulman, 2002; Iyer et al., 2010),** as well as ER stress **(Gregor and Hotamisligil, 2007)**. In addition to inducing central inflammation **(Milanski et al., 2009; Gupta et al., 2012; Morselli et al., 2014)**, palmitate causes ER stress, ERK activation, and apoptosis in adipocytes **(Guo et al., 2007)** and hepatocytes **(Malhi et**

al., 2006), as well as in hypothalamic neurons **(Mayer and Belsham, 2010).** In agreement with this, we found that in astrocytes palmitic acid increased IL6 and CHOP production in a dose dependent manner. Astrocytes are the predominant source of IL-6 in the CNS **(Van Wagoner and Benveniste, 1999)** and the PA-induced increase in IL-6 expression could be part of an inflammatory response, as IL-6 is one of the effectors of the neuroinflammatory cascade **(Rothwell and Hopkins, 1995; Allan and Rothwell, 2003)**. However, IL-6 could also be functioning as a neuroprotective beneficial signal, avoiding brain damage and enhancing neuronal survival **(Van Wagoner et al., 1999)**. Indeed, an increase in IL6 in astrocytes and microglia has also been shown to have a protective role from diet induced obesity, preventing further weight gain and limiting the adverse hypothalamic consequences **(Hidalgo et al., 2010).** Moreover, IL-6 functions as a neuroendocrine signal and could be acting as an anorectic signal to inhibit food intake in response to FA excess **(Ropelle et al., 2010).**

The increase in CHOP production by palmitic acid suggests ER stress, which has been linked to obesity, with increased levels of FFAs known to contribute to the development of ER stress in other cell types **(Oyadomari and Mori, 2004; Ozcan et al., 2004; Nakatani et al., 2005; Ozcan et al., 2006; Szegezdi et al., 2006; Ron and Walter, 2007; Zhang et al., 2008).** Increased ER stress can induce cytokine production, and thus induce inflammatory processes that can lead to insulin resistance **(Ozcan et al., 2004; Ozcan et al., 2006; Shi et al., 2006)** and can also lead to apoptotic cell death **(Oyadomari and Mori, 2004; Karaskov et al., 2006; Szegezdi et al., 2006).**

Palmitic acid also stimulated the release of nitrites and nitrates to the culture media in a dose dependent manner, which can have a cytotoxic role on the surrounding cells under pathological circumstances **(Moncada et al., 1991; Zhang et al., 1994a)**. Nitric oxide production increases in pancreatic β-cells in response to palmitic acid **(Shimabukuro et al., 1997)**. Nitric oxide is proposed to link ER stress and inflammation **(Gotoh and Mori, 2006)** and is associated with insulin resistance **(Kaneki et al., 2007).** There is evidence of induction of CHOP by nitric oxide **(Kawahara et al., 2001; Gotoh et al., 2004)**, which is consistent with our results**.** Studies have indicated that *in vitro,* hypothalamic neurons do not have an inflammatory response to saturated

fatty acids, although an ER stress response does occur **(Choi et al., 2010)**. Prolonged HFD is reported to induce neuronal apoptosis **(Moraes et al., 2009)** and to decrease the neuronal number in the hypothalamus **(Thaler et al., 2012).** A similar response was also observed in neural stem cells (NSCs), which differentiate into neurons and astrocytes, after exposure to palmitic acid **(Yuan et al., 2013).** Therefore, FAs induce stress in hypothalamic astrocytes with these glial cells releasing factors that could regulate or damage surrounding neurons and thus indirectly affect neuronal regulation of energy balance.

Astrocytic production of IGF-1 is involved in neuroprotection **(Madathil et al., 2013; Genis et al., 2014)** and IGF-1 protects astrocytes from oxidative stress **(Davila et al., 2016).** PA reduced IGF-1 mRNA levels in astrocytes, with this reduction being more apparent in females at higher doses. Whether this decrease in IGF-1 production is related to the increased ER stress in response to PA, as well as whether there is a decreased protective effect of astrocytes on the surrounding neurons, remains to be demonstrated.

The increase in CHOP mRNA levels in response to PA was significant in males, but not in females. In addition, the release of nitrites and nitrates and the increase in IL-6 expression was higher in males than females, although this did not reach statistical significance. This is probably due to the large variability in some groups, and the large number of groups being analyzed. Indeed, if the sexes are analyzed separately, some changes do become significant. Moreover, increasing the number of experiments could possibly reduce the variability in these results.

7.2 Palmitic and oleic acids interaction

There are distinct inflammatory responses to the different fatty acids present in our diet. Likewise, saturated and unsaturated fatty acids are reported to produce different effects on energy homeostasis **(Lee et al., 2001; Karaskov et al., 2006; Gupta et al., 2012; Itariu et al., 2012; Kien et al., 2014).** Here, the response of hypothalamic astrocytes to oleic acid was significantly different from that seen in response to palmitic acid.

Oleic acid did not produce the dramatic decrease in GFAP seen in response to PA exposure, nor were IL-6 or CHOP expression levels or the release of nitrites and nitrates increased by OA. Moreover, when OA was added, it was capable of blocking the effects of PA on GFAP, IL-6 and CHOP mRNA levels. However, although the increased release of nitrites and nitrates that was found after PA exposure was unapparent with OA, the combination of both fatty acids still stimulated the release of nitrites and nitrates, which was significant in males but not in females.

This suggests that OA does not trigger the inflammatory, ER stress or certain cytotoxic effects on astrocytes that are seen with PA and supports the fact that saturated and unsaturated FAs can have different impacts on energy homeostasis **(Lee et al., 2001; Beeharry et al., 2003; Karaskov et al., 2006; Benoit et al., 2009; Milanski et al., 2009; Cintra et al., 2012; Gupta et al., 2012; Itariu et al., 2012; Kien et al., 2014).** Here we show that some of these differential effects of fatty acids could possibly be mediated by astrocytes.

The observation that CPT1A mRNA levels were differentially regulated by palmitic and oleic acids in astrocytes is of interest. This enzyme is essential for fatty acid oxidation, as it is the first and rate-limiting step in the formation of palmitoylcarnitine **(Lee et al., 2011)**. Palmitoylcarnitine, or acyl-carnitine, is then moved to the inner mitochondrial membrane through a specific shuttle system to be oxidized. The fact that palmitic acid is less capable than oleic acid of inducing CPT1A in astrocytes, and thus of initiating fatty acid oxidation in these cells, could contribute to its toxicity. In combination with OA, palmitic acid could be more freely shuttled into the mitochondria and oxidized due to the increased levels of CPT1A and this could decrease its toxicity.

Interestingly, OA increased the expression of IGF-1, with this elevation being significant in males, but not in females. Although PA alone had no effect on the expression of this neuroprotective factor, it inhibited the effect of OA in males. In contrast in females, PA reduced IGF-1 mRNA levels and OA blocked this reduction. Further studies are necessary to determine the mechanisms by which fatty acids modulate IGF-1 production in astrocytes, including whether this is a primary

response to the fatty acids, or an indirect response through previous modulation of other factors. Indeed, these studies were done at 24 hours of exposure to fatty acids. However, it is of interest that this neuroprotective factor **(Genis et al., 2014)** is differently modulated by these fatty acids in males and females.

In contrast to the studies with PA alone, here we found a decrease in TNFα mRNA levels in response to both PA and OA, with the combination of these fatty acids being additive. The fact that the variability in the results of the first experiments was greater could explain this differential result as there was also a tendency to decrease in the previous study. This decrease in TNFα mRNA levels was unexpected, as this cytokine has been directly associated with the inflammatory response to fatty acids, however TNFα is also reported to be inhibited by IL-6; therefore, it could be that the increased production of IL-6 in response to PA is exerting an inhibitory effect on TNFα levels **(Oh et al., 1998; Calder et al., 2013).**

7.3 Protective effects of estrogens against palmitic acid

The central anti-inflammatory effects of estradiol have been widely studied **(Arevalo et al., 2010; Cerciat et al., 2010; Guo et al., 2012)**. However, less is known regarding the effect of estradiol specifically in hypothalamic astrocytes in overnutrition or obese conditions. Here we found that PA decreased the expression of ERα in primary astrocytes of both sexes, but estradiol had the opposite effect and was able to block the effect of PA, especially in female astrocytes. It has been reported that female hypothalamic astrocytes express higher levels of ERα in in response to estradiol stimulation **(Kuo et al., 2010)**. This was in accordance to the observations of Morselli et al **(Morselli et al., 2014),** despite that they employed hypothalamic astrocytes from mice, the estradiol concentration used was higher, the PA concentration lower and the treatment exposuretime was only 8 hours.

Pretreatment of astrocytes with estradiol reversed some of the changes produced by palmitic acid, such as IL6 and CHOP production, showing protective effects in both sexes. Neuroprotection and anti-inflammatory effects through ERα has been shown in astrocytes and neurons **(Barreto et al., 2009; Spence et al., 2013;**

Arevalo et al., 2015). Moreover, estrogens are known to be inhibitors of inflammatory cytokines such as IL6 and IL1β in peripheral tissues and brain **(Koka et al., 1998; Bruce-Keller et al., 2000).**

Although IGF-1 production tended to decrease after PA, no effect of estradiol was found. As IGF-1 is produced by astrocytes as a neuroprotective factor and as protection against oxidative stress **(Genis et al., 2014),** it would be coherent to think that estradiol may protect astrocytes and thus surrounding neurons by modulating the release of IGF-1. However, although female astrocytes showed a slight increase, there was no significant increase in IGF-1 levels after estradiol pre-treatment , although with an increased "n" the effect could possibly be found to be significant. Likewise, the anti-inflammatory cytokine IL-10 showed a light increase in response to estradiol alone and estradiol plus PA compared to PA alone in females, but not in males. These results suggest that estradiol may mediate its protective effects in females by stimulating the release of IGF-1 and IL-10 in response to PA, althoug further experiments are needed to confirm this hypothesis.

These data indicate that estradiol could play an important role as an anti-inflammatory or anti-ER stress factor in astrocytes when exposed to saturated fatty acids. In males, estradiol was less capable of protecting against the decline in ERα expression induced by PA, which could possibly decrease the ability of estradiol to impede neuronal damage, as previously shown **(Carbonaro et al., 2009)**. The increase in IL-6 and CHOP expression induced by PA were similarly reversed by estradiol in males and females. However, as *in vivo* females have higher circulating levels of estradiol than males, this mechanism could be involved in the higher level of protection against high fat diets observed in females.

7.4 Palmitic acid and the Toll receptor 4 antagonist RS-LPS

Activation of TLR signaling cascades produce a wide array of proinflammatory cytokines, including IL-1β, IL-6 and TNF-α, in addition to reactive oxygen and nitrogen species mediators such as NO **(Takeda and Akira, 2015)**. In response to PA, the most striking results that we observed were the increase in CHOP, IL-6 and the

amount of nitrites and nitrates released to the media, which are signs of inflammation and oxidative stress. Importantly, oxidative stress activates mechanisms that result in glia-mediated inflammation, which can produce secondary neuronal damage. As saturated fatty acids have been proposed to activate TLR4 in the hypothalamus **(Milanski et al., 2009)**, we asked whether PA activated TLR4 in astrocytes. It is well known that TLR4 is activated by lipopolysaccharide (LPS) **(Poltorak et al., 1998).** Whereas LPS stimulates TLR4 in microglia, the main cells responsible for the immune response in the CNS to activate an inflammatory response **(Qin et al., 2005; Rivest, 2009),** lower constitutive expression of TLR4 is found in astrocytes. However, TLR4 activation in astrocytes is also reported to activate proinflammatory processes mediated by the NFκB, MAPK and Jak1/Stat1 signaling pathways **(Carpentier et al., 2005; Gorina et al., 2011)**. We used a form of LPS, RS-LPS, that is purported to antagonize TLR4 **(Kutuzova et al., 2001)** to determine if hypothamic astrocytes respond to PA through this receptor. However, not only did RS-LPS not block the effects of PA, but it also stimulated IL-6, CHOP, IL-1β, Nfκbia (IκBα) mRNA levels and this was additive with the effects of PA. This could indicate that in astrocytes the expression of these factors in response to PA is not dependant on TLR4 activation. Inflammation could be activated in astrocytes through another TLR, such as TLR3, which is reported to be more highly expressed in these glial cells than is TLR4 **(Farina et al., 2005). This receptor** mediates the release of a wide variety of predominantly anti-inflammatory and neuroprotective, as well as pro-inflammatory factors, IL-6 among them **(Bsibsi et al., 2006)**, whereas TLR4 mainly triggers the release of pro-inflammatory mediators such as TNF-α **(van Noort and Bsibsi, 2009).** What is clear is that RS-LPS is not acting as a TLR4 antagonist in these cells, but as at least a partial agonist. Moreover, the effects of PA and RS-LPS appear to be additive.

Astrocytes from cerebral cortex are reported to release proinflammatory cytokines in response to TLR4 activation **(Gorina et al., 2011)** and the TLR4 antagonist used here has been shown to attenuate inflammation in microglial cells by reducing TLR4 and NF-κB and MAPKs signaling pathways **(Gaikwad and Agrawal-Rajput, 2015).** However, the activation of TLRs in astrocytes and microglia is not the same, as

astrocytes respond later than microglia to activation of TLRs **(Rivest, 2003; Owens, 2005)**. Thus, the mechanism of activation could be different and could possibly explain the lack of an antagonist response to this factor in astrocytes.

7.5 Intracellular signaling in astrocytes in response to PA

We found that male astrocytes rapidly increased the amount of STAT5 in response to PA. The STAT family has a central role in inflammatory reactions and is stimulated by multiple cytokines to regulate the expression of inflammatory mediators. Particularly, STAT5 has been shown to be activated by glycogen synthase kinase-3 (GSK-3) in primary cultures of cerebral cortical astrocytes to trigger inflammatory responses **(Beurel and Jope, 2008; Wang et al., 2011)** and PA induces GSK-3 activation in cultures of primary hepatocytes **(Ibrahim et al., 2011).** Thus, STAT5 activation appears to be one of the inflammatory signaling pathway activated by PA. In addition, STAT5 participates in the regulation of glycogen storage in astrocytes **(Bosier et al., 2013)** and the increase found in STAT5 could indicate an increase in glycogen storage in response to fatty acids, however if GSK-3 is activated to induce STAT5 increase and trigger inflammation, glycogen synthase would be inactivated by GSK-3 and thus glycogen storage inhibited. Further studies are necessary to determine the implication of this signaling pathway in astrocytes response to nutrient–derived signals.

Stress and inflammation can also activate NFκB, ERK and JNK pathways in astrocytes leading to the synthesis and release of inflammatory mediators **(Gao and Ji, 2010; Gorina et al., 2011).** NFκB and MAPKs play an important role in triggering microglia activation and subsequent secretion of inflammatory mediators **(Vallabhapurapu and Karin, 2009).** However, we found no changes in the activation of these pathways. This could be due to the degree of variability found in these result . Moreover, as only one time-point after exposure to PA is reported here, it is possible that some of these pathways are activated either more rapidly, or at a later time-point.

VII. CONCLUSIONS

1. Males and females respond differently to increased food intake even during the neonatal period; thus, the sex differences in metabolic control cannot be solely attributed to post-pubertal differences in circulating gonadal steroid levels.

2. Neonatal overnutrition induces significant changes in circulating insulin, leptin, and adiponectin levels during a critical period of hypothalamic development. Hence, modifications in the levels of these hormones, some of which are known to influence hypothalamic development, could be involved in the long-term metabolic effects of early overnutrition.

3. The hormonal changes induced by overnutrition differed between males and females during the neonatal period. The greater increase in serum insulin, as well as in testosterone levels in males, during this critical developmental period, could be involved in the differential long-term metabolic outcomes observed between the sexes.

4. The long-term effects of neonatal overnutrition change throughout development, with males being more susceptible to metabolic alterations during adulthood.

5. Increased weight gain during the perinatal period was not associated with any indication of systemic inflammation, which could be due to the ability of adipose tissue to expand due to hyperplasia during early development. This observation supports the fact that obesity during childhood is different from that seen in adults.

6. The increased bodyweight in adult male rats was associated with significant elevations in the hypothalamic levels of the inflammatory cytokine TNFα and the astroglial marker GFAP, as well as the number of GFAP+ astrocytes. However, there was no increase in other markers of astrocyte activation, such as vimentin, or in microglial markers or inflammatory signaling. This suggests that the inflammatory and gliosis responses in the hypothalamus may occur gradually in conditions of increased weight gain that are not due to ingestion of diets, such as those high in fats, that have direct effects on inflammatory processes and on glial cells.

7. Hypothalamic astrocytes respond differentially to palmitic acid and oleic acid, with oleic acid protecting against the inflammatory processes induced by palmitic acid. Thus, the harmful effects of a high fat diet will depend on the relative composition of the different fatty acids and not only the quantity.

8. Higher circulating levels of estrogens in adult females can have protective effects on astrocytes against palmitic acid.

VIII. CONCLUSIONES

1. Machos y hembras responden de forma diferente al incremento de la ingesta de alimentos, incluso durante el periodo neonatal. Por consiguiente, las diferencias sexuales en el control metabólico no pueden atribuirse únicamente a las diferencias postpuberales en los niveles circulantes de esteroides gonadales.

2. La sobrenutrición neonatal induce cambios significativos en los niveles circulantes de insulina, leptina y adiponectina durante un periodo crítico del desarrollo hipotalámico. Por tanto, las modificaciones en los niveles de estas hormonas, algunas de las cuales son conocidas por influenciar el desarrollo hipotalámico, podrían afectar los efectos metabólicos a largo plazo de la sobrenutrición temprana.

3. Los cambios hormonales inducidos por la sobrenutrición difieren entre machos y hembras durante el periodo neonatal. El mayor incremento en insulina sérica, así como de los niveles de testosterona, en machos durante este periodo crítico de desarrollo, podría intervenir en la diferenciación de los efectos metabólicos a largo plazo observados entre ambos sexos.

4. Los efectos a largo plazo de la sobrenutrición neonatal se modifican durante el desarrollo, siendo los machos más susceptibles a las alteraciones metabólicas durante la época adulta.

5. El incremento de la ganancia ponderal durante el periodo perinatal no se asoció con ningún signo de inflamación sistémica. Este hecho podría deberse a la capacidad del tejido adiposo a expandirse debido a la hiperplasia durante el desarrollo temprano. Esta observación apoya el hecho de que la obesidad durante la infancia es diferente a la que se aprecia en adultos.

6. El incremento del peso corporal en las ratas adultas se asoció con elevaciones significativas en los niveles hipotalámicos de citoquina TNFα y del marcador astroglial GFAP, así como del número de astrocitos GFAP+. No obstante, no se evidenció incremento de otros marcadores de la activación astrocítica, tales como vimentina, o marcadores de microglía o de señalización inflamatoria. Esto sugiere que las respuestas inflamatorias y de gliosis en el hipotálamo pueden acontecer gradualmente en situaciones de ganancia ponderal que no son debidas a la ingesta de

dietas, como es el caso de aquellas ricas en grasas, que tienen efectos directos sobre los procesos inflamatorios y sobre las células de la glía.

7. Los astrocitos hipotalámicos responden de forma diferente a los ácidos palmítico y oleico. En efecto, el ácido oleico es capaz de proteger contra los procesos inflamatorios inducidos por el ácido palmítico. Por consiguiente, los efectos nocivos de una dieta con alto contenido graso, dependerán de la composición relativa de los diferentes ácidos grasos y no únicamente de su cantidad.

8. Los niveles circulantes más elevados de estrógenos en las ratas hembra adultas pueden tener efectos protectores en los astrocitos contra el ácido palmítico.

IX. BIBLIOGRAPHY

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