University of Southampton

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Clinical Neurosciences

Mouse Maternal High-Fat Diet Impairs Metabolism, Brain Cytoarchitecture, and Hippocampal Neurogenesis in the Adult Offspring Mouse.

by

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Obesity is a global health problem, and the number of reproductive-age women with obesity continues to increase. Cases of maternal obesity induced by a high-fat diet (HFD) have increased in recent years. Different studies have described that maternal HFD can affect the normal development of several organs, making offspring more susceptible to certain diseases. Animal obesity models suggest that a maternal HFD during gestation is a risk factor for developing physiological and behavioural dysfunctions in offspring. We test the hypothesis in mice that maternal HFD without obesity, during pregnancy and lactation, changes the cortical and hippocampal structure and cellular organisation and modifies metabolic-related parameters, and neurogenesis in the adult hippocampus.

Female mice were fed different diets from conception: normal fat diet (NFD), HFD throughout gestation and lactation (HFD) or embryonic HFD (Emb-HFD: HFD for 3 days, NFD thereafter). After weaning, the offspring were maintained on NFD. Our data showed that the HFD and Emb-HFD groups developed metabolic disturbances in adulthood and had a greater density of astrocytes and microglia cells, in the cortex and hippocampus. Similarly, we observed that the offspring of mothers fed a HFD had a higher density of new-born neurons and a reduced density of mature neurons in the dentate gyrus, indicating that exposure to a maternal HFD can generate changes in adult neurogenesis.

In many cases we observed a graded response (NFD<Emb-HFD<HFD) suggesting a very early induction of diet-induced responses, well before any neural differentiation, with exacerbated effects upon continued HFD challenge. This suggests that not only diet during pregnancy is crucial for proper glial cell density and neurogenesis, but also the time of exposure is critical to determine the size of the effects in the offspring brain, caused by maternal HFD.
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Research Thesis: Declaration of Authorship

Print name: DIEGO ARMANDO OJEDA PEDRAZA

Title of thesis: Mouse Maternal High-Fat Diet Impairs Metabolism, Brain Cytoarchitecture, and Hippocampal Neurogenesis in the Adult Offspring Mouse.

I declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission.

Signature: ................................................................. Date:.................................
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Definitions and Abbreviations

AdipoR: Adiponectin receptor.

ADHD: Attention-deficit hyperactivity disorder.

BBB: Blood–brain barrier.

BDNF: Brain-derived neurotrophic factor.

BMI: Body mass index.

BSA: Bovine serum albumin.

BW: Body weight.

CA: Cornu ammonis.

CNS: Central nervous system.

CP: Cortical plate.

DAPI: 4’,6-diamidino-2-phenylindole.

DCX: Doublecortin.

DG: Dentate gyrus.

DM2: Type 2 diabetes mellitus.

DOHaD: Developmental origins of health and disease.

DS: Donkey serum.

E3.5: Embryonic day 3.5.

EC: Entorhinal cortex.

EGFR: Epidermal growth factor receptor.

Emb: Embryonic.

FA: Fatty acid.

FFAs: Free fatty acids.

GABA: Gamma-aminobutyric acid.

GCL: Granule cell layer.

GFAP: Glial fibrillary acidic protein.

Glut: Glucose transporter.

HFD: High-fat diet.

Iba1: Ionized calcium-binding adapter molecule 1.

ICM: Inner cell mass.
Definitions and Abbreviations

**InsR**: Insulin receptor

**IL**: Interleukins.

**IPC**: intermediate progenitor cell.

**kDa**: Kilodalton.

**mRNA**: Messenger ribonucleic acid.

**NeuN**: Neuronal Nuclei.

**NFD**: Normal Fat Diet.

**NF-κB**: Nuclear factor of kappa-light-chainenhancer of activated B cells.

**NGF**: Nerve growth factor.

**nIPC**: Neuronal intermediate progenitor cells

**NPC**: Neuronal progenitor cell.

**NSC**: Neural stem cell.

**ObR**: Leptin receptor

**OPC**: oligodendrocyte precursor cells

**PB**: Phosphate buffer.

**PBS**: Phosphate buffered saline.

**PFA**: Paraformaldehyde.

**PND**: Postnatal day.

**RGC**: Radial glial cell

**RMS**: Rostral migratory stream

**ROS**: Reactive oxygen species

**SGZ**: Subgranular zone.

**Sox2**: Sex determining region Y box.

**SVZ**: Subventricular zone.

**TBS**: Tris buffered saline.

**TE**: Trophoderm.

**TNF**: Tumour necrosis factor

**VZ**: Ventricular zone.
Chapter 1. Literature Review

1.1 OBESITY

Obesity is defined as an excessive accumulation of body fat, caused by the disruption of energy balance, which occurs when intake exceeds the body's energy expenditure\(^1\). Obesity is a complicated problem because multiple factors are involved in its development. Obesity is considered a significant health problem, where different tissues are affected, such as adipose tissue, liver, muscle, pancreas, and brain. Obesity has been associated as a risk factor of coronary heart disease, hyperlipidaemia and type 2 diabetes mellitus (DM2)\(^5\).\(^6\).

1.1.1 Diagnosis and Classification

The Body Mass Index (BMI) defines obesity in clinical practice. The BMI relates body weight in kilograms to height in meters squared and is calculated according to the formula BMI = weight [kg] / height [m]\(^2\). Following the World Health Organization (WHO) criteria, classification of obesity is based on the BMI value, where a BMI of 25.0 to 29.9 kg/m\(^2\) is defined as overweight, and a BMI of 30 kg/m\(^2\) or higher as obesity\(^7\). It was estimated that in 2014 about 375 million women and about 266 million men were obese in the world, affecting both developed and developing countries\(^8\).

![Prevalence of overweight and obesity](image)

**Figure 1.1 Prevalence of overweight and obesity**

(a) (BMI≥25 kg/m\(^2\)) and obesity (b) (BMI≥30), by age and sex, 2013. ■ Developed country, women. ▲ Developed country, men. △ Developing country, women. ▼ Developing country, men. — Global, Women. — Global, men. It was taken from Ng M et al., 2014\(^9\).
Moreover, it is possible to define obesity taking into account the percentage of body fat, categorizing an individual, as an obese subject with a percentage of body fat more significant than 25% for males and greater than 33% for women\textsuperscript{7,10}. The health risks associated with obesity not only depend on the total adiposity but also its distribution; therefore the evaluation of the body fat distribution is recommended\textsuperscript{11,12}.

### 1.1.2 Pathogenesis of obesity

Obesity is a multifactorial disease; environmental and genetic factors influence its aetiology. Obesity is defined as a chronic, complex, multifactorial disease, developed by an interaction between genes and the environment. Obesity is attributed to a constant energy imbalance between high caloric intake and a decrease in energy expenditure\textsuperscript{13}. It seems that the change undergone by society in recent years in terms of lifestyle, including different eating habits and more sedentary lifestyle, has a significant impact on the development of this disease\textsuperscript{14,15}.

### 1.1.3 Environmental or lifestyle factors

The weight gain in population in recent years is due to changes in lifestyle during the modernization process, such as new technologies, and passive leisure, which have modified food, diets and physical activity\textsuperscript{13}. Weight gain is due to caloric surplus, as a result of declining physical activity. Feeding behaviours depend on many factors (religious, olfactory, visual, emotional) since the individual does not see food only as a nutritious object but also something that has a symbolic significance\textsuperscript{16}. Lack of exercise or sedentary lifestyle is an essential factor linked to the development of new technologies associated with the evolution of society in search of comfort and minimal physical effort\textsuperscript{17}.

Certain social and economic conditions can be linked to obesity. For example, not having access to safe areas for exercise, learning unhealthy ways to cook, not having enough money to buy fresh fruits and vegetables or unprocessed and packaged foods. Although in recent years genetic factors have been identified that explain the susceptibility of some individuals to obesity, the increase in obesity that has occurred in the last decades is due mainly to essential changes in the diet, in physical activity and other socio-cultural factors\textsuperscript{18,19}.

### 1.1.4 Genetic Factors

Obesity does not show a classical heritage model, and it is highly dependent on environmental factors\textsuperscript{20}. Numerous studies have shown that predisposition to obesity and its associated
conditions, are more similar between genetically related individuals than in those unrelated. The phenotypes associated with obesity have a significant narrow-sense heritability (h²), being this trait the proportion of the variability that is attributable to genetic factors. Values can range from zero (variation does not have a genetic contribution) to one (all variations are due to genetic differences). In the case of BMI, the h² has values of 40-70% in different human studies. Therefore, these studies allow concluding that not only genetic factors are involved in the aetiology of obesity, but also other factors as environmental factors could be playing a fundamental role in the development of this disease.

1.1.5 Genetics of obesity in childhood

Obesity in childhood is a typical phenotype of several syndromes caused by chromosomal abnormalities or genetic mutations. These genetic alterations do not explain the increase in cases of obesity in recent years; however, the study of these forms of obesity has given valuable information on identifying relevant metabolic pathways involved in the development of this condition.

There are approximately thirty syndromes with obesity, which are usually accompanied by mental retardation, dysmorphic body and other characteristics. The best-characterized forms are Prader Willi, Bardet Biedl, Albright osteodystrophy, Adler syndrome, fragile X syndrome, Cohen syndrome, among others. Some of these syndromes are associated with chromosomal abnormalities, and others are monogenic forms with pleiotropic effects.

1.1.6 Metabolic syndrome

Obesity is associated in most cases with endocrine, cardiovascular and osteoarticular pathology, mainly related to biological, social and cultural factors. Obesity is a major public health problem that contributes significantly to the leading causes of death, cardiovascular diseases and cancer. Many of the comorbidities of obesity are reflected in the so-called metabolic syndrome. Metabolic syndrome is the association of a set of risk factors for developing heart diseases and Type 2 diabetes mellitus; some of these risk factors are:

- Arterial hypertension
- Increased blood sugar levels
1.1.7 Energy balance

Classically, adipose tissue has been considered as a reservoir of energy. However, not only acts as a store of fat molecules but also, it synthesizes and releases various hormones related to both metabolism and the regulation of food intake\textsuperscript{28}. The total energy expenditure consists of\textsuperscript{29,30}:

a) Resting energy expenditure (energy used for the normal functioning of cells and organs in the post-absorptive state and at rest).

b) Thermic effect of food (increase in energy expenditure associated with the digestion, absorption and increased sympathetic nerve activity after food intake)

c) Energy consumed with physical activity (energy expenditure resulting from the voluntary and involuntary mechanical activity).

The energy intake control and total energy expenditure are under the influence of genetic and environmental factors\textsuperscript{29-32}. The perception of hunger and the decision to start a meal involves complex and poorly understood interactions between genetic, social factors, environment, mental signals, and circadian rhythms\textsuperscript{33,34}. The process is variable, and although several endogenous peptides have been identified, the explanation for the decision to start eating is not yet clear\textsuperscript{33-35}.

1.2 Effect of a high-fat diet on individuals

Dieting has now become a crucial element in the prevalence of obesity in the world. The western diet is a term used to describe a diet based on red meat, refined sugars, high-fat foods and refined grains\textsuperscript{36}. This type of diet often contains large amounts of saturated (SFA; found in baked good, cheese, and fatty meat), and trans-fatty acids (TFA), and a reduced concentration of n-3 polyunsaturated fatty acids (PUFAs)\textsuperscript{36,37}. Obesity and health problems can be side effects from long-term consumption of this diet.

The term “High Fat Diet” (HFD) refers to a diet with a high percentage of fat around 30% to 80%, while a healthy intake of fat should be less than 30 percent of calories\textsuperscript{38,39}. Dietary fat intake has been related to the increase in adiposity and for inducing obesity. According to epidemiological
studies of obesity in humans, HFD usually contains an increase in fat (35%-45%) that is generally accompanied by a reduction of carbohydrates (45%-55%), but with constant amounts of protein (15% - 20%). That is one of the reasons to suggest a positive relationship between the increase in dietary energy from fat (especially in western society) and the proportion of people with obesity.41

The association between fat and obesity is not limited to human beings. In rats, the increase of fat in the diet leads to an increase in weight, demonstrating that the amount of fat in the diet is an essential element for weight gain.42 It has been described that diets with a content higher than 30% of total energy as fat lead to an increased risk of obesity.43 Researchers have used varying percentages of fat in diets; nonetheless, this has generated some controversies between different studies. A concentration of 13% of total fat in a high-energy diet seems to be the minimum amount and 85% the maximum used.43,44 However, a diet containing animal fat at 40% of energy, with a low amount of n-3 fatty acids appear to be the best method to induce obesity in rat models.43,45

1.2.1 HFD and hormones

Energy imbalance can modify the expression of different hormones such as leptin, ghrelin and insulin.46

1.2.1.1 Leptin

Leptin is a crucial hormone in controlling food consumption and regulating body weight.47 Leptin is produced by adipose tissue, and elevated plasma leptin has been found to increase energy consumption and decrease food intake.47 Plasma leptin levels in humans reach their maximum peak at midnight and their lowest peak at midday, having a behaviour based on the circadian rhythm.48 In rats, the maximum peak of leptin is achieved in the active phase (night) and the lowest peak in the rest phase (daytime).49 A high percentage of fat in the diet is sufficient to increase plasma leptin levels when compared to animals fed a control diet, both in humans and animal models as a consequence of obesity-induced by HFD. Additionally, it is possible to identify that the animals exposed to an HFD reduced the expression of the hypothalamic leptin receptor gene, causing the increase of leptin in plasma and generating leptin resistance.50

1.2.1.2 Ghrelin

Ghrelin a peptide produced by the cells in the fundus of the stomach stimulates the pituitary to release the growth hormone.50 Ghrelin levels increase before and decrease after food intake.55 Three peaks of ghrelin have been determined in humans at 08:00 hrs, 12:00hrs-13:00hrs and
Chapter 1

17:00hrs-19:00hrs, then ghrelin concentrations reach their basal levels after food ingestion\textsuperscript{16}. On the other hand, obese people have lower fasting plasma ghrelin levels, and a lower suppressive effect of ghrelin secretion after eating compared to the levels of a non-obese person\textsuperscript{17,58}. Also, it has been reported that in an obesity status, an HFD has a milder suppressive effect on ghrelin secretion than observed in a high carbohydrate diet\textsuperscript{59}. Studies in obese animals identified that the plasma ghrelin concentrations and ghrelin gene expression are reduced, alongside an alteration of the suppression of ghrelin after food intake, never reaching a satiety stage\textsuperscript{60,61}. This suggests that fat-rich diets are not able to regulate ghrelin secretion, which may be related to the observed hyperphagia behaviour observed in animal models. Currently, the impact of HFD on ghrelin plasma levels is unclear, and further studies should be performed to clarify this potential association. Recent studies suggested that ghrelin could be a critical factor by which the gut microbiota influence the energy balance of the host, therefore altered microbiota could lead to an increase in ghrelin secretion and obesity\textsuperscript{62-64}.

1.2.1.3 Insulin

Insulin in the brain is involved in different processes such as memory, learning, synaptic plasticity and neuronal survival\textsuperscript{65}. Insulin may activate different pathways downstream of the insulin receptor, which is involved in the processes mentioned above. The first one is the IRS-PI3K-Akt pathway, which once activated stimulates the expression of insulin-sensitive glucose transporters (GLUT4). The second is the Shc-Ras-ERK pathway, which regulates the expression of genes responsible for synaptic plasticity and cell differentiation\textsuperscript{66,67}.

HFDs have been reported to affect adequate glucose tolerance and alter the action of insulin in reducing blood glucose concentration\textsuperscript{68,69}. Obese individual cause by an HFD showed insulin resistance, possibly by a reduction in insulin receptors, mainly if the diet contains a fat percentage higher than 40% of total energy. This is perhaps caused by alterations in the composition of the cell membrane, affecting the affinity of the insulin receptors\textsuperscript{68,69}. Additionally, the increase of adipose tissue generates stress in the endoplasmic reticulum, causing the production of cytokines that affect the cells' response to insulin\textsuperscript{70}. 

6
1.2.2 Oxidative stress

Oxidative stress is a state of the cell in which intracellular oxide-reduction homeostasis is altered, that is, the balance between pro-oxidants and antioxidants. This imbalance occurs due to excessive production of reactive oxygen species (ROS), reactive nitrogen species (RNS) and deficiency in antioxidant mechanisms, leading to cell damage\textsuperscript{71,72}. All cells in humans are exposed to large amounts of ROS and RNS; nevertheless, different antioxidant pathways protect cells from oxidative damage. This protection system consists of a set of enzymes such as catalase, superoxide dismutase, glutathione peroxidase, vitamins (such as A, C and E), and carotenoids\textsuperscript{73}. However, an inefficient deactivation or elimination system leads to the accumulation of ROS and RNS in the cells, damaging proteins, lipids and nucleic acids\textsuperscript{74,75}. These damages can generate protein oxidation, lipid peroxidation and glycated products leading to a degeneration of neurons\textsuperscript{74}.

HFD has been reported to induce oxidative stress, leading to deterioration in cognition performance, and reduced levels of brain-derived neurotrophic factor (BDNF)\textsuperscript{76}. BDNF modulates hippocampal plasticity and hippocampal-dependent memory\textsuperscript{77}. A diet rich in fat can generate a more severe oxidative process according to the content of fat in the diet. Rats fed a diet containing 60% calories from fat showed oxidative damage at the hippocampus level, and impaired retention after T-maze task, compared to rats fed a diet containing 41% calories from fat\textsuperscript{78}. Similarly, mice fed HFD (45% Kcals from fat) had elevated levels of superoxide, peroxynitrite, ROS, and impairment on cognition after measuring associative learning (fear conditioning test and freezing behaviour) compared to mice fed a 10% Kcals of fat\textsuperscript{79}. These studies reveal the relationship between HFD and oxidative stress and the impact they have on cognitive function.

1.2.3 Insulin resistance

McNelly and colleagues observed a direct effect of HFD on insulin after overweight rats developed insulin resistance, following exposure for 12 weeks to an HFD (45% calories from fat). Additionally, these rats showed poor performance in behavioural flexibility (delayed matching to position-DMTP/non-matching to position tasks DNMT, test for spatial memory) compared to the control group. This data demonstrated the impact of insulin resistance on behaviour\textsuperscript{80}. The insulin receptor is expressed in metabolically active brain regions such as the hippocampus, cerebral cortex, hypothalamus, and cerebellum\textsuperscript{81}.

However, subsequent studies identified that there were no changes in the hypothalamus, hippocampus, or cortex in insulin signalling-related proteins, in rats after an HFD feeding (45% of calories from fat)\textsuperscript{82}. However, insulin has been associated with memory disorders, impaired
hippocampal neurogenesis, reduced hippocampal dendritic spine density, decreased BDNF levels and decreased cognitive performance\textsuperscript{83-86}.

\subsection*{1.2.4 Inflammation}

Pro-inflammatory cytokines such as IL-1, IL-6 and TNF-\(\alpha\) are found to lead the inflammatory response in the brain, in response to a stimulus. An interesting feature of these cytokines is their ability to cross the blood-brain barrier (BBB)\textsuperscript{87-89}. Pro-inflammatory cytokines are generated in both the brain parenchyma (microglial cells and astrocytes) and the endothelial cells of the BBB. IL-1 and IL-6 receptors are localized throughout the brain, but in the hippocampus (an essential region for learning and memory) is where they are most concentrated\textsuperscript{87}. High concentrations of IL-1, for example, have a detrimental role in memory and learning as IL-1 may alter hippocampal development, as well as affect BDNF expression and neurogenesis\textsuperscript{87-89}.

Different animal models have been used to elucidate the role of HFD concerning neuroinflammation. A knockout mouse model for low-density lipoprotein receptor (LDLR) (LDLR\textsuperscript{-/-}) allowed to identify in the hippocampus of these mice, after the 8-week intake of an HF and high cholesterol diet, microglia and astrocytes activation and increased mRNA expression of TNF-\(\alpha\), IL-1-\(\beta\), and IL-6\textsuperscript{90}. Years later, Pistell et al., in middle-aged (12 months old) C57BL/6 mice fed HFD showed low performance in the T-maze and increase of pro-inflammatory cytokines (TNF-\(\alpha\), IL-6) in the cortex. However, these findings were dependent on fat percentage, since the only mice fed a diet with 60\% calories from fat showed inflammation. In contrast, those fed with 45\% calories from fat did not exhibit these effects\textsuperscript{91}.

Different studies have identified microglial activation in neuroinflammation after exposure to an HFD\textsuperscript{92-94}. Microglia cells play an essential role in the brain especially at the level of protection, exerting their function as macrophages in the brain\textsuperscript{95,96}. Cells of the microglia are mainly in the quiescent state waiting for the appropriate stimulus to be activated. Microglial cells produce both pro- or anti-inflammatory cytokines and chemokines according to the stimulus\textsuperscript{95}. If the stimulus is chronic, the activation of the microglia cells is maintained, and they release toxic free radicals, IL-10 and TGF-\(\beta\). IL-10 is essential to counteract damage derived by excessive inflammation by inhibiting the release of pro-inflammatory cytokines from microglial cells\textsuperscript{97}. TGF-\(\beta\) has an important neuroprotective role as anti-inflammatory and anti-apoptotic mediator\textsuperscript{98}. However, it is unclear if microglial activation after exposure to an HFD causes neuronal death, or if microglial activation occurs after neuronal death to remove cell debris.
In HFD rodent models, the basal gene expression of pro-inflammatory markers (IL-1β, IL-6, or TNFα) appear to be similar to the control diet group, however, the HFD group gave a more sensitive response after being exposed to an immune challenge (intraperitoneal LPS injections)\(^{99,100}\). Moreover, the basal expression of different proteins (Iba1, GFAP, IL-6, TNFα, and MCP-1) in the whole brain could be elevated after changing the fat content in diet from 41% to 60% of calories or reduced BDNF protein expression\(^{91,99,100}\). BDNF is a neurotrophin that plays an essential role in the growth, maintenance, and survival of many types of neurons\(^{101}\). The consumption of HFD decreases the level of BDNF in the hippocampus of rats, which has a significant impact on hippocampal synaptic plasticity and neurogenesis\(^{102}\).

### 1.2.5 HFD and Cognition

Cognitive functions such as attention, working memory, verbal fluency, and memory seem to be affected by HFD consumption\(^{103}\). The definition of dementia is based primarily on the progressive deterioration of two or more cognitive function. The diagnosis is not a simple task, and it requires a detailed and repetitive analysis of an individual’s ability to perform specific tasks, daily activities. It evaluates changes in behaviour and personality\(^{103}\). In recent years, researchers have focused on understanding the effect of nutrition and its impact on brain functions. Each year the number of cases of obesity, dementia and diabetes have increased, which has suggested the possible association that obesity generated by the consumption of HFD increases the risk of developing dementia\(^{104,105}\).

Different studies have linked High saturated fat diets with impairment of cognitive functions. Some of them described how SFA intake in a middle-aged population (age 45 to 70) affects cognitive flexibility, memory and processing speed\(^{106}\). A similar finding showed that a six-year consumption of SFA impaired cognition affecting memory, processing speed and attention\(^{107}\). In a study where healthy men aged 22±1 year old (mean ± SE) were administered an HFD (70% fat) for five days, showed that attention and processing speed were reduced when compared to men fed a standard diet (24% fat). Additionally, HFD has been associated with attention-deficit/hyperactivity disorder (ADHD) in adolescents compared to a healthy diet pattern\(^{108}\). In humans, it has been determined an impairment performance on a cognitive task is associated with the consumption of HFD, mainly SFAs and omega-6. In contrast, low fat and omega-3 rich diet appears to play a protective role against cognitive impairment\(^{107,109,110}\).
SFAs have been linked not only with cognitive impairment but also with mood alterations in humans\textsuperscript{111}. A study in Australian women showed that a typical western diet increases the incidence of depression and anxiety, compared to those consuming a balanced diet\textsuperscript{112}. A similar study in adolescents (10-14 years) showed an association with depression symptoms and an HFD, compared to those eating a healthy diet\textsuperscript{113}. Changes in mood are not only caused by an increase of SFAs in the diet, but also by an omega-3 deficiency. Different studies have shown a negative correlation between the amount of omega-3 in serum and major depression symptoms\textsuperscript{114-116}. Omega-3 fatty acids consist of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and α-linoleic acid (ALA), among others. DHA is essential to support membrane integrity and functionality in neurons\textsuperscript{117}, which might be associated with a protected role of omega-3 in cognitive impairment and depression.

Not only are diets high in saturated fat associated with poorer memory performance or mood, but also a risk factor for the development of neurodegenerative diseases. Several studies in humans have associated HFD as a relevant factor involved in the development of these diseases. Eskelinen et al. described an association between a high SFA intake in midlife and mild cognitive impairment \textsuperscript{118}. Similar findings were observed in high SFA intake and an increased risk of dementia, particularly Alzheimer’s disease\textsuperscript{106,119-121}. This type of HFD has also been proposed to play a role in the development of Parkinson’s disease\textsuperscript{122-124}. These data suggest that diet is a factor that contributes to the development of neurodegenerative diseases.

In rodent models, HFD has been used in order to study the effects of obesity on animal cognition\textsuperscript{125-128}. Different studies have illustrated an impairment of spatial memory performance after executing behaviour tests such as the Morris water maze or novel object-place recognition, showing the impact of HFD on hippocampal-dependent behaviour tasks\textsuperscript{91,99,129,130}. For instance, an HFD short-term exposure in male Sprague-Dawley rats seems to be enough to impair hippocampal-dependent place recognition memory but not the novel object recognition test\textsuperscript{131}. Also, a detrimental effect on hippocampal-dependent memory was observed in male Wistar rats after 1-2 months of exposure to HFD, when tested using the Morris water maze task, presenting an impairment in long-term spatial reference memory\textsuperscript{99}. However, no Morris water maze deficits after 5 and 10 months on HFD were detected in male C57BL/6 mice, indicating no alteration on hippocampus after this extensive exposure to HFD\textsuperscript{129}.

Data collected on mice fed with 60% of calories of fat showed that these animals made more errors performing the T-maze test compared to the mice fed with a 41 % diet, suggesting that percentage of calories from fat plays an essential role on spatial ability and cognition\textsuperscript{91}. More understanding of the mechanisms underlying the effect of HFD on cognitive and learning is needed. Different factors
should be taken into consideration, such as HFD exposure time, model species (rat or mouse), strain, and percentage of calories from fat, among others.

### 1.3 MATERNAL OBESITY

Low social conditions increase the risk of changes in nutritional status, health, growth and development\(^\text{132}\). Particularly in the diet of pregnant women, alterations are noted, with micronutrient deficiencies and excesses of macronutrients (high in saturated fats) that result in obesity and other associated diseases\(^\text{133}\). Numerous studies have shown that obstetric pathologies and delivery complications (gestational diabetes, hypertension and preeclampsia, infections, postpartum haemorrhage, fetal macrosomia, shoulder dystocia, fetal-neonatal death, increased rate of caesarean and anaesthesia complications) are strongly linked to excessive maternal weight before pregnancy or its disproportionate increase over the same\(^\text{134-136}\).

Obesity during pregnancy is a problem not only for the immediate adverse effect on maternal health but on the developing fetus. A summary of the changes observed in some animal models of maternal HFD on the offspring are summarized in Table 1.1. The offspring of obese mothers have an increased incidence of stillbirths, neural tube defects, heart defects and abnormal growth\(^\text{137,138}\). Also, children of obese mothers before or during pregnancy have a high risk of long-term obesity, Attention-Deficit/Hyperactivity Disorder (ADHD), Type 2 diabetes mellitus, among others, when compared with children born from mothers with normal weight\(^\text{139-141}\).
<table>
<thead>
<tr>
<th>Animal model</th>
<th>Time of exposure to maternal HFD</th>
<th>Diet-induced maternal obesity</th>
<th>Fat composition</th>
<th>Consequences in the HFD offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>8 weeks prior to mating and during gestation</td>
<td>Yes</td>
<td>60% of calories from fat</td>
<td>Type 2 diabetes in F1 and higher β-cell mass in F1, Gniuli et al. 2008</td>
</tr>
<tr>
<td></td>
<td>2 weeks before mating and during gestation and lactation</td>
<td>Yes</td>
<td>36% of calories from fat</td>
<td>Decreased fetal growth and birth weight, Hartil et al. 2009.</td>
</tr>
<tr>
<td></td>
<td>8 weeks prior to mating, and during gestation and lactation</td>
<td>Yes</td>
<td>32% of calories from fat</td>
<td>Increase in fetal growth, transplacental transport of glucose and neutral amino acids, and placental GLUT1 and SNAT2 mRNA, Jones et al. 2009.</td>
</tr>
<tr>
<td></td>
<td>4 weeks before mating and during gestation and lactation</td>
<td>Yes</td>
<td>45% of calories from fat</td>
<td>Reduced mitochondrial function, increased hepatic lipogenesis, oxidative stress and inflammatory markers, Bruce et al. 2009.</td>
</tr>
<tr>
<td></td>
<td>4 weeks before mating and during gestation and lactation</td>
<td>Yes</td>
<td>45% of calories from fat</td>
<td>Increased body length, reduced insulin sensitivity and altered growth hormone GH axis, Dunn et al. 2009.</td>
</tr>
<tr>
<td></td>
<td>During gestation or lactation, and gestation and lactation</td>
<td>No</td>
<td>49% of calories from fat</td>
<td>Insulin resistance, lower Glut-2 expression, and hepatic steatosis, Gregorio et al. 2010.</td>
</tr>
<tr>
<td></td>
<td>12 weeks before mating and during gestation and lactation</td>
<td>Yes</td>
<td>60% of calories from fat</td>
<td>DNA hypomethylation in different regions of the brain, Vucetic et al. 2010.</td>
</tr>
<tr>
<td></td>
<td>6 weeks before mating and during gestation and lactation</td>
<td>Yes</td>
<td>32% of calories from fat</td>
<td>Increased lipid peroxidation, and decreased BDNF in the hippocampus, Impaired acquisition of spatial learning in the young offspring, Tozuka et al. 2010.</td>
</tr>
<tr>
<td></td>
<td>4, 12 or 23 weeks prior to mating</td>
<td>Yes</td>
<td>60% of calories from fat</td>
<td>Increased neonatal adiposity and reduced liver size at birth, Krasnow et al. 2011.</td>
</tr>
<tr>
<td></td>
<td>During gestation and lactation</td>
<td>No</td>
<td>49% of calories from fat</td>
<td>Increase visceral fat, liver steatosis and insulin resistance, Volpato et al. 2012.</td>
</tr>
<tr>
<td></td>
<td>1 week prior to mating, and during gestation and lactation</td>
<td>No</td>
<td>35% of calories from fat</td>
<td>Metabolic syndrome, liver lipid accumulation and activation of c-Jun N-terminal kinases (JNK) consistent with the development of non-alcoholic fatty liver disease, Ashino et al. 2012.</td>
</tr>
<tr>
<td></td>
<td>3 weeks before mating and during gestation and lactation</td>
<td>Yes</td>
<td>60% of calories from fat</td>
<td>Increased anxiety-like behaviours, increased BDNF, GABAA receptor alpha2 subunit and 5-HT1A receptor mRNA levels in the hippocampus, Peleg-Raibstein et al. 2012.</td>
</tr>
<tr>
<td></td>
<td>6 weeks before mating and during gestation</td>
<td>Yes</td>
<td>58% of calories from fat</td>
<td>Delayed impairments to anxiety-like behaviours and stress coping strategies, increased mRNA levels of the glucocorticoid receptor (Nr3c1) in the hypothalamus, Balsevich et al. 2016.</td>
</tr>
<tr>
<td>Time Period</td>
<td>Maternal HFD</td>
<td>Offspring Effect</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>--------------</td>
<td>----------------------------------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 weeks before mating and during gestation and lactation</td>
<td>Yes</td>
<td>Increased body weight, serum leptin and insulin, and reduced hypothalamic leptin-dependent STAT-3 phosphorylation&lt;sup&gt;155&lt;/sup&gt;. Ferezou-Viala et al 2007.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>From embryonic day 6 to postnatal day 15</td>
<td>No</td>
<td>Increased expression of orexigenic peptides, and increased proliferation of neuroepithelial and neuronal precursor cells&lt;sup&gt;156&lt;/sup&gt;. Chang et al 2008.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 weeks before mating and during gestation</td>
<td>Yes</td>
<td>Increased serum leptin and insulin, and regulation of levels of appetite-regulating neuropeptides gene&lt;sup&gt;157&lt;/sup&gt;. Gupta et al 2009.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 weeks before mating and during gestation and lactation</td>
<td>Yes</td>
<td>Increased oxidative and inflammatory signalling in the brain, and cognitive decline&lt;sup&gt;158&lt;/sup&gt;. White et al 2009.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 weeks before mating and during gestation and lactation</td>
<td>Yes</td>
<td>Increased body weight, microglial activation markers were increased in the hippocampus at birth, impairment in anxiety and spatial learning&lt;sup&gt;159&lt;/sup&gt;. Bilbo et al 2010.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>During gestation</td>
<td>No</td>
<td>Increased birth weight, over-production of glucose and histone modification in the liver&lt;sup&gt;160&lt;/sup&gt;. Strakovsky et al 2011.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>From embryonic day 2 to lactation</td>
<td>No</td>
<td>Increased body weight, adiposity, plasma leptin concentration, impaired glucose tolerance, and reduced phosphorylated STAT&lt;sup&gt;160&lt;/sup&gt;. Sun et al 2012.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 weeks before mating and during gestation and lactation</td>
<td>Yes</td>
<td>Impaired reversal learning and altered leptin signalling&lt;sup&gt;161&lt;/sup&gt;. Wu et al 2013.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 weeks before mating and during gestation and lactation</td>
<td>Yes</td>
<td>Increased body weight, impaired spatial learning&lt;sup&gt;163&lt;/sup&gt;. Moser et al 2017.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 weeks before mating and during gestation and lactation</td>
<td>Yes</td>
<td>Sexual dimorphism of the hippocampal-dependent function&lt;sup&gt;164&lt;/sup&gt;. Robb et al 2017.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>From embryonic day 2 to lactation</td>
<td>No</td>
<td>Decreased expression of Insr, Lepr, and GLUT1. Impaired object recognition memory and impaired spatial memory&lt;sup&gt;165&lt;/sup&gt;. Cordner et al 2019.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.1 Effect of maternal HFD on the offspring in rodent models.**
1.3.1 Maternal obesity and immune response

Analysis of obese mothers has identified lipid accumulation in the placenta, which generates an increased expression of pro-inflammatory cytokines such as IL-1, IL-6, and TNFα\textsuperscript{166,167}. The placenta in obese women, presents structural changes, mainly by changing the thickness and the surface of the placenta, and chronic activation of endothelial cells\textsuperscript{168}. These changes are able to generate a reduction in the placental blood flow, activating different pro-inflammatory cytokines such as Granulocyte-Macrophage-Colony-Stimulating factor (GM-CSF), IL-2, IL-4, IL17, Monocyte Chemoattractant Protein-1 (MCP-1), and TNFα\textsuperscript{169}. Placental inflammation associated with gestational diabetes has been a risk factor for delivering babies that are large-for-gestational-age and affects the nutrient transport\textsuperscript{170,171}.

Obesity has a degree of mild inflammation, caused by an accumulation of macrophages in adipose tissue\textsuperscript{172-175}. These macrophages express the cluster of differentiation (CD) 14 and/or CD86 and correlate with increased BMI\textsuperscript{173}. The high number of resident macrophages in adipose tissue secretes pro-inflammatory markers such as interleukin 6 (IL-6), c-reactive protein (CRP), and TNFα, reaching circulation and generating a state of mild chronic inflammation\textsuperscript{172-175}. An estimated number of 20 to 30 million macrophages are accumulated per kilogram of fat excess in humans, disturbing the normal functioning of insulin and leptin signalling, affecting the lipid metabolism and encouraging the storage of these\textsuperscript{176}. It has been observed that this degree of chronic inflammation has a negative effect on various organs such as the brain\textsuperscript{177-179}.

Additionally, different studies have reported that different pathways may be involved in placental inflammation, candidate routes are p38 mitogen-activated protein kinase (p38-MAPK), signal transducer-activated transcription factor (STAT3), nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB) and c-Jun-N-terminal kinase (JNK)\textsuperscript{180,181}. The study of obese pregnant women to assess whether maternal BMI is associated with elevated pro-inflammatory cytokines and activation of placental inflammatory pathways, indicated that maternal BMI activates the placental p38-MAPK and STAT3 inflammatory pathways\textsuperscript{180}. Cytokines involved in p38-MAPK activation are TNFα and MCP-1, and STAT3 activation involved TNFα and IL-6\textsuperscript{180,182}.

Another possible mechanism for the development of placental inflammation is the Toll-like receptor 2 and 4 (TLR2, TLR4) pathway, which can be activated by an excess of fatty acids due to increased adiposity. TLRs are activated to detect pathogens or foreign substance, such as lipopolysaccharides (LPS) of Gram-negative bacteria\textsuperscript{176}. Activation of TLR-4 pathway appears to be generated by the fatty acids, they act similarly to LPS, and may additionally activate the NF-κB and JNK pathways, generating placental inflammation, together with increased pro-inflammatory
cytokines such as TNFα, IL-6, IL-8. Over-activation of TLR, leads to the accumulation of ROS and inflammation due to an increase of misfolded proteins in the endoplasmic reticulum, increasing stress, which leads to cell damage. Placental inflammation also can alter the cytokine levels in the fetus, affecting the development of the fetal immune system, with health consequences later in life (allergies).

1.3.2 Maternal obesity and Neuroinflammation

The BBB has a specialized microvascular endothelium, which limits the entry of different blood components from the peripheral circulation to CNS. However, different studies have demonstrated that high-energy diets influence the integrity of the BBB as pro-inflammatory markers are able to increase the permeability of the BBB since IL-1β decrease the expression of the tight junction proteins. Obesity caused by an HFD induces downregulation of different proteins at the BBB, such as transport, cell metabolism, cytoskeletal, and scaffolding adaptor proteins. Cytoskeletal proteins are essential for an adequate formation and function of the tight junction proteins. Vimentin and tubulin are proteins downregulated at the BBB in obese mice models. Vimentin controls the tight junction alignment and the transport of low-density lipoprotein-derived cholesterol, so downregulation of vimentin in obesity might affect the membrane fluidity inhibiting the alignment of the tight junction proteins. Tubulin plays a critical role in tight junction complex functions and BBB transport, so downregulation of this protein might cause impairment of the transport function in BBB. Another key player at the BBB permeability is the vascular endothelial growth factor (VEGF), but long term exposure to HFD increases VEGF expressing, resulting in an alteration of the integrity of the BBB.

The immune system in the CNS plays an essential role in the proper development of the brain. In the early stages of development, activation of the immune system could alter neuronal function and impair processes such as memory and learning. The possible mechanisms that explain this phenomenon are that early activation of the immune response alters the normal development of neural pathways associated with memory and learning, or that an early activation can re-program the immune response in the adult, exacerbating inflammation and releasing a large number of pro-inflammatory cytokines, which would indirectly affect essential neural processes for cognition.

Neuroinflammation, due to this inflammatory response can alter the normal physiological processes in the CNS, for example, neurons in this kind of environment can experience excitotoxicity and neurodegeneration. Neuroinflammation can affect different regions such as
the hippocampus and prefrontal cortex, causing gliosis and neuronal damageren. Glial fibrillary acidic protein (GFAP) a marker for astrocyte, is the main intermediate filament protein expressed in mature astrocytes and is involved in cell motility, proliferation, vesicle trafficking, and controlling neuronal outgrowth183. Ionized calcium-binding adaptor molecule 1 (Iba1) is frequently used as a marker for microgliosis and is involved in the remodelling of the actin cytoskeleton that allows for motility, proliferation, and phagocytosis in activated microglia192. Chronic activation of microglia and astrocytes causes abnormal synaptic remodelling as microglia acts in synaptic pruning, and disruption of neuronal networks through the formation of compact glial scar tissues, respectively193,194.

Bilbo et al. showed that maternal obesity- caused by the consumption of HFD was shown to stimulate microglia (increase in cell number and CD11b expression), and increased expression of TLR4 mRNA in the hippocampus of the offspring at the time of birth126. On the other hand, the offspring were weaned onto a control diet (P21). Then in adulthood (P85-95), these rats presented anxiety-like behaviour, based on results obtained in the elevated plus-maze test, where the offspring from HFD fed dams spent more time in closed arms compared to the open arms126. These observations suggest that exposure to maternal obesity caused by an HFD can affect or program the behavioural outcomes in the offspring early in life.

A study in mice described how maternal obesity caused by an HFD could contribute to hyperactivity in females and males, generating a phenotype similar to ADHD males195. A similar association was observed between maternal adiposity before pregnancy and children with ADHD symptoms196. Additionally, in the group of females from HFD obese dams, these mice had elevated levels of inflammation markers in the brain, such as IL-1β and TNF-α, an increased number of microglia cells, exhibiting alterations in behaviour like increased anxiety (open field test) and decrease of sociability (3-chamber social test). However, no modifications or alterations in behaviour and/or inflammation was identified in males195. These data suggest that adult behavioural alteration may occur after an insult during pregnancy and that early developmental mechanisms were altered, generating these phenotypes in adulthood.

Animal models exposed to an HFD, have allowed assessing changes in behaviour such as anxiety. One of the most commonly used methods is the elevated plus maze (EPM), in which anxiety is measured by the time spent in the open arms of the maze (indicative of less anxiety), in contrast to the time spent in the closed arms of the maze (indicative of an anxiety-like behaviour). When comparing two groups of C57BL/6L mice fed before and during pregnancy, with a 60% HFD, or a diet with a normal fat percentage, it was possible to note that maternal consumption of an HFD was sufficient to produce an anxiety-like behaviour in the offspring in comparison to the control.
Maternal HFD nearly doubled the expression of BDNF in the offspring dorsal hippocampus and increased GABAergic and serotonergic receptor expression in the ventral hippocampus.

Bilbo & Tsang (2010) found that maternal HFD increased serum levels of pro-inflammatory cytokines such as IL-6 and microglial activation in the hippocampus in the offspring. Those mice showed an increase in anxiety after the performance in the EPM. In contrast, a different study using Wistar rats did not find differences when anxiety was evaluated in the EPM. However, the HFD offspring (from obese dams) exposed only during gestation, showed reduced anxiety when scored in an open field test. When spatial memory retention was evaluated by Tozuka et al. (2010), no differences were found. However, the maternal HFD offspring exhibited impaired hippocampal BDNF production and dendritic arborisation of new hippocampal neurons.

Previously, White et al. (2009) reported that maternal obesity-induced by HFD was sufficient to impair memory retention in male offspring, even more, if the HFD diet was consistent during the lifespan, as well as inflammation (IL-6) and microglial activation in the hippocampus.

### 1.3.3 Maternal obesity and metabolic disorders

Maternal obesity caused by exposure to HFD has been described as a determinant factor for the development of metabolic disorders in the offspring, including dyslipidaemia, hypertension, chronic liver diseases and cardiovascular disease. It was observed that maternal exposure to a high-saturated fatty acids diet before and after conception, the offspring presented alterations in the vascular system. Similarly, the evaluation of plasma in the offspring of obese mothers fed with HFD increases the concentration of triglycerides and cholesterol in young and adult offspring. Therefore, this increase in the plasma levels of triglycerides and cholesterol could alter the hepatic lipid metabolism and might generate problems in the cardiovascular system.

The liver is one of the organs that could be affected in the offspring by maternal exposure to HFD. mRNA levels of some genes associated with hepatic lipid metabolism peroxisome proliferator-activated receptor-α (ppar-α) and carnitine palmitoyltransferase-1a (cpt-1a) may change by exposure to maternal HFD. Also, maternal HFD before conception altered not only the mRNA levels of these metabolic genes (ppar-α and cpt-1a) but also the levels of metabolic-related microRNAs in the liver. In the same way, it has been suggested that in the offspring of obese mothers fed with HFD, there is a reduction in the body's cardio-protective and anti-inflammatory functions.
On the other hand, studies in the mouse model have shown that offspring exposed during pregnancy and lactation to maternal HFD (from obese dams) developed metabolic syndrome. A metabolic syndrome is a group of conditions that put the individual at risk for developing heart disease and type 2 diabetes. Ashino et al., observed in those mice signs of metabolic syndrome such as increased liver mass, hepatic lipid content, insulin resistance, reduced plasma levels of triglyceride, hepatic steatosis, among others. These changes were accompanied by increased plasma levels of TNFα and IL-1β. Elahi et al., found a similar phenotype since the adult offspring from obese dams showed hypercholesterolaemia, elevated c-reactive protein levels (in females), hypertension and fatty liver. This indicates that maternal HFD could alter different organs that lead to metabolic alterations in the offspring.

In rats, like what was observed in mice, the offspring born to obese dams fed an HFD showed greater susceptibility to the development of metabolic syndrome. The phenotype found in these animals indicated an increase in liver weight, elevated glucose, triglycerides and corticosterone plasma levels, increased lipids in the liver and increased left ventricular thickness. After weaning, rats were exposed to an HF/High sucrose diet; the offspring exhibited increased body weight, increased insulin, glucose, and triglycerides plasma levels. Therefore, after exposure to a maternal HFD, the offspring may present poorer metabolic profile, if they are exposed with an adverse diet for a second time.

1.4 Developmental Origins of Health and Disease (DOHaD)

For a long time, it was considered that the fetus in utero was free from harm by external agents. In the mid-1930s, the study of death rates in England and Sweden, allowed to identify that environmental conditions during fetal life and childhood appeared to be determining the survival of each generation. In 1940, it was discovered that the rubella virus during pregnancy produced birth defects in the new-born.

In the 1960s, Neel launched the "thrifty genotype hypothesis" proposing that specific populations had a higher propensity to insulin resistance, either by selection or by genetic factors. According to this author, a random mutation that leads to insulin resistance could be adaptive and beneficial for individuals exposed to environments with food shortages, resulting in the natural selection of these individuals, with subsequent transmission of characteristics to future generations. In the same decade the birth of children born with phocomelia (malformation of the limbs) from mothers who had consumed thalidomide in the pregnancy, suggested that not only diseases in the mother affect
the fetus but also the administration of medicines\textsuperscript{208}. The most significant discovery in the last 15 years within teratology was to know that deficiency in maternal nutrition induced an increased risk of malformations in the fetus, such as folic acid deficiency and neural tube defects (anencephaly, spina bifida); moreover, the consumption of this vitamin before and during pregnancy prevented up to 85% of the defects\textsuperscript{209,210}.

In the 1970s, the offspring of women exposed to a period of food shortages (Dutch famine) during pregnancy in World War II were analysed. In adulthood, these individuals showed different patterns of body composition depending on the time in which mothers and offspring had been exposed to maternal malnutrition during fetal life. If the mother had suffered malnutrition during the last trimester of pregnancy, this group had a lower incidence of obesity than control counterparts without malnutrition. However, if this occurred in the first half of pregnancy, the incidence of obesity increased significantly in the offspring than control counterparts without malnutrition\textsuperscript{211}. These findings showed that nutrition during pregnancy is essential for normal embryonic/fetal development.

In recent decades, various research areas have suggested that the events involved in normal fetal development have long-term effects and influence health in adulthood. Currently, new factors that interact with the expression of genes in utero and establish physiological and structural patterns related to the survival of the individual are known. Some of these affects not only the individual but also affect future generations\textsuperscript{76,137,212,213}. It is thought that the stimulus or aggression in the early stages of life originates permanent consequences; this is called programming in the field of developmental biology. The stimulus may be caused by endogenous factors (hormonal signalling) or exogenous (environment)\textsuperscript{214}. During fetal life and early childhood, nutrition can affect metabolism, growth, neurodevelopmental and pathological processes (hypertension, diabetes, atherosclerosis and obesity) permanently\textsuperscript{137,215}.

1.4.1 Barker Hypothesis.

Barker and his group discovered that the small birth size, according to gestational age, is associated with increased risk of adverse events in adult life (abnormal levels of blood lipids, diabetes mellitus, hypertension and death from ischemic heart disease)\textsuperscript{138,216,217}. Intrauterine growth restriction has been identified as a marker of poor fetal nutrition, which adapts and programs the fetus and the individual to be susceptible to diseases in adulthood\textsuperscript{139}. Barker et al., in the 1980s, established that
the incidence of some adult diseases, such as stroke, Type 2 diabetes and dyslipidaemia, were related to the intrauterine environment during development (Barker hypothesis)\textsuperscript{138,139,216,217}.

Currently, this hypothesis is known as developmental origins of health and disease (DOHaD)\textsuperscript{218}. Introduction of different phenotypes by environmental variations in early stages of life, including nutrition, is associated with varying degrees of metabolic disease. Exposure to different environmental factors in early life can induce persistent changes in the fetus leading to altered epigenetic profiles, modifying the function and structure of different tissues\textsuperscript{219}. Several epidemiological studies in humans and animal models indicate that during critical periods of pre- and postnatal development, various environmental stimuli influence developmental pathways, which induce permanent changes in metabolism and susceptibility to chronic diseases. Therefore, the Barker hypothesis states that specific structures and functions of the organs perform programming during embryonic and fetal life that determines the regulation of physiological and metabolic responses in adulthood\textsuperscript{138,139,216,217}. The short-term effect of programming allows fetal survival, while long-term effect predisposes to diseases in adulthood\textsuperscript{220-222}. The alteration in nutrient availability during pregnancy results in adaptation in fetal development. This adaptation is made through hormonal adjustments by the embryo and fetus to re-establish benchmarks so that the new-born will be better adapted for an adverse environment (malnutrition)\textsuperscript{220-222}.

1.4.2 Preimplantation Period.

Embryonic development in mouse begins with fertilization, which involves the entry of a single sperm cell through the membrane of the oocyte. Once fertilization occurs, a series of mitotic divisions known as cleavages are triggered. Divisions occur sequentially and form 2- (Embryonic day 1.5), 4- (Embryonic day 2), 8-cell embryo (Embryonic day 2.5) and so on\textsuperscript{223}. At the stage of 8-cell embryo, cells develop an apicobasal polarity. This process is called compaction, where the morula (aggregate of spherical cells) forms on embryonic day 3. During the passage from 16 to 32 cells, the cells of the outer layer of the morula mature forming tight junctions, which then pump liquid from the outside of the embryo to form an internal cavity in a process called cavitation. In this stage, the embryo is called a blastocyst (around embryonic day 3.2). In the blastocyst stage, it is possible to observe two defined cell layers: an inner layer called inner cell mass, whose cells are pluripotent. The second layer is an outer layer made up of epithelial cells forming the trophectoderm\textsuperscript{224} (Figure 1.2).
Figure 1.2 Schematic representation of the periconceptional period.
From oocyte maturation to blastocyst formation and formation of embryonic and extraembryonic cell lineages later gestation. It was taken from Sun et al., 2016225.
The mature blastocyst continues to increase in size, pumping fluid into the cavity, and cell division continues, until contacting the uterus. Once the contact is done, the implantation phase begins. At this point, around the embryonic day 4, in the inner cell mass two cell lineages have been generated, the primitive endoderm that borders the cavity, and epiblast (pluripotent cells that will form the embryo) that is surrounded by the trophectoderm\textsuperscript{223,224}.

Different studies have shown how nutritional changes during embryonic development lead to an increased risk of developing long-term health problems\textsuperscript{200,222,226}. However, maternal nutritional changes have been carried out during gestation and some until weaning, and the impact of maternal nutritional changes on different specific periods of utero development is unclear\textsuperscript{226,227}. However, it seems that the earliest stages during embryonic development are the most vulnerable to changes or alterations, with the pre-implantation period being one of the most vulnerable due to extensive re-modelling of epigenetic programming, additionally few cells at this point will give rise to the entire organism so impact at this time will affect entire organism\textsuperscript{226,227}. This epigenetic programming leads to control of critical processes during development. First, to regulate the expression of genes involved in cellular differentiation in a stage-specific manner. Second to maintain the monoallelic gene expression in specific loci\textsuperscript{228,229}. All these epigenetic modifications occur via several mechanisms including DNA methylation, histone protein posttranslational modifications, chromatin remodelling, noncoding RNA regulation, and RNA editing\textsuperscript{230}.

In rats fed a low protein diet during the pre-implantation period (from conception until embryonic day 3.5). Then on a normal diet, various changes in offspring were observed, even in adulthood. The observed changes included low birth weight, hypertension, cardiovascular diseases, abnormal size of kidney and liver to the body weight\textsuperscript{231}. In mice exposed to similar maternal conditions, an increase in systolic pressure in adults, cardiovascular abnormality, a deficit in short-term and long-term memory, changes in cortical thickness in the brain were identified\textsuperscript{232-234}. These data demonstrate how maternal nutritional changes in the pre-implantation stage induce health problems in adulthood, which is in line with the DOHaD field.

A previous student in our group (Dr Francesca Lock) showed that maternal HFD (same HFD and same mouse strain used in this research) during the preimplantation period is enough to reduce the number of embryos and blastocysts recovered after uterine flushing (unpublish data). This data suggested that maternal HFD during the preimplantation period potentially delayed embryo development. In order to understand the changes generated by the HFD during the preimplantation period in the number of embryos and blastocysts, Dr Francesca Lock analysed the maternal uterine environment. In this analysis, it was identified that the total concentration of proteins in the uterine fluid did not change between mothers fed an HFD and the control mothers. However, the
concentration of the FGF2 protein (involved in angiogenesis) was increased in the uterine fluid in mothers fed an HFD, which could impact the development of the blastocyst (This data is not shown here due to copyright restrictions). Additionally, Dr Francesca Lock showed that in the uterus in the HFD dams there was a higher accumulation of lipids in the cells, which could suggest changes in the lipid signalling in these cells (This data is not shown here due to copyright restrictions). These results indicate that the preimplantation period is enough to modify the uterine environment in mothers exposed to an HFD.

On the other hand, it has been previously shown in our research group that mothers fed an HFD during the preimplantation period do not show significant differences in body weight compared to mothers fed a standard diet (unpublished data from Dr Francesca Lock, Figure 1.3). However, a reduction in the consumption of HFD was observed compared to the dams fed a standard diet during the pre-implantation period (unpublish data from Dr Francesca Lock). When analysing the different components of the diet, it was observed that the dams fed with the HFD consumed more fat, fewer carbohydrates and the same amount of protein as the dams fed with a standard diet. This data is not shown here due to copyright restrictions (unpublish data from Dr Francesca Lock).

This anorexia period may be a critical point to consider associating the impact of the preimplantation period and the impact on the offspring. This data is supporting the idea that 3.5 days exposure to an HFD was enough to affect the embryo. However, very few studies have focused on the study of maternal HFD during the pre-implantation period and its effect on development and cellular composition in different regions of the brain. In the following chapters, the relationship between HFD and the brain will be discussed in more detail.

Figure 1.3. Dam weights during the preimplantation period.
1.5 Brain Structure and Composition

This section will discuss the structure and cellular organisation of the cortex and hippocampus regions of the adult offspring brain (Figure 1.4). The other regions of the brain will not be described.

![Figure 1.4 Sagittal section of the mouse adult brain.](image)

The top lines indicate the stereotaxic coordinates of individual coronal sections with respect to a sagittal section of the adult mouse passing through the bregma. The regions that are going to be analysed in this thesis (cortex and hippocampus) are indicated in red. Lateral ventricle LV. It was adapted from Paxinos 2004.

1.5.1 The development process of the neocortex

The development process of the neocortex in mammals compared to different brain structures is widely described. In rodents, neurons are generated from embryonic day 12 (E12) to E18. Neurons are cells within the nervous system that receive signals and transmit information to other neurons, nerves cells and muscles. The next cells that populate the brain are astrocytes and appear around E18, with their highest peak around postnatal day 2 (P2). Astrocytes are the most common glial cell, and their functions are critical for the development, functioning and survival of neurons, maintenance of the BBB, and transport of nutrient. After astrocytes, the next glia cells to appear are oligodendrocytes (postnatally), once the astrocytic genesis decreases. Oligodendrocytes are responsible for forming the myelin that covers the neuronal axons in the central nervous system (Figure 1.5).
Figure 1.5 Timeline of microglia invasion, neurogenesis and gliogenesis.
Each triangle indicates the onset and peaks of the different developmental processes in the developing mouse brain (a) and human brain (b). P0 indicates the time of birth. E: embryonic; P: postnatal; gw: gestational week. It was adapted from Reemst et al. 2016 and Menassa et al. 2018.

1.5.1.1 Neurogenesis process
Neural progenitor cells in the ventricular zone can generate neurons or self-renew, exhibit interkinetic nuclear migration (INM), in which their nuclei migrate in the ventricular zone from the apical surface to the basal part in synchrony with the cell cycle (Figure 1.6). Neural progenitor cell will give rise to radial glial cells (RGCs). RGCs have two long radial oriented processes that confer apical-basal polarity: RG cells apically contact the ventricle and basally the meninges, the basal lamina and blood vessels. Around day E9 and E11 in the mouse brain, RGCs divide symmetrically into two identical RGCs. One of these cells will be in senescence status to keep the number of RG constant, and the other cell will divide asymmetrically. This asymmetric division generates a new cell named neuronal intermediate progenitor cell (nIPC).
Figure 1.6 Schematic illustration of interkinetic nuclear migration. Neural progenitor cells have an oscillatory movement associated with their cell cycle phase alongside the apical-basal axis. •Gap1 G1, •Synthesis S, •Gap2 G2, •Mitosis M. The image was taken from Yoichi Kosodo et al., 2011.242

The nIPCs divide symmetrically, enhancing the number of nIPC and contributing to the expansion of the cortex. The new neurons generated from the nIPCs migrate outside the proliferative zone, using the processes of the RG cells as a guide for migration.245,246 These new cells settle progressively in the most superficial layers following an inside-out model that leads to the formation of the six different layers of the neocortex. The deepest cortical layers (6 and 5) originate from the ventricular zone (VZ) after the asymmetric division of the RGCs. In contrast, the upper ones (4, and 2/3) originate from the symmetric division of the nIPCs of the subventricular zone (SVZ)247 (Figure 1.7).

The proper formation of the different layers of the neocortex is crucial for the generation and establishment of neural circuits and the proper functioning of the cerebral cortex. Therefore, cortical neurogenesis is a complex process that needs to be rigorously regulated, indicating that alterations during the embryonic development would affect the structure or thickness of the different layers. Although neurogenesis in the brain occurs primarily during early embryonic and postnatal development, it continues into adulthood in the SVZ where NSCs produce neurons that migrate to the olfactory bulb through the rostral migratory stream in mice248.
Figure 1.7 Schematic illustration of neurogenesis in the mouse neocortex.

Neuroepithelial cells (NP) divide symmetrically undergoing interkinetic nuclear migration (IKNM) to produce radial glial cells (RGCs). The RGCs are aligned to the ventricular area from where they extend a long radial process until reaching the basal surface. The RGCs are divided asymmetrically generating other RGCs and new neurons. The first neurons migrate radially from the ventricular zone (VZ) and settle inside the anterior plate (PP) to form the nascent cortical plate (CP), which will then become layers (L) 2 to 6 of the neocortex. Subsequently migrating neurons divide the PP into the marginal zone (MZ) and the subplate (SP). As neurogenesis proceeds, several subtypes of neurons are generated through successive asymmetric divisions of the RGCs. Early-born neurons are intended for the deep layers (L6 and L5; red), and later-born neurons are intended for the upper layers (L4, L2/3; green). Therefore, the different layers of the cortex are generated in an 'inside-out' fashion. At the end of neurogenesis, the radial scaffolding detaches from the apical surface, and the RG cells become gliogeneic, generating astrocytes (Ast), or generating Ependymal cells (EL). BV: blood vessel; CR: Cajal-Retzius neuron; DL Pyr: deep-layer pyramidal neuron; IZ: intermediate zone; UL Pyr: upper layer pyramidal neuron, WM: white matter. The image was taken from Kwan et al., 2012.249
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1.5.1.2 Gliogenesis

During the late embryonic period, there is a transition between neurogenesis and gliogenesis, which leads to the generation of the first glial cells (astrocytes and oligodendrocytes) around day E18 in mouse and gestational week 20 in humans.

1.5.1.3 Astrocytes

Astrocytes present many functions that are critical for the development, functioning and survival of neurons, such as maintenance of ionic homeostasis, maintenance of the BBB, and synthesis, uptake and release of glutamate. Thus, it has been seen that neurons cannot survive without close interaction with astrocytes in the brain\textsuperscript{193,239}. Astrogenesis in mice starts around E18 and lasts at least until approximately postnatal day 7 (PND 7), where most RG cells separate from the ventricle and become astrocytes. This separation generates morphological changes that lead to bipolar RGCs to become unipolar after ventricular detachment, and finally taking astrocytic morphology (multipolar)\textsuperscript{250}.

The increasing number of astrocytes in the cortex in the new-born is linked to the progressive disappearance of RGCs. However, the precise timing of astrocyte production is unclear due to the lack of specific markers to differentiate astrocytic precursors from multipotent RGCs\textsuperscript{251}. Astrocytes divide symmetrically and locally before final differentiation, an observation that supports the existence of astrocytic intermediate progenitor cells (aIPCs)\textsuperscript{248}. Astrocytes generated by aIPCs undergo a similar migration to the inside-out model described for neurons\textsuperscript{252}.

Astrocytic migration gives rise to two different types of astrocytes: fibrous and protoplasmic astrocytes. Fibrous astrocytes are in the white matter (brain region containing myelinated axons) and classically present a "star-like" shape with dense glial filaments, which can be labelled with GFAP marker. Protoplasmic astrocytes have irregular processes and typically have few glial filaments, and they are found in the grey matter (brain region containing neuronal cell bodies and glial cells)\textsuperscript{253}. The branched structure that astrocytes acquire during terminal differentiation allows them to contact blood vessels and neuronal synapses. Mature astrocytes are mainly produced during postnatal development and are maintained in the resting state; however, they can proliferate in response to injury\textsuperscript{193,239}.
1.5.1.4 Oligodendrocytes

The oligodendrocytes are the cells that form the myelin that cover the neuronal axons and allow the salutatory conduction through the formation of Ranvier's nodules. Myelin is a specialized membrane that works as an electrical insulator, surrounding an axon, increasing axon’s membrane resistance and reducing the membrane capacitance\textsuperscript{254}. Myelin makes the propagation of action potentials more efficient and rapid\textsuperscript{254}. Oligodendrocytes originate from RGCs, through oligodendrocyte intermediary progenitor cells (OIPCs). OIPCs divide symmetrically to form oligodendrocyte precursor cells (OPCs), which then migrate and distribute uniformly throughout the brain at birth\textsuperscript{255}. During the first two postnatal weeks, the OPCs generate the primary wave of mature oligodendrocytes. However, proliferating OPCs persist in the adult brain, distributed both in grey matter, where they constitute 2-3% of the total cells, as in the white matter, where they represent 8-9% of the total cells\textsuperscript{255,256}. The population of adult OPCs remains relatively stable throughout life due to the balance between apoptosis and cell proliferation. NSCs, located in adult SVZ, but not in SGZ, have been shown to produce oligodendrocytes\textsuperscript{257}.

1.5.1.5 Microglia origin

Microglia are the immune cells of the CNS, and their main functions are to detect and eliminate any possible pathogens, as well as cell debris present in the parenchyma\textsuperscript{258,259}. Unlike the other glial populations (astrocytes and oligodendrocytes), microglia cells have a mesodermal origin. The mesoderm represents the middle layer of the trilaminar embryo layers and will form connective tissues and muscle in the body\textsuperscript{260}. However, even today, there are different hypotheses about their exact origin\textsuperscript{261}. Four cell types have been described with the capacity to become microglial cells: fetal macrophages, monocytes, hematopoietic progenitor cells, and mesodermal progenitors\textsuperscript{262}. The appearance of microglia in murine brain originates from primitive myeloid progenitor cells (primitive macrophages and erythrocytes) in the yolk sac at E8.5. These macrophages generated in the yolk sac migrate to the brain in early stages of embryonic development, and then generate the pool of microglia cells in the adult brain\textsuperscript{263}. Yolk sac derived macrophage precursors migrate and colonize the mouse embryo, approximately between days E8 and E10 (Figure 1.8), the brain being the first colonized organ\textsuperscript{263,264}. The first microglia progenitor cells in the mouse brain can be identified on day E9 prior to neurogenesis and gliogenesis occurred, which suggest that an inadequate microglia development or migration could influence other cell population in the brain\textsuperscript{265}. 
Figure 1.8 Microglia migration in the mouse embryonic brain.

The precursors of the microglia are generated in the yolk sac around day E7. The colonization of the CNS begins around days E8 - E10.5. A coronal plane representation of the embryonic mouse brain showing the second microglial invasion that occurs at E14.5 and microglial cells migrate to populate different layers of the cortex and different regions of the brain. CP: cortical plate, M: meninges, NCx: neocortex, V: ventricle. Adapted from Mosser et al., 2017.

The first invasion of CNS by microglia occurs during embryonic development, in which cells colonize the brain and increase their cell number. This gradual increase in the number of microglia cells is due to the rapid proliferation of microglia progenitor cells. Taking into account that the vasculature in the brain has not been developed at this embryonic time, it has been described that microglia invade the brain via extravascular routes. Two possible routes have been proposed, the first is through the meninges by crossing the pial surface and the second is from the ventricles,
where microglia cells are floating or coupled to the wall of the ventricle. Through these pathways, the microglial cells can reach the parenchyma and invade the brain.

Second brain invasion occurs between days E14 and E16, thanks to the migration of new microglial cells from the periphery. In this second invasion, the number of cells increases, but it is not due to an increase in proliferating cells since by day E14.5 the number of microglia progenitor cells decreases. The proliferation of microglial cells continues to increase slowly, and by day E17.5 microglial cells disperse throughout the brain. After embryogenesis, microglia self-renew constantly in order to maintain the pool of microglia cells throughout life.

Microglial activation generates morphological and functional changes, in which three types of microglia can be observed—first, the quiescent or resting microglia, which have many fine and elongated branches. Second, the activated microglia, which have shortened and thickened ramifications, and are the central cells responsible for the synthesis of pro-inflammatory proteins under pathological conditions—finally, the ameboid microglia, which have a globular morphology. Apart from these morphological modifications, microglial cells are classified based on their functions either as pro-inflammatory microglia named M1 or as immunosuppressive microglia named M2. M1 as pro-inflammatory cells, they produce different cytokines and interleukins such as IL-1β, TNF-α, IL-6, CD16/32, CD86, CD40, inducible nitric oxide synthase (iNOS), reactive oxygen species (ROS), and proteases in response to the inflammatory process. M1 cells are the first line of defence against and infection or injury in the brain, promoting the destruction of the pathogens but also causing neuronal damage due to the neurotoxicity induced.

On the other hand, M2 microglia helps to control the inflammatory process realising anti-inflammatory factor (IL-4, IL-10, IL-13, and TGF-β), generating an immunosuppressive response and promoting tissue repair, and extracellular matrix (ECM) reconstruction. Also, M2 cells promote neuronal survival releasing neurotrophic factors such as insulin-like growth factor 1 (IGF-1). An imbalance of M1/M2 microglia has been observed in different CNS disorders such as multiple sclerosis, Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis, and major depressive disorder.
1.6 Neocortex

The cerebral cortex is an extremely complex structure, in which the various sensory organs are represented in some areas or zones known as primary sensory areas. From the sense of smell to vision, each sensory modality has its cortical territory, which maintains a relatively constant location in all mammals. The cerebral cortex has been divided into two main types: isocortex and allocortex. The isocortex or neocortex corresponds to that part of the cortex with stratification in six layers, while the allocortex, represented by the archicortex (hippocampus and fascia dentata) and paleocortex (olfactory cortex) exhibit a single sheet structure composed of three layers.

The cortex of mammals is composed of two main types of cells, neuron and glia cells—the neurons in the cortex exhibit different forms in their morphology of dendrites and axons. Neurons can be classified as unipolar, bipolar, or multipolar, with multipolar morphology being the most common in the cortex (Figure 1.9).

![Figure 1.9 Structural Classification of Neurons.](image)

Unipolar neurons have a short single process. Bipolar neurons have one axon and one dendrite. Multipolar many extensions from the cell body. a) Cell body; b) Axon; c) Dendrites. Adapted from Loadot et al., 2015.

In the cortex, 75-80% of the neurons correspond to excitatory pyramidal neurons; the remaining 10-25% correspond to inhibitory interneurons. There is another group of non-pyramidal excitatory neurons called stellate cells, and they show a star-like shape formed by dendritic processes. Neurons in the cortex can be divided into two groups, neurons that have spines (generally excitatory) that correspond to around 70-80% and are mainly pyramidal and spiny.
stellate neurons. The remaining 20-30% correspond to neurons lacking spines (generally inhibitory) such as a chandelier, basket, and double bouquet neurons\textsuperscript{279}.

### 1.6.1 Cortex structure

One of the most widely used descriptions is Brodmann's which distinguishes, in general, six layers numbered from layer 1 (outermost layer) to the layer 6 (deepest, adjacent to the white matter) (Figure 1.10), showing that all the layers have differences in cell density and cell types as follows:

![Figure 1.10 Mouse neocortical layers.](image)

**A.** schematic representation of an adult mouse cortex. The cortical layers are formed in an inside-out gradient. New cells are in the deepest layers. **B.** image from 12µm thick DAPI-stained showing different cortex layers. Scale bar: 250 µm. L1 (layer 1), L 2/3 (layers 2/3), L4 (layer 4), L5 (layer 5), L6 (layer 6), wm (white matter). Blue circles represent subplate cells. Adapted from Hoerder-Suabedissen A et al., 2015\textsuperscript{280}. 
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- Layer 1 (Plexiform or molecular layer) is characterized by having a very low cell density. Approximately 10% of the whole thickness of the cortex is occupied by Layer 1, which is the most superficial layer. In this layer are very few neurons, about 85% of the neurons are inhibitory. However, this layer presents a large number of axons and dendrites that come from neurons located in the other layers\textsuperscript{281}. Layer 1 neurons have been suggested to be involved in feedback interactions via the thalamus, with this circuitry deemed important in attention and associative learning\textsuperscript{282}.

- Layer 2/3, unlike layers 2 and 3 in primates, in the mouse cortex these two layers are counted as a single layer because these layers are less differentiated and thinner\textsuperscript{279,283}. Layer 2 has a very high density of small pyramidal cells, and layer 3 is subdivided into two sublayers a surface sublayer 3a that presents small pyramidal cells and a deep sublayer 3b, which is populated by big pyramidal cells\textsuperscript{281}. Layers 2 and 3 are mainly composed of pyramidal neurons. The size of the pyramidal neurons increases from one layer to the other, that is, in layer 2, the pyramidal neurons have a smaller size, and in layer 3 a larger size. Most neurons in layer 3 are pyramidal neurons (approximately 75%) and have long axons, which extend horizontally, allowing them to connect to other neurons in the same layer. The rest of the pyramidal neurons connect with layer 4 interneurons and layer 5 pyramidal neurons\textsuperscript{281}.

- Layer 4 has a high density of granular cells that differentiates it from adjacent layers 3 and 5. This layer allows the cortex to be divided into supra- and infragranular layers. Layer 4 is usually thick in the sensory area. The neurons located in layer 4 connect pyramidal neurons and interneurons of layers 2/3 through their axons. Another characteristic of this layer is afferent axons from other structures of the brain, such as the thalamic region end in this layer. The cell composition of layer 4 in the visual cortex changes according to the species. For example, in mice, the layer 4 is mainly composed of pyramidal neurons, but in cats and monkeys, mainly spiny stellate neurons are observed in this layer\textsuperscript{281,284}.

- Layer 5 presents pyramidal cells of medium and large size. The layer 5 is composed mainly of pyramidal neurons, which connect their axons, especially with the white matter, some connect interneurons of layers 2/3 and some efferent axons with the spinal cord. In rats, it has been described that the pyramidal neurons of layer 5 receive inhibitory input from layers 2/3 and receive excitatory input from all layers\textsuperscript{281,284}.

- Layer 6 is located next to the white matter. It contains a large number of pyramidal cells modified with polymorphic morphology. The pyramidal neurons of this layer are connected to layers 4 and 5 through their axons. However, 50% of the neurons in this layer send afferents to other areas of the cortex\textsuperscript{281,284}.
1.6.2 Hippocampus

The hippocampus is a bilaterally arranged brain structure and plays an essential role in cognitive processes. Anatomically, the hippocampus comprises six cortical areas: the subiculum, the presubiculum, parasubiculum, the entorhinal cortex, the Cornu Ammonis (CA) or Ammon’s horn that is subdivided into the CA1, CA2 and CA3 regions, and the dentate gyrus (DG) (Figure 1.11). The three-dimensional shape of this structure is relatively complicated since its longitudinal axis doubles in an inverted "C". It extends from a dorsal (septal) to the ventral (temporal) axis in rodents, corresponding to a posterior to the anterior axis in humans. The pyramidal cell layer is constituted by the CA1, CA2 and CA3 regions. The stratum pyramidale is a cell layer located below the CA1, CA2 and CA3 regions, and the stratum oriens is located deep the pyramidal layer. This area in the hippocampus contains apical dendrites of pyramidal cells and interneurons. Toward the hippocampal fissure, and superficial to the pyramidal cell layer, there are three regions: the stratum lucidum, the stratum radiatum, and the stratum lacunosum-moleculare. The stratum lucidum is located next to the CA3 region but is absent in CA2 and CA1, and the stratum radiatum below stratum pyramidale.

![Figure 1.11 A schematic map of the trisynaptic pathway in the hippocampus.](image)

Schematic illustration of a coronal section of an adult hippocampus. Cornu Ammonis (CA); Dentate Gyrus (DG); Entorhinal Cortex (EC); Lateral Perforant Path (LPP); Medial Perforant Path (MPP); Mossy Fibres (MF); Schaffer Collaterals (SC); Subiculum (S). Taken from Pattern et al. 2015.
The outer layer of the DG is called the molecular layer and contains afferent axons and dendrites of the intrinsic cells of each structure. In the hippocampus proper, this layer has traditionally been divided into the stratum radiatum and the stratum lacunosum-moleculare. The middle layer called the granular layer in the DG, and the pyramidal layer in the hippocampus contains the main neurons of each structure. The inner layer, called the multiform layer, has the axons of pyramidal and granular cells, intrinsic neurons, and many glial elements. This layer is also called stratum oriens in the hippocampus and hilus in the DG. The innermost part of the hippocampus is a layer of myelinic axons that arise from the cell bodies located in the subiculum and the hippocampus. This layer, called alveus, continues with the fimbria of the hippocampus, which in turn becomes the fornix. The fornix is the main connection path between the hippocampus and other brain structures.

The hippocampus contains two main cell regions. At the end of the subiculum is located the regio superior, composed of cells of small size, corresponding to the CA1 region. Towards the Dentate Gyrus, there is the region inferior formed by larger cells, corresponding to the CA3 region, along with a small transition zone called the CA2 region. The primary neurons of the CA regions are pyramidal glutamatergic neurons, named based on their morphology, constituting approximately 90% of all neurons in the hippocampal CA regions. The cells are arranged in a single layer of densely packed cell bodies forming the pyramidal layer. The arrangement of cell bodies and dendritic and axonal projections to the hippocampus is seen in transverse sections of the brain, forming layers which are bent and folded over one another.

In adult hippocampal neurogenesis takes place in the dentate gyrus. This has a layered organization. In particular, the neuronal precursors are in the subgranular zone (SGZ), which is delimited by the granule cell layer and hilus. The granular neurons of dentate gyrus present their afferent to the molecular layer and their efferent to hilus and CA3. In turn, the molecular layer comprises three sublayers (internal, medial and external) with axons of different origin providing the specificity of the stimuli that reach each portion of the dendritic tree of the granular cells.

In addition, these sublayers of the molecular layer have different connectivity along the septotemporal axis: while the region that projects to the dorsal hippocampus of rodents receives projections of the neocortex, the region that projects to the ventral hippocampus receives afferents from the prefrontal cortex, the amygdala, the hypothalamus, and other subcortical nuclei. This segregation at the synaptic inputs contributes to the marked anatomical and functional difference that characterizes the longitudinal axis of the hippocampus.
1.7 Neurogenesis in the Adult Mammalian Brain.

Adult neurogenesis is the process of formation of new neurons from neural stem cells (NSCs) throughout the adult life of the individual, upon completion of its development. This process, which is commonly accepted nowadays, was discussed until just 50 years ago. In the early twentieth century, Santiago Ramón y Cajal showed that the brain tissue was composed of individual nerve cells, discovery for which he received the Nobel Prize in 1906. Cajal concluded in his study that neurons are generated exclusively during the prenatal stage of development\textsuperscript{292}. In the 1960s, Joseph Altman described for the first-time neurogenesis. However, it took 30 years to talk about neurogenesis again, due to several factors—first, neurogenic areas needed to be characterized\textsuperscript{293,294}—second, the presence of NSCs in adult mammals that could also be isolated and cultured in vitro were discovered in 1992\textsuperscript{295}, along with the work of Peter Eriksson et al. (1998) defending neurogenesis in humans\textsuperscript{296}.

The adequate microenvironment for the generation of neurons, known as the stem cell niche, consists of several components. First, the precursor cells (stem cells, progenitor cells), which are characterized mainly by two properties: unlimited self-renewal and to be multipotent, that is, to have the ability to differentiate into several cell types\textsuperscript{297,298} (\textbf{Figure 1.12}). Secondly, the neurogenic niche is also composed of astrocytes, blood vessels, microglia, and extracellular components, such as the extracellular matrix\textsuperscript{297,298}. The interaction of precursor cells with all these components plays a critical role in the regulation of adult neurogenesis and may affect proliferation, self-renewal, cell differentiation, survival and migration\textsuperscript{299}. In order to generate new neurons, it is essential to establish an adequate microenvironment, in which stem cells can interact with other components such as astrocytes, microglia, blood vessels and extracellular matrix.
Figure 1.12 Process of self-renewal and differentiation of neural stem cells.

Neural stem cells divide for self-renewal and/or giving rise to progenitors, which subsequently undergo differentiation processes to generate neurons (neurogenesis) or glial cells (gliogenesis). During development, neural stem cells first generate neurons, and later glial cells (astrocytes and oligodendrocytes).

1.7.1 Neurogenic regions in the adult mammalian brain

Currently, the existence of only two neurogenic active regions in the adult mammalian brain has been demonstrated, the subventricular zone (SVZ), located in the adjacent cell layers to the ependymal layer, lining the walls of the lateral ventricles; and the subgranular zone (SGZ) located in the hippocampal dentate gyrus\(^{300,301}\). Over the past 15 years, some researchers have proposed new neurogenic regions in the adult brain, and they are still under intense debate. This is the case for the olfactory bulb (OB) where, besides receiving and incorporating new neurons from the SVZ, new neurons could be generated locally\(^{302,303}\).

1.7.1.1 Neurogenesis in the Ventricular-Subventricular zone

The ventricular-subventricular zone (V-SVZ) is an important neurogenic niche in the adult brain since it generates glial cells (astrocytes or oligodendrocytes) and olfactory bulb interneurons\(^ {304}\). In this region the NSCs or type-B cells, characterized by self-renewal ability and multipotency, divide, generating rapid amplification progenitors or type-C cells (or TAP Transit Amplifying Progenitors). Type-B cells are classified according to their location, in B1 and B2. B1 cells are found mainly in the lateral ventricle, and type-B2 cells are located near the inferior striatal parenchyma\(^ {304}\). Once type-
B cells divide, they give rise to type-C cells; which are capable of dividing a larger number of times than NSCs, giving rise to neuroblasts, whose cell fate is determined towards the neuronal lineage. Type-C cells divide and give rise to migratory neuroblasts (type-A cells) that migrate from the lateral ventricle to the olfactory bulb through the rostral migratory stream (RMS)\textsuperscript{305,306}. In the RMS, the neuroblasts form a chain and migrate towards the olfactory bulb through a tube formed by astrocytes. Once the neuroblasts exit the SVZ into the RMS, they start expressing the polysialated glycoprotein neuronal cell adhesion molecule (PSA-NCAM). The direction in which neuroblasts migrate is given by chemorepulsion of the ventricular zone and chemoattraction of the olfactory bulb\textsuperscript{307}.

The migration rate of type-A cells through RMS depends mainly on the degree of maturation; older neuroblasts migrate faster than younger ones. These neuroblasts migrate tangentially forming a chain along the rostral migratory stream (RMS) wrapped by astrocytes assembled in a tubular form\textsuperscript{301,308}. Once they reach the core of the OB, these immature neurons leave the RMS and migrate radially to the granular and periglomerular layers, which gather different subtypes of inhibitory interneurons that are integrated into the structure of the OB\textsuperscript{309}.

Once the neuroblasts reach their target region the olfactory bulb, they are classified into two types of GABAergic interneurons, granule neurons and periglomerular neurons\textsuperscript{310}. These two types of interneurons have receptors for the inhibitory neurotransmitter GABA and function as local inhibitors. These new cells enter the current circuit to replace the old cells that underwent apoptosis. It is believed that the renewal of cells in the olfactory bulb may enhance the differentiation between odours\textsuperscript{311}. In more detail, the cytoarchitecture of SVZ cell subtypes comprises the following (Figure 1.13):
Figure 1.13 Schematic representations of the neurogenesis in the ventricular-subventricular zone (SVZ).

Sagittal (A), and coronal (B) sections, showing the localization of the ventricle. C) Cytoarchitecture of ventricular-subventricular (VZ-SVZ) stem cell niche. Type-B stem cells (astrocyte-like NSCs) give rise to transit-amplifying progenitors (TAP) or type-C cells progenitors, which in turn will give rise to neuroblasts or type-A cells and oligodendroglia cells. In addition, the ependymal cells (type-E cells) are observed on the ventricular wall and contact both the cerebral-spinal fluid (CSF) in the ventricle and type-B cells. Adapted from Lim et al., 2016.
**Type-B cells:** They are the NSCs of SVZ. They have a morphology that allows them to contact simultaneously both the light of the ventricle and therefore be in contact with the cerebrospinal fluid (CSF) through their apical process, as well as blood vessels, through their basal process ending in a vascular feet structure\(^{305,312}\). Some authors subdivide this population in Type-B1 if they are closely associated with ependymal cells and type-B2 if they are in the underlying striatal parenchyma. In any case, the type-B cells are largely quiescent, although they are able to enter the cell cycle and proliferate, representing 12% of proliferating cells in the SVZ\(^ {313}\). Since the type-B cells express Glial Fibrillary Acidic Protein (GFAP), they are also frequently referred to as SVZ astrocytes, and they also express the transcription factor Sox2 (Sex Determining Region Y-box2), the glutamate transporter GLAST (Glutamate Aspartate transporter) characteristic of radial glia cells, and the intermediate filament of undifferentiated cells Nestin\(^ {314-316}\). Once the cells are in the cell cycle, they express division markers such as Ki67, regardless of whether the division is symmetric, to self-renew, or asymmetric, to give rise to the type-C or TAP cells\(^ {317}\).

**Type-C cells:** Also called Transit-Amplifying Progenitor cells or TAPs. They are located next to the type-B cells, and preferably near blood vessels. These cells complete their cell cycle in approximately 18 to 25 hours\(^ {318}\). However, the proportion of dividing cells is higher than Type-B cells and represents 52% of the dividing cells of SVZ\(^ {313}\). This type of cells expresses EGFR but lose GFAP expression. The majority of cell progeny of these cells is Type-A cells, whose cell fate is committed to the neuronal lineage.

**Type-A Cells:** Denominated neuroblasts or neural progenitors, are in the RMS migrating from the SVZ to the OB. They express markers of immature neurons such as βIII-Tubulin, Doublecortin (DCX) and PSA-NCAM (Polysialylated-Neural Cell Adhesion Molecule). Although these neuroblasts have a higher degree of differentiation than type C cells, they retain the ability to proliferate as they migrate through the RMS forming chains\(^ {307}\). They represent 15% of dividing cells in the SVZ\(^ {313}\). When they reach the OB, they finally differentiate into inhibitory interneurons and integrate into the granular or periglomerular cell layers.

**Type-E cells:** refer to ependymal cells that form the epithelium physically separating the SVZ from the cavity of the lateral ventricles. Initially, they were considered the NSCs of the SVZ, however subsequently this hypothesis was rejected since they are quiescent cells and do not exhibit properties of NSCs in vitro\(^ {313,319}\). They are characterized by the presence of microvilli and frequent cilia on the ventricular surface. Recently it has been shown that these cells are organized to form pinwheel structures, whose centre is the point where the Type-B or NSCs cells contact the ventricle through their apical processes\(^ {305,320}\).
1.7.1.2 Neurogenesis in the Subgranular Zone (SGZ)

Similar to the neurogenic process that takes place in the SVZ, SGZ contains NSCs called Type-1 cells or radial NSCs. These cells divide to give rise to rapid amplification cells called Type 2 or non-radial progenitors, representing an intermediate step to the formation of neuroblasts (also called type-3 cells). These neuroblasts give way to immature neurons that migrate a short distance into the granular cell layer (GCL) to complete their maturation and generate mature granule neurons. Thus, the following cell subtypes would cover the cytoarchitecture of the SGZ (Figure 1.14).

**Type-1 cells** are the DG hippocampal NSCs. They are also called radial glia cell progenitors given their morphology. A single radial projecting process through the GCL characterizes them. Like the SVZ NSCs, these cells appear to have self-renewal capacity by symmetrical divisions, although this matter is still controversial.

**Type-2 cells:** also called transit-amplifying progenitor (TAP) or radial progenitors, their characteristics at proliferative activity and immunoreactivity are similar to the type-C cells from the SVZ. They are positive for TBR2, Ngn2, Sox2 markers. Some authors classify this population in 2a or 2b based on the differential expression of Sox2 and DCX/PSA-NCAM factor. When Sox2 expression decreases, DCX expression increases, indicating the passage of the cells from 2a to 2b. The fundamental difference between the two cell types is that the Type-2b cells have a more limited ability to divide as well as a higher degree of differentiation.

**Type-3 cells:** also called neuroblasts, have the same immunoreactive characteristics than Type-A cells of the SVZ. However, in this case, they continue their differentiation program to immature neurons in the SGZ. They express, among other markers, DCX and β-III-Tubulin.

Newly formed neurons: They could be subdivided into immature granule neurons and mature granule neurons. First, in the same manner, as in OB, they have markers such as DCX and β-III-Tubulin. As they mature, they leave the cell cycle and express calretinin. A few days later, they express calbindin and NeuN. In addition, they extend their dendrites to the molecular layer, while projecting their axons through the Hilus to the CA3 region. Thus, they contact pyramidal neurons in that region, and finally, they integrate into the existing neuronal circuit in the DG.
Figure 1.14 Adult neurogenesis in the dentate gyrus of the hippocampus in mice.

A) Sagittal sections, showing the localization of the hippocampus and dentate gyrus. B) The sequence of the different cell types in adult hippocampal neurogenesis. Five events of neuronal development can be identified according to morphology, proliferative capacity, and expression of markers, in an estimated timeline. DG: Dentate gyrus; GCL: granule cell layer; SGZ: subgranular zone; GFAP: glial fibrillary acidic protein; DCX: doublecortin; NeuN: neuronal nuclei; Prox-1: “Prospero homeobox protein; PSA-NCAM: the polysialylated form of the neural cell adhesion molecule NCAM; Sox2: sex-determining región Y-box2; Tbr2: T-box brain protein 2. Adapted from Duan X et al., 2008.
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1.7.1.3 The niche as a functional unit

The niche is a specialized microenvironment where the NSCs are present, and which can keep these cells in an undifferentiated state, thus preserving their ability to self-renew and generate new progeny such as neurons and glial cells. Niches are regions with unique structural and signalling characteristics. Therefore, Shihabuddin et al. tested how progenitor cells derived from non-neurogenic regions of the brain were able to generate specific neurons when they are implanted in the neurogenic niches of the brain. This data indicates that NSCs represent a crucial model for cellular therapy, and neurogenic niches have specialised microenvironments to support neural differentiation.

Several factors characterize the neurogenic niche. First, the neurogenic niche has a unique architecture with the proximity of the cells that compose it, adhesions between those cells, and anchorage and intercellular communication regulation provided by the extracellular matrix. Second, the NSCs developmental regulation in a niche is generally independent of the adjacent regions, due to possible mechanisms that restrict local growth. Third, the cells that form the niche are endothelial cells, astrocytes, microglia, ependymal cells (in the SVZ) and mature neurons (in the SGZ). These cells located in a niche have essential unique characteristics. For example, astrocytes from the niche secrete pro-neurogenic signals, whereas astrocytes from non-neurogenic regions promote neither proliferation nor neuronal differentiation. These characteristics of niche cells are due at least in part, by expression and secretion of specific molecules that play an essential role in the regulation and maintenance of the niche. These molecules are Wnt, Notch, Sonic Hedgehog or BMP5, growth factors, neurotrophins, different types of neurotransmitters, among others.

1.7.1.4 Adult hippocampal neurogenesis events

In the process of generating new neurons in the SGZ (lasting eight weeks in mice), it is possible to observe five events (proliferation, differentiation, migration, dendritic and axonal contact and integration):

1. Proliferation. Neurogenesis in the hippocampus is possible because of the presence of stem cells, which proliferate generating neural progenitor cells. In rodents, the duration of the mitotic cycle of the precursors lasts between 12 and 24 hours, which leads to the production of approximately 8,000 to 10,000 new neurons per day. Since the dentate gyrus consists of approximately one million granular cells (35% of the total hippocampus), this phenomenon can generate just under 1% of the total granular cells each day. However, it has been estimated that under normal conditions,
the death rate of the new cells generated is around 60-80% during their first month of life\textsuperscript{326,336}. Probably, in the end, only those new neurons able to establish functional synaptic contacts with other neurons survive, just as they do during development\textsuperscript{326}.

At this stage, the cell precursor phase takes place. The first stage of precursors (type-1 cells) has properties suggestive of glial cells, including radial glial morphology. The body of the cells is in the SGZ, and the dendrites extend into the GCL. Type-1 precursors produce intermediate progenitor cells, or type-2 cells, with high proliferative activity. A subset of these cells express glial markers, but their morphology has different characteristics than the radial cells (type-2a cells), another subset of cells express neuronal markers (type-2b cells)\textsuperscript{337}. These developing type-2 cells receive the first GABAergic synaptic inputs, being particularly sensitive to such stimulation, and type-1 cells respond to these stimuli by increasing cell proliferation\textsuperscript{338}. From type-2b cells arise type-3 cells, also called neuroblasts, which represent the stage of transition between proliferative cells (with division capacity) and post-mitotic cells (morphologically maturing towards immature neuron). Neuroblasts have migratory cell properties, reaching their final position in the external region of the granule cell layer\textsuperscript{339}.

Then, in the early survival phase, the excess of precursor cells is removed from the neuronal lineage generated in the previous phase. Most of these cells fail to make connections and die before their full integration into the hippocampal circuit. The exact mechanism by which this process is induced is still unknown. Although the early cell survival rate depends on the species and even the strain within the same species of study\textsuperscript{340}, in all cases cells surviving this phase is likely to survive for a long time.

2. Differentiation. Neural progenitor cells can produce multiple types of cells in the central nervous system, such as neurons, astrocytes, or oligodendrocytes\textsuperscript{341,342}. Predominantly new neuronal phenotype cells (75%) are generated, mainly glutamatergic granular cells, but also some interneurons in the GABAergic basket (14%). A smaller proportion of cells differentiate into astrocytes (15%), or oligodendrocytes. These ratios are relative and may vary according to factors such as age, species or exceptional circumstances, like injury or disease\textsuperscript{326,343}.

3. Migration. The new hippocampal neurons do not migrate great distances. However, this migration occurs between the SGZ, and the granular layer, which is located a few micrometres away in the dentate gyrus, where these cells mature and integrate into existing circuits\textsuperscript{326}.

4. Dendritic and axonal targeting. Ten to 14 days after being born, the new neurons generated extend their dendrites into the molecular layer and send their axon to CA3, where they later contact pyramidal neurons\textsuperscript{326,342}. At this point, the post-mitotic maturation phase takes place. Type-3 cells
(and possibly some type-2b) exit the cell cycle and as immature neurons initiate post-mitotic differentiation into granule cells. In this phase, the maturation of new neurons takes place. These cells are characterized by a rounded nucleus and slightly triangular soma with a branched apical dendrite. Once the axonal connectivity with CA3 is established, the first glutamatergic synapses occur, coinciding with a period of increased plasticity and the development of dendritic spines. At the same time, synapses are established in the spines, dendrites and somas of the new neurons (synaptogenesis).

5. Synaptic integration. At the end of the maturation phase, a second critical phase of survival and stabilization of the new neurons occurs (late survival phase). Only those new neurons that complete their development and maturation and integrate into the cerebral circuits survive to this stage; otherwise, they degenerate. In fact, at this stage, the new neurons generated do not show any properties that allow them to be distinguished from existing granular cells. The new cells establish their functional synaptic contacts and complete their maturation to become mature granular cells. At this time, they receive afferent inputs from the entorhinal cortex in the dendrites located in the molecular layer. At the same time, they establish contacts with their target cells, with CA3 pyramidal neurons and hilus interneurons.

1.7.1.5 Functional relevance of adult neurogenesis

Some of the questions that came with the discovery of adult neurogenesis were whether these newly formed neurons are incorporated into the existing neural circuitry of both the OB and hippocampus and if they had any role in those circuits. In the case of neurogenesis in the SVZ, it is known that immature neurons migrate to the OB through the RMS, and they become part of the OB circuit. For the SGZ, the new neurons derived from NSCs matured can integrate into the GCL with the same characteristics as neurons generated in the embryonic stage. Although the exact functions of the new neurons are not entirely known, it is believed that the dorsal region of the DG is related to learning and memory.

In contrast, the ventral region is related to affective behaviours. In learning and memory, there are several hypotheses about what the role of adult neurogenesis in both processes could be. The first hypothesis relates to their potential role in pattern separation or discrimination tasks. This feature refers to the ability to discriminate similar experiences as different or similar events, and this is a characteristic of episodic memory. The first event marks a pattern in mature neurons while in the second, newly formed neurons are storing the differential information between the first event and the second to identify them as different. In the absence of neurogenesis, common
information from both events and differential information is accumulated in mature neurons. This event increases hippocampal activity, interfering between the pattern of the first event and the second, so they are not perceived correctly\textsuperscript{353,354}.

Another hypothesis is the involvement of neurogenesis in the SGZ with spatial coding processes. Spatial coding refers to the ability to associate two events, little or nothing like each other due to temporal proximity. Therefore, two independent events that occur close in time will be stored in the new neurons. In the case of the third event with a time separation from the first two, for example, three months after, the new neurons that would store the event are not the same from events 1 and 2 because they have already matured. This causes the first and second events perceived interrelated, but the third is perceived independently\textsuperscript{355}. One last hypothesis would be related to the level of resolution of the memory. This refers to the ability to remember more details of an event. Since mature neurons accumulate older memories and new neurons, recent memories, memory might have a higher resolution in the presence of active\textsuperscript{356}.

Adult neurogenesis can be affected by extrinsic factors that alter either positively (increasing the production of new neurons) or negatively (decreasing said production) neurogenesis. On the one hand, there are positive regulators of neurogenesis, such as physical exercise or environmental enrichment\textsuperscript{357,358}. In many cases, these positive regulators have been essential to demonstrate the relationship between adult neurogenesis, memory and learning of individuals. Therefore, it has been observed that neurogenesis improves the performance in various hippocampus-dependent behaviour tests such as water-maze, Y-maze, novel object recognition, among others\textsuperscript{359,360}.

On the other hand, there are negative regulators, including ageing, obesity or inflammation. Ageing is the most potent negative regulator of adult neurogenesis in all species\textsuperscript{361}. Therefore, during ageing, animals not only get worse outcomes in the behavioural tests compared to young individuals but also ageing alters critical signalling pathways in maintaining the neurogenic niche\textsuperscript{362}. Certain diseases cause neurogenic changes, some of these diseases are brain tumours\textsuperscript{363}, epilepsy whose seizures produce a massive but aberrant increase of neurogenesis\textsuperscript{364}, depression, schizophrenia and certain neurodegenerative diseases such as Parkinson\textsuperscript{365} and Alzheimer's disease\textsuperscript{366}. 
Chapter 1

1.8 Brain Metabolism

The cellular function and the maintenance of any tissue depend on an adequate regulation between metabolite supply and cellular energy metabolism. In the central nervous system, this regulation is essential for the proper functioning of the brain, since the brain is a very dynamic organ with a high energy demand, consumed principally during neuronal activity367.

The human brain represents about 2% of body weight, consuming approximately 20% of total oxygen metabolism, and interestingly its metabolic activity is remarkably constant, no matter how much motor or mental activity is demanded368. It has been described that 75 to 80% of the energy of the brain is consumed by neurons, mainly at synapses in the restoration and maintenance of neuronal membrane potentials, although it is also used for maintenance of the glutamate-glutamine cycle, neurotransmitter synthesis, and vesicle recycling among others369,370. However, because the brain has a heterogeneous cell population, it has been described that astrocytes also play an important role in the metabolism of the brain and in the regulation of blood flow, while the role of oligodendrocytes and microglia in the regulation of metabolism has not been explored in detail371,372.

1.8.1 Astrocytic metabolism

Traditionally, astrocytes have been described as glycolytic and oxidative regulators in the CNS. The oxidation process consists of the generation of approximately 30 ATP molecules per glucose molecule (Figure 1.15). In the oxidation process, once glucose is completely oxidized, pyruvate and lactate metabolites are generated, then in the mitochondria, the tricarboxylic acid cycle (TCA), the transfer of electrons in the respiratory chain, the consumption of oxygen, the production of CO2 and water, occur in order to produce ATP373. In the glycolysis process, glucose generates two molecules of ATP and pyruvate, at low oxygen levels, pyruvate produces lactate and NAD+, which is a fundamental cofactor for maintaining a glycolytic flux, but at physiological levels of oxygen, lactate can be produced in the brain by a process known as the Warburg Effect373,374.
Figure 1.15 Glucose metabolism.

Glucose enters cells through glucose transporters (GLUTs) and is phosphorylated by hexokinase (HK) to produce glucose-6-phosphate (glucose-6P). Glucose-6P gives rise to fructose-6-phosphate (fructose-6P) by glucose-6-phosphate isomerase (GPI). Fructose-6P by phosphofructokinase (PFK) produces fructose-1,6-bisphosphate (fructose-1,6-P2). Fructose-1,6-P2 produces one molecule of glyceraldehyde-3-phosphate (GA3P) and one molecule of dihydroxyacetone phosphate (DHAP). GA3P will produce two molecules of pyruvate, ATP and NADH. Pyruvate can then enter mitochondria, where it is metabolized through the tricarboxylic acid cycle and oxidative phosphorylation, producing ATP. Under hypoxic conditions or depending on the cellular metabolic profile, pyruvate can also be reduced to lactate by lactate dehydrogenase. It was adapted from Magistretti et al. 2015.
Astrocytes play a fundamental role in the flow of energy substrates to neurons due to both their strategic location and their metabolic versatility. Astrocytes extend their processes around the capillaries of the cerebral parenchyma, in which glucose from the circulation is found. Hence, astrocytes play an essential role in the distribution of nutrients from the blood to other brain cells. The use of glucose by astrocytes mainly involves the production of lactate and pyruvate, which do not necessarily have to be metabolized through the tricarboxylic acid cycle (TCA) but are mostly released into the extracellular environment. Glucose can also be incorporated into lipids, amino acids and glycogen, and is a precursor to some neurotransmitters such as GABA, glutamate and acetylcholine. The brain can utilize under certain circumstances specific substrates to produce energy in the brain such as lactate, acetate, fatty acids and ketone bodies.

The first step is the entry of glucose into the astrocytes by facilitated diffusion through the plasma membrane, a process that is carried out by several glucose transporters, GLUT1 (astrocytes and oligodendrocytes), GLUT3 and GLUT4 (neurons cells) and GLUT5 (microglial cell). All brain cells express GLUT1, but it is highly expressed in astrocytes and in endothelial cells of capillaries, which makes this transporter the main carrier involved in the import of glucose into the brain from the blood, whereas GLUT3 is restricted to neurons but has a higher affinity for glucose.

After entering the cells, glucose phosphorylation takes place, astrocytes express (mostly in the mitochondria) hexokinase 1, which transforms glucose into glucose 6-phosphate. This new molecule can be metabolized via glycolysis, take an alternative route via pentose phosphate pathway, or be stored as glycogen. The amount of glucose metabolized via the pentose phosphate pathway in cultured astrocytes is small compared to that metabolized by the glycolytic pathway. Besides, the glycolytic activity of cultured astrocytes is predominantly anaerobic, and lactate is considered the main metabolic product. Lactate maintains cognitive function in hypoglycaemia and hypoxia. Pyruvate also maintains synaptic activity and neuronal morphology during glucose deficiency. Astrocytes provide the neighbouring neurons with monocarboxylates (lactate and pyruvate) during high energy requirement or lack of glucose situations. The use of these monocarboxylates as energy substrates requires oxidative metabolism, which is compromised in cases of anoxia but can take place if glycolysis is inhibited. At low glucose levels, astrocytes can maintain neuronal activity for an extended period via monocarboxylate release.

Uptake of monocarboxylates, such as lactate, pyruvate, and ketone bodies, must be transported into the extracellular space via monocarboxylate transporters (MCT). The isoform MCT1 is expressed in vascular endothelial cells and astrocytes, and MCT2 is expressed primarily in hippocampal and cortical neurons, MCT4 in astrocytes. In the cells, lactate is converted to...
pyruvate, which is an efficient metabolic source of ATP, since one molecule of pyruvate provides 15 molecules of ATP after its oxidation mediated by pyruvate dehydrogenase and via TCA cycle\textsuperscript{373}. The enzyme responsible for this conversion is lactate dehydrogenase (LDH), whose expression and isoforms vary in different tissues. There are observations that the LDH-5 isoform, which promotes the conversion of pyruvate to lactate, is highly expressed in astrocytes, while the LDH-1 isoform promotes pyruvate production, is found mostly in neurons\textsuperscript{392,393}. These data support the idea that astrocytes would process glucose via glycolysis generating lactate, which, once released into the extracellular space is subsequently captured by neurons, and converted to pyruvate, and subsequently metabolized in the TCA cycle to generate ATP\textsuperscript{384,385}.

Astrocytes play an essential role in neuronal activation by maintaining neurotransmitter stores through the glutamine-glutamate cycle, which allows glutamate to be recycled by converting glutamate to glutamine by glutamine synthase\textsuperscript{394}. Glutamate released at the synapse is taken up by astrocytes via transporters, primarily Na\textsuperscript{+} dependent glutamate transporters GLT-1 (EAAT2) and GLAST (EAAT1)\textsuperscript{367,395}. This glutamate transport in astrocytes is driven by an electrochemical Na\textsuperscript{+} gradient: a glutamate molecule is co-transported with three Na\textsuperscript{+} ions into the cell and as a consequence, a K\textsuperscript{+} ion is released. This leads to an increase in the intracellular concentration of Na\textsuperscript{+}, which is balanced by the action of the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase. This process is an ATP-consuming process and involves the activation of glycolysis and lactate production\textsuperscript{367,395}. In this way, we can see that the coupling between neuronal metabolic activity, glutamate release, glycolysis in astrocytes, and lactate exchange, are essential steps to generate energy in neurons. This set of processes is known as astrocyte-neuron lactate shuttle model\textsuperscript{396} (Figure 1.16)
Figure 1.16 Schematic representation of the astrocyte-neuron lactate shuttle model.

GLUT1: glucose transporter 1, GLUT3: glucose transporter 3, MCT1: monocarboxylate transporter 1, MCT4: monocarboxylate transporter 4, GS: glutamine synthetase; Glu: glutamate; Gln: glutamine; GluR: glutamate receptor; EAATS: excitatory amino-acid transporters; GLS: glutaminase; LDH1, LDH5: isoenzymes of lactate dehydrogenase. It was adapted from Mason et al. 2017.396.
1.8.1.1 Glycogen metabolism

Glycogen is the primary energy reserve in the brain. It is mostly located in astrocytes, although it is also present in the choroid plexus cells and ependymal cells, as well as in some NSCs. The brain can be considered as a glycogen storage organ, whose function could be to provide glucose during physiological activity. Glycogen turnover is extremely fast in astrocytes and correlates with the activity of neighbouring neurons. The degradation of glycogen in the astrocytes is activated by various neurotransmitters (glutamate, norepinephrine, serotonin and histamine) via adenosine monophosphate (cAMP) and Ca\(^{2+}\). The breakdown of glycogen is controlled by phosphorylation of glycogen phosphorylase, causing it to move from its inactive form to the active phosphorylated form. A decrease in glucose levels causes a loss in glycogen storage, while insulin and similar growth factors increase the levels of glycogen stored in astrocytes. Alternatively, glucose from glycogen degradation can be metabolized via the pentose phosphate pathway to generate NADPH and protect astrocytes from oxidative stress.

1.8.1.2 Insulin metabolism in the brain

Initially, studies on the effect of insulin on the CNS began to observe its relationship with glucose metabolism. At the end of the 1950s, different studies showed that insulin was able to increase glucose uptake in spinal cord tissue, and also from different regions of the brain, such as the pituitary, and the choroid plexus, suggesting that glucose metabolism could be insulin dependent. As mentioned previously, the glucose transport in the brain depends on GLUTs, but it has not been described that insulin is involved in this transport. However, it has been described that insulin is used as a regulatory neuropeptide. Studies have shown how intracerebroventricular insulin injection decreased food intake and body weight in primates, while intravenous administration of insulin did not affect food intake in humans. These studies allowed to suggest that insulin, similar to leptin, could have a role in the regulation of energy and satiety in the CNS. These data were confirmed in a knockout mouse model of the insulin receptor, since these animals presented obesity induced by an increase in food intake, and insulin resistance, demonstrating the role that insulin plays in the regulation of feeding behaviour.

Insulin is transported from the bloodstream to the BBB, and then to the CNS, through a saturable transport system. Once the insulin passes through the BBB, it is coupled to its receptor which is abundant but not homogeneously distributed in the brain, regions such as the olfactory bulb, hypothalamus, cerebral cortex, cerebellum, hippocampus, and striatum have the highest concentration of the receptor. The insulin receptor (IR) which is highly abundant in neurons, is a protein composed of two subunits, an alpha subunit (extracellular), with a ligand-binding site, and
a beta subunit (transmembrane) with tyrosine kinase activity. Similarly, IGF-1 receptors (IGF-1R) were detected throughout neurons and glia cells and are composed of two subunits (α and β) that belong to the receptor tyrosine (Tyr) kinase superfamily. Interestingly, insulin and IGF-1 can bind and activate both IR and IGF-1R.

The binding of insulin to the α subunit of the IR activates the β subunit of the receptor, autophosphorylates the three tyrosine residues, becoming a fully active receptor. The insulin receptor substrate (IRS) can activate several pathways, including the pathway mediated by phosphatidylinositol 3-kinase (PI3K) that is the integrator of brain insulin signals. PI3K is a heterodimer with a catalytic and regulatory subunit that binds IRS through its Src homology-2 (SH2) domains; this activates the PI3K converting phosphatidylinositol 4,5 bisphosphate (PIP2) into phosphatidylinositol 3, 4, 5- triphosphate (PIP3). PIP3 binds to protein kinase B (PKB/Akt) which phosphorylates the Ser136 of the pro-apoptotic BAD protein (inactivated when phosphorylated) and prevents its binding to BCL-2, avoiding starting a pro-apoptotic cascade. PKB can also inhibit the pathway of caspases (apoptotic proteins), thus inhibiting apoptosis.

The glycogen synthase kinase-3 (GSK3) phosphorylates proteins associated with microtubules (such as Tau proteins which are a protein involved in the stabilization of microtubules) in the neurons; precisely, the hyperphosphorylation of Tau is associated with neurodegenerative processes, and insulin via the PI3K-PKB pathway regulates this phosphorylation process. In neurons, insulin can also activate the MAP kinase (mitogen-activated protein kinase) pathway, which is involved in synaptic plasticity, cell death (after oxidative stress) and N-methyl D-aspartate (NMDA) receptor-mediated excitotoxicity. All these pieces of evidence indicate that insulin acts in the brain as a neurotrophic factor.

1.8.1.3 Leptin metabolism

Leptin is a 146 amino acid peptide hormone after enzymatic cleavage and synthesized by adipocytes of white adipose tissue. This hormone is secreted into the bloodstream and binds to specific cell surface receptors, triggering the activation of specific intracellular signalling pathways. Leptin controls appetite by binding to leptin receptors in the hypothalamus, decreasing the synthesis and release of different neuropeptides. Leptin was identified during the search for a genetic mutation causing obesity in mice. Friedman and colleagues cloned the ob gene in mice and observed that in the homozygous state (ob/ob), this loss-of-function mutation caused hyperphagia and obesity in these animals. Subsequently, they named the hormone encoded by the ob gene leptin.
Leptin receptor (ObR) belongs to class I cytokine receptor family and encodes six different isoforms of the receptor, called ObRa to ObRf. These isoforms originate by alternative splicing of the original messenger RNA and share an extracellular domain and a common transmembrane domain\textsuperscript{412,413}. The intracellular domain is, on the contrary, variable and characteristic of each isoform. Thus, there are several short forms (ObRa, ObRc, ObRd and ObRf), one long (ObRb) and one soluble (ObRe). The long-form ObRb has the structural elements to be involved in signalling transduction\textsuperscript{414}. The \textit{db/db} mouse\textsuperscript{413} did not express the long ObRb isoform, while the other receptor isoforms were identified\textsuperscript{414,415}. ObRb is generally expressed in various hypothalamic areas of the CNS, including the arcuate nucleus, ventromedial hypothalamus, dorsomedial hypothalamus, paraventricular nucleus, and lateral hypothalamus\textsuperscript{416}. \textit{ObRb} (\textit{-/-}) mice develop severe hyperphagia, obesity and diabetes, mimicking the phenotype observed in \textit{db/db} and \textit{ob/ob} mice\textsuperscript{417}. The \textit{db/db} mouse model of leptin deficiency is a model of type 2 diabetes mellitus. The \textit{ob/ob} mice exhibit a mutation in the leptin gene, which makes unable to bind to its receptors. These data indicate that the metabolic actions of leptin depend mostly, if not exclusively, on the activation of ObRb in the SNC.

ObRb and activates intracellular signalling pathways such as JAK (janus kinase)/STAT (signal transducer and activator of transcription). The binding of leptin to ObRb determines the dimerization of the receptor and the binding of JAK2 to its cytoplasmic domain. Then, JAK2 phosphorylates tyrosines within the ObRb and triggers the activation of distinct downstream signalling pathways\textsuperscript{414}. The activation of ObRb promotes phosphorylation and dimerization of STAT3 (mediated by JAK2), which once activated translocates to the nucleus. In the nucleus, STAT3 binds to specific DNA sites to modulate gene transcriptional with necessary implications for the regulation of metabolism and body energy balance\textsuperscript{411,418}. JAK2 phosphorylates and activates IRS-2 (insulin receptor substrate-2), inducing the activation of PI3K (phosphoinositide 3-kinase). Since insulin receptor-dependent (RI) signal transduction is mediated by PI3K, IRS-2 constitutes a junction point between leptin and insulin signalling pathways\textsuperscript{419} (\textbf{Figure 1.17}).

We have described how different types of cells and molecules work together to maintain normal brain function. However, genetic factors, nutritional status, and the environment that individual has been raised modify the development of the nervous system. Therefore, this chapter will discuss how external factors can program permanent changes in physiology and metabolism that increase risks of diseases later in life.
Leptin binds to the extracellular domain of ObRb and induces ObRb dimerization and phosphorylation and activation of cytoplasmic associated JAK2 kinases. This association between ObRb and JAK2 enhances JAK2 kinase activity, resulting in phosphorylation of Y985, Y1077, Y1138 and autophosphorylation of JAK2. This phosphorylation activates downstream molecules such as STAT3 and IRS-2. STAT3 will subsequently become phosphorylated by JAK2 and translocate as dimers to the cell nucleus where it will modulate the expression of the target genes. IRS-2 is a junction point between leptin and insulin signalling pathways. JAK2: Janus kinase 2, IRS-2: insulin receptor substrate-2, PI3K: phosphoinositide 3-kinase, STAT3: signal transducer and activator of transcription 3, Tyrosine: Y. It was adapted from Wauman 2017[420].
1.9 Project Rationale

Previous studies have demonstrated the impact that maternal diet during the preimplantation period of development can have on the health of the offspring. A piece of growing evidence highlights the importance of the time around conception (periconceptional period) and the time from fertilisation to the time of implantation of the blastocyst in the uterus (preimplantation period)\[^{421-423}\]. This preimplantation period in mouse takes over four days and in humans 5 to 6 days\[^{424}\]. This period is characterized by different morphological and metabolic changes, and epigenetic modifications\[^{425}\]. It is remarkably sensitive to the environment, and alterations or disruptions within this window could have implications for the health of an organism and its predisposition to disease.

In our research group, previous studies have shown that a low-protein diet restricted only to the preimplantation period in mice and rats affects embryo development\[^{231,232,426-429}\]. This protein restriction has been associated with hypertension, anxiety-related behaviour, cardiovascular disease, and an increase in body fat in the offspring.

The consumption of HFD increases every year. Here, we have indicated that maternal HFD associated with obesity during pregnancy is detrimental to fetal development and increases the risk of many chronic diseases in the offspring, such as ADHD, autism spectrum disorders, depression and anxiety. Studies have shown that maternal obesity caused by HFD during pregnancy and lactation compromises brain development with lasting consequences on cognitive function in the offspring. However, the importance of maternal HFD during pregnancy and lactation in the absence of obesity for brain development in the offspring is unknown. Also, the impact of HFD on offspring health during the preimplantation period has not studied yet in in vivo models. Here in this project, we want to probe when during pregnancy, the offspring is more vulnerable to an HFD, either during the preimplantation period or after gestation and lactation.

The overnutrition model was used here (HFD) is relevant to mimic today’s pregnancies and may provide a useful insight towards developing guidance for the human population. The impact of maternal HFD in the offspring will be studied using three diet groups, the NFD, the Emb and the HFD group. At 26 weeks of age, the offspring of the NFD, Emb and HFD groups will be studied to observe differences in terms of metabolism in the brain and liver in chapter 3 (since these two organs were available).

This study will address the effect that these diets NFD (standard chow diet) and the HFD on the cytoarchitecture in the brain, especially in the cortex and the hippocampus. Initial studies will
determine the effect of the time of the maternal diet on different cell densities in the brain. (Chapter 4).

Maternal obesity has been studied for its effects on adult neurogenesis. It is unclear whether the effects seen in a maternal HFD model in the absence of obesity can alter adult neurogenesis in the offspring. Neurogenesis will be studied based on the cell density of the different type of cells for stem cells to mature neurons (Chapter 5).

It is hoped that by the end of this work, understanding the changes that occur in the offspring after exposure to a maternal HFD in the absence of obesity allow us to understand the impact of maternal diet and possibly develop preventive and preventive measures in the future.


1.10  Aims and Hypothesis

Exposure to a maternal HFD and associated obesity during pregnancy leads to a higher risk for children of developing diabetes, cardiovascular diseases, obesity and behavioural disorders. We propose that maternal HFD during gestation and lactation, or just during the periconceptional period, in absence of obesity, affects brain structure and development in the offspring mice. This may contribute to metabolic alterations and neuroinflammation in the adult offspring.

The project aim is therefore to evaluate the effect of maternal HFD in absence of obesity, on anatomy, structure and cell composition of the offspring brain.

The specific aims are as follow:

- **Aim 1:** To assess the effects of the maternal HFD on offspring metabolism, focusing on energy balance, and physical activity.

- **Aim 2:** To characterise any long-term neurodevelopmental consequences in adult offspring of dams given an HFD by analysing the cortex and hippocampus.

- **Aim 3:** To study the effects of the maternal HFD in adult neurogenesis in the offspring brain, focusing on the NSCs in the lateral ventricle and dentate gyrus.
Chapter 1
2.1 Breeding

Dr Judith Eckert and Oliver Hutton (Faculty of Medicine, University of Southampton) oversaw the breeding of the mice used for this research, before the start of my project. The mice strain used for the analysis was outbred MF1. MF1 animals were used to maintain a maximal level of heterozygosity, similar to what is seen in human populations\(^4^{30}\). Mice were bred in-house (University of Southampton Biomedical Research Facility) and maintained under a 12-hour light-dark cycle, at constant temperature (\(22\pm2^\circ\)C) with food and water available ad libitum. All mice were fed with a standard lab chow diet. Non-obese virgin dams at 7.5 -8.5 weeks old were left to mate overnight, and in the morning, the vaginal plug was checked to confirm sexual intercourse. The morning of plug confirmation was marked as day 0.5. This project was carried out under the UK Home Office License PPL 30/3001, and all mice and experimental procedures were conducted using protocols approved by, and in accordance with, the UK Home Office Animal (Scientific Procedures) Act 1986 and local ethics committee at the University of Southampton.

2.1 Maternal Diets

After pregnancy confirmation, eight dams were assigned to a diet group, and their diet was switched accordingly at day 0.5 during gestation and lactation, totalling 18 litters. The pregnant females could develop their pregnancies to term. The embryonic high fat diet group (Emb) was switched from the high-fat diet back to standard lab chow diet after 3.5 days of gestation (Figure 2.1). Once born, the number of pups were not adjusted per litter to avoid unexpected physiological changes due to the alteration of the litter size\(^4^{31-433}\). Litter size was not significantly different between the diet groups (measurements described in detail in chapter 3) and was included as a cofactor during the statistical analysis. After weaning, they were fed with the standard lab chow diet until 26 weeks of age. This part of the thesis was carried out by Dr Judith Eckert and Oliver Hutton.

The normal fat diet used was RM1 (Rat and mouse no.1 maintenance) obtained from SDS (Special Diet Services, Essex, UK) and contained 7.5% Kcal fat, 17.5% Kcal protein, 75% Kcal carbohydrates (Tables 2.1 and 2.2). The high-fat diet used was also obtained from SDS (diet code: 824053) and contained 45% Kcal fat, 20% Kcal protein, 35% Kcal carbohydrate (Tables...
Specific micro and macronutrient composition of the different diets is provided in Appendix A and B.

<table>
<thead>
<tr>
<th>Lipids</th>
<th>% weight (g) (source)</th>
<th>Normal fat diet (Standard chow diet) 3.53 kcal/g</th>
<th>High-fat diet (HFD) 4.54 kcal/g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 (soybean oil)</td>
<td>23.5 (lard, soybean oil)</td>
<td></td>
</tr>
<tr>
<td>% energy (kcal)</td>
<td>7.4</td>
<td>45</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Proteins</th>
<th>% weight (g) (source)</th>
<th>18 (whey, soya)</th>
<th>23.6 (casein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% energy (kcal)</td>
<td>17.5</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Carbohydrates</th>
<th>% weight (g)</th>
<th>70</th>
<th>46.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>% energy (kcal)</td>
<td>75.1</td>
<td>35</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1 Energy composition of the normal fat diet and the High-fat diet.
Data obtained from Special Diet Services (SDS). Standard chow diet (NFD) (RMI, Code number 801151). HFD (Code 824053).

<table>
<thead>
<tr>
<th>Fatty Acids breakdown (%energy)</th>
<th>NFD</th>
<th>HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saturated Fatty Acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C12:0 Lauric %</td>
<td>0.02</td>
<td>0.09</td>
</tr>
<tr>
<td>C14:0 Myristic %</td>
<td>0.14</td>
<td>0.44</td>
</tr>
<tr>
<td>C16:0 Palmitic %</td>
<td>0.31</td>
<td>4.08</td>
</tr>
<tr>
<td>C18:0 Stearic %</td>
<td>0.04</td>
<td>1.89</td>
</tr>
<tr>
<td><strong>Monounsaturated Fatty Acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14:1 Myristoleic %</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>C16:1 Palmitoleic %</td>
<td>0.09</td>
<td>0.09</td>
</tr>
<tr>
<td>C18:1 Oleic %</td>
<td>0.77</td>
<td>6.30</td>
</tr>
<tr>
<td><strong>Polyunsaturated Fatty Acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:2(ω6) Linoleic %</td>
<td>0.69</td>
<td>3.10</td>
</tr>
<tr>
<td>C18:3(ω3) Linolenic %</td>
<td>0.06</td>
<td>0.35</td>
</tr>
<tr>
<td>C20:4(ω6) Arachidonic %</td>
<td>0.13</td>
<td>0.01</td>
</tr>
<tr>
<td>C22:5(ω3) Clupanodonic %</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Table 2.2 Fat breakdown of the normal fat and the high-fat diet.
Data obtained from Special Diet Services (SDS). Standard chow diet (NFD) (RMI, Code number 801151). HFD (Code 824053).
<table>
<thead>
<tr>
<th></th>
<th>NFD</th>
<th>HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macro Minerals (%energy)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>0.73</td>
<td>0.60</td>
</tr>
<tr>
<td>Total Phosphorus</td>
<td>0.52</td>
<td>0.23</td>
</tr>
<tr>
<td>Sodium</td>
<td>0.25</td>
<td>0.16</td>
</tr>
<tr>
<td>Chloride</td>
<td>0.38</td>
<td>0.28</td>
</tr>
<tr>
<td>Potassium</td>
<td>0.67</td>
<td>0.61</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.23</td>
<td>0.08</td>
</tr>
<tr>
<td><strong>Micro Minerals</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron</td>
<td>159.30</td>
<td>58.50</td>
</tr>
<tr>
<td>Copper</td>
<td>11.50</td>
<td>8.76</td>
</tr>
<tr>
<td>Manganese</td>
<td>72.44</td>
<td>13.30</td>
</tr>
<tr>
<td>Zinc</td>
<td>35.75</td>
<td>71.75</td>
</tr>
<tr>
<td>Cobalt</td>
<td>634.10</td>
<td>0.00</td>
</tr>
<tr>
<td>Iodine</td>
<td>1202.69</td>
<td>254.84</td>
</tr>
<tr>
<td>Selenium</td>
<td>298.99</td>
<td>181.42</td>
</tr>
<tr>
<td><strong>Vitamins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin A</td>
<td>8554.27</td>
<td>4936.44</td>
</tr>
<tr>
<td>Vitamin D3</td>
<td>621.70</td>
<td>7836.60</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>84.10</td>
<td>97.31</td>
</tr>
<tr>
<td>Thiamine</td>
<td>8.58</td>
<td>7.36</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>4.33</td>
<td>6.37</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>7.49</td>
<td>30.85</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>2.59</td>
<td>0.00</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>0.79</td>
<td>2.45</td>
</tr>
</tbody>
</table>

Table 2.3 Macro and micronutrients of the normal fat and the high-fat diet.
Data obtained from Special Diet Services (SDS). Standard chow diet (NFD) (RMI, Code number 801151). HFD (Code 824053).
All mice at 7.5 - 8.5 weeks old were left to mate overnight. Female mice were fed different diets from conception: normal fat diet (NFD); high fat diet (HFD) throughout gestation and lactation (HFD) or embryonic HFD (Emb) comprising HFD for 3.5 days and NFD after that. After weaning, all offspring were maintained on NFD. Six male, and six female brains were collected per group. PND: postnatal day.
2.2  **Body Weight Gain**

Individual maternal body weight before, after delivery and after lactation was measured and an average weight of each experimental group was calculated. Body weight of the offspring was monitored every week, and the average weight of each experimental group was calculated. This part of the thesis was carried out by Oliver Hutton.

2.3  **Indirect Calorimetry**

Indirect calorimetry was measured using an Oxylet (Panlab, Cornella, Spain). The mice were moved to a different room between 3 and 5 pm, where they would be isolated for the 24-hour analysis. Each mouse was placed in an individual metabolic chamber at 25°C (Oxylet LE 1335 Physiocage, PANLAB). Each cage has a food and drink dispensers that quantify food and drink consumption through weight changes detected by load cells. All mice had *ad libitum* access to food and water throughout the study.

After three hours of acclimation time, oxygen consumption and carbon dioxide production were analysed every 10 minutes using the Oxylet LE 405-gas analyser (PANLAB). The respiratory gas analysis was carried out using Metabolism software 2.1.02 a computer-assisted data acquisition program (Oxylet; Panlab, Cornella, Spain) over to 24-h period. Energy expenditure (EE) was calculated according to the following formula: 

\[
EE (\text{kcal} / \text{day} / \text{kg of body weight}^{0.75}) = \nu O_2 \times 1.44 \times (3.815 + (1.232 \times RQ)^{0.34}).
\]

The respiratory quotient (RQ), which is the ratio of CO$_2$ produced to O$_2$ consumed while food is metabolized, was calculated by dividing the vCO$_2$ produced by the vO$_2$ consumed (RQ = VCO$_2$ / VO$_2$).

The system calculated the activity of each animal by detecting changes of potential and kinetic energy produced by the animal in its movements. The system measures the amount of force exerted by a particular activity which is detected by the weight transducer. The transducer is located below the cage to detect all activity variations (Oxylet LE 1335 Physiocage, PANLAB). Oliver Hutton did these measurements.
2.4  Tissue Collection

2.4.1  Adult brain and liver

At 26 weeks of age, the animals were killed (between 9 am, and 11 am), and their brains and livers were removed. Six female and six male brains and livers from each of the three diet groups, 36 in total, were randomly selected, ensuring each was from a different litter. Mice were not transcardially perfused. The brains were cut sagittally along the midline, and half of the brain was directly fixed by placing them in 4% paraformaldehyde (PFA) for 2 hours. Then samples were cryoprotected into 30% sucrose solution until specific gravity between the solution and the brain was equilibrated at 4°C (brain sank to the bottom of the flask), to avoid ice crystal formation and prevent tissue damage. The olfactory bulb was removed from all brains. The other hemisphere of the brain (the cortex and the hippocampus were dissected out), and the liver (left lobe) frozen in liquid nitrogen and stored at -80°C for future analysis (western blotting and RT-PCR). The left liver lobe of the adult offspring was selected since this lobe seems to be more sensitive to maternal dietary alterations. This part of the project was carried out by Oliver Hutton and Dr Sandrine Willaime-Morawek.

The selected hemisphere was embedded in Tissue Tek (OCT embedding compound; Miles) which preserves the antigenicity for target antigens. Before embedding the brains, a mould with a cylindrical shape (1 cm in diameter) was made with aluminium foil, and the bottom was filled with OCT (about 0.5 cm depth). The hemisphere was placed with the frontal side facing down into the OCT filled the mould with the medulla/cerebellum facing upwards. It was ensured that the brains sat vertically so that the coronal slices were not angled. The OCT/mould was then immersed in a beaker filled with isopentane for snap-freezing the tissue. Isopentane has a high thermal conductivity, which helps tissue sample freezes faster and evenly. The OCT/mould was placed in a foam cooler filled with crushed dry ice until the tissue was frozen. An effort was made to remove any bubbles from OCT, so they did not interfere with the cutting. All hemispheres were wrapped in aluminium foil and stored at -80°C until processed.

2.4.2  Postnatal day one animals

Postnatal day 1 (PND1) animal bodies were fixed with 4% formaldehyde solution and stored at room temperature in a 70% ethanol solution. The animals were decapitated, and the brains were flushed with 1XPBS, removed from the skull, and then slowly rehydrated in 50%, and 30%, 1X PBS, 24 hours each step. Then brains were cryoprotected by transferring them to
15% sucrose and then 30% sucrose solution in PBS until they sank, at 4°C. After overnight cryoprotection, tissues were equilibrated in 50:50 30% sucrose in PBS:OCT (Tissue Tek) for 1 hour and subsequently oriented and embedded in OCT. Tissue blocks were stored in -80°C.

### 2.5 Brain section cutting

The adult brains were cut using a cryostat at -24°C at a thickness of 14 μm. For cortical analysis, coronal brain cryostat sections (14 μm thick) were made between bregma 1.42 mm and bregma 0.26 mm, according to the stereotaxic coordinates of the mouse brain atlas. For hippocampal analysis, coronal brain sections (14 μm thick) were made between bregma -1.22 mm and bregma -2.70 mm for hippocampal samples, and from bregma 1.10 mm to bregma 0.14 mm for lateral ventricle. PND 01 brain tissue was sectioned at 14 μm thickness onto Superfrost Plus slides (Fisher Scientific) on a Leica cryostat and stored at -20°C. Coronal brain sections were made between bregma 3.39 mm and bregma 3.63 mm for hippocampal samples, according to the stereotaxic coordinates of the P0 mouse brain atlas.

Brains were cut into coronal sections and mounted in microscope slides. Each microscope slide contained eight brain slices and a superscript number of 1 to 5 referring to the number of the microscope slide within each set, for a total of 8 sets. This arrangement of the brain slices across the microscope slides can be seen in Figure 2.2. For each brain, we obtained a total of 40 microscope slides (8 sets with 5 microscope slides each) with eight brain slices each. For example, for C1M1 we got the following eight sets C1M1: 1<sup>1</sup>-<sup>5</sup>, 2<sup>1</sup>-<sup>5</sup>, 3<sup>1</sup>-<sup>5</sup>, 4<sup>1</sup>-<sup>5</sup>, 5<sup>1</sup>-<sup>5</sup>, 6<sup>1</sup>-<sup>5</sup>, 7<sup>1</sup>-<sup>5</sup>, 8<sup>1</sup>-<sup>5</sup>.

The slides were labelled as follows:

- First is a letter that refers to the diet the mother: C - control fat diet; H - HFD group; and E - Emb group.
- The second digit, a number, refers to the litter within each diet group that the mouse came from.
- The third letter is either an M or an F referring to the gender of the mice.
- The final number refers to the number of the mouse within the litter.

However, the codes were then randomized by an external individual who kept the identity of the animals hidden until the analysis of collected data.
Chapter 2

2.6 Immunostaining

One slide was selected from each of the 36 brains from the anterior cerebral cortex and one from the dorsal hippocampus. Therefore, six slides were selected per group for each of the staining. An additional slide was chosen to act as a negative control. The remaining slides were kept at -20°C for further analysis. The washing solution (PBST) consisted of PBS (0.1M; pH 7.4) and 1% Triton X-100. This buffer was used in all washes and incubations.

Before starting, slides were thawed for 20 min at 37°C. Citrate buffer (ThermoFisher Scientific, UK) was used for heat shock antigen retrieval using an 800watts microwave at half power for 25 minutes. Slides were then placed under cold running water for 3 minutes and washed in PBS 3 times for 5 minutes, and let the slides dry out at 37°C for 5 minutes. Sections were drawn around with an ImmunoPen® (Merck Millipore), which forms a hydrophobic barrier retaining the antibodies, minimizing the waste of antibodies. Permeabilization of the cell membrane for antibody entry was carried out by the addition of 300μl of PBST (PBS and 1% Triton X-100) to each of the slides. The slides were then incubated for 20 minutes at room temperature. The PBST from the Permeabilization stage was tipped off, and 300μl of blocking solution PBST and 10% Donkey Serum (Sigma-Aldrich, UK) was added to each slide to reduce nonspecific binding. The slides were then placed into an incubation chamber at 37°C for 1 hour. The sections were then incubated with a primary antibody overnight at 4°C. Primary antibodies were diluted in blocking buffer. The concentration of all antibodies was optimized before analysis. Then 500μl of the primary antibody solution was added to each slide. The primary antibodies used here are (Table 2.3):

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
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<td>12</td>
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<td>13</td>
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<td>21</td>
<td>26</td>
<td>22</td>
<td>27</td>
<td>23</td>
<td>28</td>
</tr>
<tr>
<td>31</td>
<td>36</td>
<td>32</td>
<td>37</td>
<td>33</td>
<td>38</td>
</tr>
<tr>
<td>C1M1</td>
<td>1²</td>
<td>C1M1</td>
<td>1²</td>
<td>C1M1</td>
<td>1³</td>
</tr>
</tbody>
</table>

Figure 2.2 Diagram showing the arrangement of each brain sections across one of the eight sets;

This method was essential in ensuring there was a selection of anteroposterior levels on each slide and that five equivalent slides were obtained for different staining to be compared on consecutive sections. The sections were stored at -20°C before further analysis.
<table>
<thead>
<tr>
<th>Target</th>
<th>Antibody Type</th>
<th>Distributor</th>
<th>Product ID</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DAPI</strong></td>
<td></td>
<td>Calbiochem</td>
<td>CAS 28718-90-3</td>
<td>1:1000</td>
</tr>
<tr>
<td><strong>DCX. Doublecortin</strong></td>
<td>Goat anti-mouse pAb</td>
<td>Santa Cruz</td>
<td>Sc-8066</td>
<td>1:200</td>
</tr>
<tr>
<td><strong>GFAP. Glial Fibrillary Acidic Protein.</strong></td>
<td>Rabbit anti-mouse mAb</td>
<td>Dako</td>
<td>Z0334</td>
<td>1:500</td>
</tr>
<tr>
<td><strong>Iba1. Ionized Calcium Biding Adapter molecule 1.</strong></td>
<td>Rabbit anti-mouse mAb</td>
<td>Wako</td>
<td>019-19741</td>
<td>1:200</td>
</tr>
<tr>
<td><strong>NeuN. Neuron-Specific Nuclear protein.</strong></td>
<td>Rat anti-mouse mAb</td>
<td>Merck Millipore</td>
<td>MAB377</td>
<td>1:200</td>
</tr>
<tr>
<td><strong>Sox2. Sex Determining Region Y-Box 2.</strong></td>
<td>Goat anti-mouse mAb</td>
<td>Santa Cruz</td>
<td>Sc-17320</td>
<td>1:200</td>
</tr>
<tr>
<td><strong>S100b. S100 calcium-binding protein B</strong></td>
<td>Mouse anti-mouse mAb</td>
<td>Abcam</td>
<td>Ab52642</td>
<td>1:200</td>
</tr>
</tbody>
</table>

Table 2.4 Primary antibodies used in immunohistochemical techniques.

The following day, the sections were washed (3 times for ten minutes each) and incubated with the corresponding secondary antibody. Secondary antibodies solution was prepared at a 1 in 200 concentration in blocking buffer. Alexa Fluor® 488-donkey anti-mouse (Merck Millipore), Alexa Fluor®, 488-donkey anti-rabbit (Merck Millipore), Alexa Fluor®, 568-donkey anti-mouse (Merck Millipore), Alexa Fluor®, 568-donkey anti-rabbit (Merck Millipore), and Alexa Fluor®, 568-donkey anti-goat (Merck Millipore) were used depending on the primary antibody. Then 500μl of the secondary antibody solution was added to each of the slides. All slides were then covered in a closed container in the dark and placed in an incubation chamber at 37°C for 2 hours. Then the slices were then washed three times for 5 minutes.
Next, nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (1μg/ml diluted in PBST buffer). Then 500μl of the DAPI solution was applied to each of the slides and left for 5 minutes in a closed container in the dark at room temperature. The slides were then washed three times for 5 minutes per wash with 0.1M PBS. All slides were then mounted with Mowiol®4-88 Reagent (Calbiochem, Darmstadt, Germany) and a glass coverslip. The slides dried overnight in the fridge at 4°C. Images were captured using a Leica DM5000 B fluorescence microscope coupled to a Leica DFC300FX microscope camera. Negative controls, where the primary antibody was omitted, were included in every assay and showed no immunofluorescence signal. ImageJ was used for quantification in the different brain areas.

## 2.7 Cell Density

### 2.7.1 PND 01 analysis

PND1 brains were analysed by counting positive cells in the different regions of interest in the hippocampus and the cortex (Figure 2.3). A part of this assay was done in collaboration with Callum Robins (BM student, Faculty of Medicine, University of Southampton). The camera on this microscope captured images in greyscale intensity and then converted the images back to colour in the LAS-X software (Leica UK Ltd, Milton Keynes). For each brain, we collected 2 or 3 images for each cortical or hippocampal area analysed at x40 magnification. Each area measuring 340μm x 260μm (88400μm²) were placed covering the entire hippocampus and cortex, and positive Iba1 cells were counted within these regions of interest (ROIs) (Figure 2.4). The total number of cells counted was divided by the total area for each sample – giving a normalised cell density.
Figure 2.3  Iba1 staining in the cortex, and hippocampus of the PND01 mouse brain. 
The coronal plane of the PND01 mouse brain. Images show (a) DAPI staining, (b) Iba1 positive cells, and (c) merged between DAPI and Iba1 cells. Scale bar 500μm.
Figure 2.4 Iba1 staining in the cortex, and hippocampus (Dentate gyrus, CA1 and CA3).
The coronal plane of the PND 01 mouse brain. The first column is DAPI staining, middle column Iba1 positive cells, and the right column is merged between DAPI and Iba1 cells. Region of interest is the area in yellow. Scale bar 100 µm.

Postnatal day 1 (PND1) brain sections were analysed by the fraction of a given ROI with the GFAP signal, and the mean intensity of GFAP signal was measured using ImageJ (Figure 2.5). The regions of interest were determined based on the granular cell layer (GCL), and the hilus in the dentate gyrus. We used the auto-threshold algorithm to find an appropriate threshold value for each ROI of each picture. For each sample, 3 to 4 pictures were taken, and the average of the auto-threshold
values was used to take the value from the averages of each brain’s Area Fraction and Mean Intensity. This assay was done in collaboration with medical student Callum Robins (Faculty of Medicine, University of Southampton) (Figure 2.6).

Figure 2.5 GFAP staining in the cortex, and hippocampus of the PND01 mouse brain. The coronal plane of the PND01 mouse brain. Images show (a) GFAP staining, (b) DAPI positive cells, and (c) merged between DAPI and GFAP cells. Scale bar 500µm.
Figure 2.6 GFAP expression in the dentate gyrus and hilus at PND1.

a. The top row is showing the coronal plane of the area analysed in the granule cell layer (white). b. The middle row is showing the coronal plane of the area examined in the hilus (yellow). c. The bottom row shows the coronal plane of both granule cell layer and hilus. Magnification x40. Scale bar 100 µm.
2.7.2 Cortex density

The counting method used in this thesis consisted in an unbiased counting frame, counting the positive cells that crossed 2 sides of the box (inclusion zone), while the other two sides of the box were ignored and not included in the quantification (forbidden zone), according to the following criteria\textsuperscript{437} (Figure 2.7):

- Any positive cell that touches the forbidden lines, crosses the forbidden lines or is outside the counting frame is not counted.
- Any positive cell that is within the counting frame, touch an inclusion line from inside or outside the counting frame, or cross the inclusion line, will be included in the count.
- If a cell touches or crosses both an inclusion line and a forbidden line, it is not included in the counting.

![Figure 2.7 Counting frame showing the counting rules.](image)

The forbidden lines in red, and inclusion lines in black. Counted objects are indicated in green, and not included objects in dark blue.

Images of the cortex were taken from the brain slices from each of the diet groups. In each section, images of a single focal plane of the somatosensory cortex were captured using x10 magnification. To ensure consistency across the images for each one the samples, three different ROIs were drawn per layer for a total of 15 ROIs (Figure 2.8). ImageJ was used to manually count positive cells (DAPI\textsuperscript{+},
NeuN⁺, GFAP⁺, S100β⁺ and Iba1⁺) present within an ROI with a set area of 52250µm² (346µm x 151µm). Sample codes and experimenter were blinded prior to the analysis, to avoid experimenter-induced bias. Positive cells (NeuN⁺, GFAP⁺ and Iba1⁺) were counted on the set area, and cell densities were calculated as the number of positive cells per mm². Three measurements were taken per layer per image and averaged. Cell counting was performed as follows:

**Figure 2.8 Quantification of cell density.**
Image shows DAPI (blue) staining of the coronal plane of the cortex in the brain. Each layer of the cortex is marked by an arrow and labelled. The boxes represent the area in which the cells were counted, three measurements per layer. Each box represents the same area, and the same method was used for each marker, NeuN, GFAP, Iba1 and DAPI. Scale bar 200µm.
2.7.3 Hippocampus cell density

Labelled cells in the dorsal hippocampal were counted in the dentate gyrus, CA1 and CA3. Images were taken in different areas containing GFAP, Iba1 and Sox2 positive cells: in the dentate gyrus including the Granular cell layer (GCL), Subgranular zone (SGZ) and Hilus (Figure 2.9), CA1, and CA3 regions. In each section, images of a single focal plan of the dentate gyrus, the CA1, and CA3 regions were captured using x40 magnification. The quantification of positive cells for the GFAP⁺, Iba1⁺ and Sox2⁺ cells was performed by counting manually with ImageJ, by an experimenter blind to the experimental conditions. Twenty-one regions of interest were placed in the different regions of the dentate gyrus, each one with a different area. The different areas for each region of interest were 2450μm² (35µm x 70µm) in the GCL, 1750μm² (25µm x 70µm) in the SGZ and 4900μm² (70µm x 70µm) in the hilus.

![Figure 2.9](image)

**Figure 2.9 Quantification of cell density in the hippocampus.**
A. Coronal sections, showing the localization of the hippocampus and dentate gyrus (DG). B. Coronal plane of the areas representing different regions in the DG where the cells were counted. C. Coronal plane of the areas represent different regions in the Hilus of the DG where the cells were counted. Images show DAPI staining (blue). Scale bars are 100µm in B and C. CA1: Cornu Ammonis 1, CA3: Cornu Ammonis 3, DG: Dentate gyrus, GCL: Granular cell layer, SGZ: Subgranular zone.
The quantification for NeuN and DCX markers, was performed by counting positive cells with ImageJ in the different layer of the dentate gyrus. Three slices per animal and 21 non-overlapping fields were analysed at x40 magnification. For quantitative analysis of GFAP⁺/Sox2⁺ and GFAP⁻/Sox2⁺ located in the SGZ were counted at x63. In each region, the same area was used to ensure consistency across the images. Cells were counted on each region of interest and cell densities were calculated as the number of positive cells per mm².

### 2.7.4 Cortical layer thickness

Coronal DAPI-stained adult mice brain sections from 6 males and 6 females per group were used for this analysis. Cortical thickness was evaluated from different coronal brain sections considering 3 defined points in the right cortical hemisphere. Images were acquired at rostral and central level of the somatosensory cortex, with a Plan semi-Apochromat x10/0.3 objective (Figure 2.10). Cortex thickness measurements were done using the ImageJ software.

![Quantification of cortical layer thickness](image)

**Figure 2.10 Quantification of cortical layer thickness.**

Each of the layers is depicted, showing where the layers were measured during quantification. The yellow bracket represents the full thickness of the cortex. Image shows DAPI staining (blue). Scale bar 250µm.
2.8 Protein Analysis

2.8.1 Total protein extraction

Individual cortex and hippocampal tissues dissected from the second hemisphere (NFD: n = 12; Emb: n=12; HFD: n = 12) were manually homogenized on ice in 300-500 µl of lysis buffer, adjusting the buffer volume according to the tissue size (cortex is bigger than hippocampus). The lysis buffer, radioimmunoprecipitation assay buffer (RIPA buffer), contained 150 mM sodium chloride (NaCl), 0.5% (v/v) Sodium deoxycholate, sodium dodecyl sulphate (SDS, 0.1 %), 50mM Tris-Cl (ph:8.0), 1% (v/v) NP-40, and a CalBiochem protease inhibitor cocktail III EDTA free (1:100, Merck Millipore, 539134-1ML) for inhibiting proteases as well as aminopeptidases. Lysates were centrifuged at 20,000x g for 30 minutes at 4°C. Supernatants were collected and stored at -20°C for later use.

2.8.2 Quantification of protein concentration

Total protein concentration was determined using the Pierce™ BCA Protein Assay (ThermoFisher Scientific, cat 23227). Protein determination by BCA is a highly sensitive method. This method combines the reaction of the proteins with Cu²⁺ in an alkaline medium (producing Cu³⁺) with a reagent for the detection of Cu³⁺ highly selective and sensitive called bicinchoninic acid.

Bicinchoninic acid (BCA), in the form of sodium salt is water-soluble, and is a highly sensitive and specific reagent for the Cu⁺ ion. The macromolecular structure of the protein and four specific peptides (cysteine, cystine, tryptophan and tyrosine) have been reported to be responsible for colour formation. Quantification by BCA combines the Biuret reaction (reaction of the protein with Cu²⁺ in an alkaline medium to produce Cu³⁺). The reaction product has a purple colour, formed by the interaction of two molecules of BCA with the Cu⁺ ion. This product is soluble in water and exhibits a strong absorbance at 562nm.

Samples were mixed with the BCA Working Reagent (ThermoFisher Scientific, cat 23227), following the manufacturer’s instructions and incubated for 30 minutes at 37°C. For each assay, a standard curve was prepared with increasing concentrations, from 0 to 2000 μg/ml of bovine serum albumin (BSA). The standard curve and unknown samples were pipetted into a 96-well plate and the absorbance measured at 562 nm in a TECAN Infinite M200 spectrophotometer.
2.8.3 **Western blotting**

Three parts of an appropriate amount of total proteins (10 or 20 μg depending on the protein to be analysed), was diluted with one-part 4x Laemmli buffer (Bio-Rad, 1610747). Laemmli contained Tris-HCl (277.8 M, pH 6.8), glycerol (44.4 %, increases sample density), SDS (4.4%, give proteins same negative charge), bromophenol blue (0.02 %, migration indicator) and β-mercaptoethanol (355nM, Bio-Rad, 1610710; reduces disulphide bonds) and denatured in a thermoblock for 7 minutes at 100°C.

The protein was resolved using gradient gels by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Proteins were separated electrophoretically (100V at room temperature for 60min) in a 4-20% Mini-Protean® TGX Precast gel (4568093, Bio-Rad, UK) gel and proteins were blotted onto polyvinylidene fluoride (PVDF) membranes (0.22µm or 0.45 via µm pore size) performing a wet transfer (80 V for 60 minutes at 4°C). Each membrane was then blocked with Tris-buffered saline (TBS, 20 mM) containing 0.1% Tween 20 and 5% non-fat dried milk for 1 hour at room temperature and incubated overnight at 4°C under agitation with a primary antibody. Primary antibodies used a listed-on table 2.4:

<table>
<thead>
<tr>
<th>Target</th>
<th>Antibody Type</th>
<th>Distributor</th>
<th>Product ID</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH. glyceraldehyde 3-phosphate dehydrogenase</td>
<td>Rabbit anti-mouse pAb</td>
<td>Sigma-Aldrich</td>
<td>G9545</td>
<td>1:10000</td>
</tr>
<tr>
<td>GAPDH. glyceraldehyde 3-phosphate dehydrogenase</td>
<td>Mouse anti-mouse mAb</td>
<td>Sigma-Aldrich</td>
<td>G8795</td>
<td>1:1000</td>
</tr>
<tr>
<td>GFAP. Glial Fibrillary Acidic Protein.</td>
<td>Rabbit anti-mouse mAb</td>
<td>Dako</td>
<td>Z0334</td>
<td>1:10000</td>
</tr>
<tr>
<td>NeuN. Neuron-Specific Nuclear protein.</td>
<td>Rat anti-mouse mAb</td>
<td>Merck Millipore</td>
<td>MAB377</td>
<td>1:5000</td>
</tr>
<tr>
<td>S100b. S100 calcium-binding protein B</td>
<td>Mouse anti-mouse mAb</td>
<td>Abcam</td>
<td>Ab52642</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

Table 2.5 Primary antibodies used for western blotting.
Membranes were washed and then incubated for 1 hour at room temperature with the corresponding secondary antibody:

- IRDye®800CW Conjugated Goat (polyclonal) Anti-rabbit; 1:10,000 (LI-COR, 925-32211).
- IRDye®680RD Conjugated Goat (polyclonal) Anti-mouse, 1:10,000 (LI-COR, 925-68071).

All solutions were prepared in 5% non-fat milk/TBS-T. The Odyssey imaging system was used to quantify the fluorescence intensity of infrared dye–conjugated secondary antibodies (LI-COR). Optical density measurements were quantified using Image Studio Lite version 5.2. Image Studio Lite calculates the average of the pixels in the frame. To acquire the final value for pixel quantification, a signal value was calculated as the sum of the pixel intensities in a frame (in that case, a cassette band) and subtracting the background value. Gel loading variability was normalized with the GAPDH protein and data were normalized on each gel. This means that the optical density of each band normalized to corresponding GAPDH band.

All antibodies were validated. In order to determine the amount of protein that would produce a clear signal without saturation, all the antibodies were characterized by probing cortex tissue samples over a series of different protein amounts (Figure 2.11). It was also necessary to show that in the absence of protein or primary antibody, no signal was detected.

![Figure 2.11](image.png)

**Figure 2.11 GFAP Protein loading optimization**

A range of 5-20µg of cortex homogenate was used for Western-blotting with GFAP (top) and GAPDH (bottom) antibodies detection to determine the optimal protein amount to load.
2.9 Quantitative real-time PCR

2.9.1 RNA isolation

Total RNA was isolated from Snap frozen liver and Snap frozen brain cortex and hippocampus using a RNeasy Lipid Tissue mini kit (Qiagen, UK) according to the manufacturer’s instructions. Briefly, tissue of interest was immediately homogenized using a handheld electric homogeniser with the QIAzol reagent, to dissolve the new homogenized solution into DNA, RNA and protein. Then we added chloroform in order to separate the mixture into RNA, DNA and organic phases. The RNA was precipitated and eluted from the midi columns with two aliquots of 250µl each of RNAse free water. The isolated RNA was quantified using a Nanodrop ND-1000 spectrophotometer (LabTech UK), and only samples with an adequate RNA concentration (A260/A280 ≥1.8) and purity (A230/A260≥2.0) were selected for reverse transcription. RNA samples were stored at -80°C until use.

2.9.2 cDNA synthesis

1µg of RNA from each sample was reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad, UK) following the manufacturer’s protocol. Briefly, 1µg of RNA was added to 4µl of 5x iScript reaction mix and 1µl of iScript reverse transcriptase and RNA free water to make the volume to 20µl (Table 2.5). This 20µl-solution was placed in a PTC240 tetrad 2peltier thermal cycler at 25°C for 5 minutes, then at 48°C for 20 minutes for reverse transcription, and finally in order to inactivate the enzyme the solution was incubated at 95°C for 1 minute. The resultant cDNA was kept at 4°C until cDNA was diluted in 1 in 5 RNAse free water and stored at -20°C for later use in qPCR.

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x iScript Reaction Mix</td>
<td>4µl</td>
</tr>
<tr>
<td>iScript Reverse Transcriptase</td>
<td>1µl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>11µl</td>
</tr>
<tr>
<td>RNA template 1µg</td>
<td>4µl</td>
</tr>
</tbody>
</table>

Table 2.6 Components of reverse transcription mix.
2.9.3 Quantitative polymerase chain reaction assay (qPCR)

We decided to use qPCR for our analysis, since this technique has been described as a powerful method to assess transcriptional differences between samples. In order to perform the different qPCR-based assays, the first step we did was to optimize the tests following the MIQE guidelines\textsuperscript{441,442}. In this way the design of the primers, the optimization of the assays, and qPCR standards were carried out following the MIQE guidelines.

RNA levels of different markers were quantified using highly specific primers, table 2.6 (Sigma Aldrich, or Primer Design, UK). Gene-specific primers were designed according to the parameters recommended by the guidelines:

- Each primer must be between 9 and 40 nucleotides base length.
- Annealing temperature of the primers must be between 58°C and 60°C, and the annealing temperature difference between the primers does not exceed 2°C.
- The length of the amplicon should be between 50 and 150 nucleotides to prevent the formation of secondary structures and produce efficient amplification.
- The GC content should be maintained within 30-80% with no more than two GC residues within the last five nucleotides at the 3’ end.
- Where possible one primer of each pair recognised an exon-exon boundary to prevent the amplification of genomic DNA.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Name</th>
<th>Accession No.</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AdipoR1</strong></td>
<td>Adiponectin receptor 1</td>
<td>NM_001306069</td>
<td>Forward: AGTGCATGTTGGTGGTACAACAAC&lt;br&gt;Reverse: AGACCCCGGACACCAAGAG</td>
</tr>
<tr>
<td><strong>AdipoR2</strong></td>
<td>Adiponectin receptor 2</td>
<td>NM_001355692</td>
<td>Forward: GGTGCGACCTTCCAGGACCC&lt;br&gt;Reverse: AGAGCAGGATGTGTCTGTTGGCT</td>
</tr>
<tr>
<td><strong>Bax</strong></td>
<td>BCL2-associated X protein</td>
<td>NM_007527.3</td>
<td>Forward: CGTGTTGCCCTTCTCTAC&lt;br&gt;Reverse: CCAGCCACCTGGTCTTG</td>
</tr>
<tr>
<td><strong>Bcl2</strong></td>
<td>BCL2 apoptosis regulator</td>
<td>NM_009741.5</td>
<td>Forward: ATGACTGAGTACCTGAGCC&lt;br&gt;Reverse: ATGCAGGGCTGATTAGTTTC</td>
</tr>
<tr>
<td><strong>Bdnf</strong></td>
<td>Brain derived neurotrophic factor</td>
<td>NM_001048139.1</td>
<td>Forward: TCTACTTCGTTGCTGAGAG&lt;br&gt;Reverse: AGACCCCTGAGCAGCC</td>
</tr>
<tr>
<td><strong>Cd11b</strong></td>
<td>Integrin alpha M</td>
<td>NM_001082960.1</td>
<td>Forward: TCCGGTACCATCAACACAT&lt;br&gt;Reverse: GGTGAATGTAATCCGAGACT</td>
</tr>
<tr>
<td><strong>Cd68</strong></td>
<td>CD68 antigen</td>
<td>NM_001291058.1</td>
<td>Forward: TTCTGCTTGGAATAAGG&lt;br&gt;Reverse: GAGAAATGCGGCCGAAGT</td>
</tr>
<tr>
<td><strong>Csfr1</strong></td>
<td>Colony stimulating factor 1 receptor</td>
<td>NM_001037859.2</td>
<td>Forward: GCAGTACCACCATCCACTTGTA&lt;br&gt;Reverse: GTGAGACACTGTCCCTCAGGTCG</td>
</tr>
<tr>
<td><strong>Cx3cr1</strong></td>
<td>Chemokine (C-X3-C motif) receptor 1</td>
<td>NM_009987.4</td>
<td>Forward: TGAAGTGACCCTGATCTCG&lt;br&gt;Reverse: AATAACAGGCTCAGCAGAAC</td>
</tr>
<tr>
<td><strong>Gfap</strong></td>
<td>Glial fibrillary acidic protein</td>
<td>NM_001131020.1</td>
<td>Forward: TCCGGGAAAGCAACACAC&lt;br&gt;Reverse: CAGCCTAGGTTGTTTACAT</td>
</tr>
<tr>
<td><strong>Glut1</strong></td>
<td>Solute carrier family 2 (facilitated glucose transporter), member 1</td>
<td>NM_0011400.3</td>
<td>Forward: AGAAGAGGGTCGGGACAGTA&lt;br&gt;Reverse: AAGTTTGAGGTCAGTGAAG</td>
</tr>
<tr>
<td><strong>Glut3</strong></td>
<td>Solute carrier family 2 (facilitated glucose transporter), member 3</td>
<td>NM_0011401.4</td>
<td>Forward: GCCCGAGAGTCAGAGGTTCT&lt;br&gt;Reverse: ACCAG太平洋GCAACCATAC</td>
</tr>
<tr>
<td><strong>Glut4</strong></td>
<td>Solute carrier family 2 (facilitated glucose transporter), member 4</td>
<td>NM_001359114</td>
<td>Forward: GTGTTCTGGTGCTCTTATG&lt;br&gt;Reverse: TGCCACATGAACCAGGGAA</td>
</tr>
<tr>
<td><strong>Glut5</strong></td>
<td>Solute carrier family 2 (facilitated glucose transporter), member 5</td>
<td>NM_019741.3</td>
<td>Forward: TCTTTGTTGAGGCTGTTGG&lt;br&gt;Reverse: GACAATGACACAGAATGGCTG</td>
</tr>
<tr>
<td><strong>Glut8</strong></td>
<td>Solute carrier family 2, (facilitated glucose transporter), member 8</td>
<td>NM_019488.5</td>
<td>Forward: CAGCTGATGTTGCTACGG&lt;br&gt;Reverse: CCAGGCCACTCGAGGAC</td>
</tr>
<tr>
<td><strong>Iba1</strong></td>
<td>Allograft inflammatory factor 1</td>
<td>NM_001361501.1</td>
<td>Forward: GTTCTGAAGGGGATGCTG&lt;br&gt;Reverse: CATTCTCAAGATTGCGAGATC</td>
</tr>
<tr>
<td><strong>Ifg1R</strong></td>
<td>Insulin-like growth factor 1 receptor</td>
<td>NM_010513.2</td>
<td>Forward: CACTTGATGACGTCTCC&lt;br&gt;Reverse: GAGAATTTCTCTACATCATTCATC</td>
</tr>
<tr>
<td><strong>Il-1b</strong></td>
<td>Interleukin 1 beta</td>
<td>NM_008361.4</td>
<td>Forward: GAAGAGGCTCATCTCTGGA&lt;br&gt;Reverse: TTCATCTGGGAGGCTGATG</td>
</tr>
<tr>
<td><strong>Il-4</strong></td>
<td>Interleukin 4</td>
<td>NM_021283.2</td>
<td>Forward: CCTGCGTCTTTGCTGAAATG&lt;br&gt;Reverse: TTTAAGTGATGAGCTGAGAC</td>
</tr>
<tr>
<td><strong>Il-6</strong></td>
<td>Interleukin 6</td>
<td>NM_001314054.1</td>
<td>Forward: CTCGGCAAGACTTCCCATCC&lt;br&gt;Reverse: TGAAGTCTCCTCTCCGGACT</td>
</tr>
</tbody>
</table>
Table 2.6 (continued).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Name</th>
<th>Accession No.</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Il-10</strong></td>
<td>Interleukin 10</td>
<td>NM_010548.2</td>
<td>Forward AGGCGCTGTCATCGATTTCCTC  &lt;br&gt; Reverse TGCTCAGTCGCTCCTGCTCTTA</td>
</tr>
<tr>
<td><strong>Il-18</strong></td>
<td>Interleukin 18</td>
<td>NM_001357221.1</td>
<td>Forward ACGTTTCCAGGACACAAACA  &lt;br&gt; Reverse CAACCCCTCCCCACCTAATC</td>
</tr>
<tr>
<td><strong>InsR</strong></td>
<td>Insulin receptor</td>
<td>NM_001330056</td>
<td>Forward TTCTTCTAGGAGGCTACCTCG  &lt;br&gt; Reverse GTCCAAGGCAATAAAAAAGATAGTT</td>
</tr>
<tr>
<td><strong>ObRa</strong></td>
<td>Leptin receptor a</td>
<td>NM_008493.3</td>
<td>Forward GAACTCTCTAGGACACACTGAGTA  &lt;br&gt; Reverse CTGTTTCCTCCTCAAAATGTA</td>
</tr>
<tr>
<td><strong>ObRb</strong></td>
<td>Leptin receptor b</td>
<td>NM_008493.3</td>
<td>Forward GCAGTGAATCGTGATATTG  &lt;br&gt; Reverse CAAAGCTGATCGACACTGTAAT</td>
</tr>
<tr>
<td><strong>Psd95</strong></td>
<td>Discs large MAGUK scaffold protein 4</td>
<td>NM_001109752.1</td>
<td>Forward GTACCTAAGGTGCCAAGC  &lt;br&gt; Reverse CTACCTGTCAGGCTGAG</td>
</tr>
<tr>
<td><strong>S100b</strong></td>
<td>S100 protein, beta polypeptide, neural</td>
<td>NM_009115.3</td>
<td>Forward TGGGCTCTTGTTGGGAACTTCT  &lt;br&gt; Reverse TGATCATGCCGGAGGT</td>
</tr>
<tr>
<td><strong>Sox2</strong></td>
<td>SRY-box transcription factor 2</td>
<td>NM_011443.4</td>
<td>Forward AGAAAGAGACTTGGTGGATAAGCT  &lt;br&gt; Reverse TGATCATGCCGGAGGT</td>
</tr>
<tr>
<td><strong>Synapsin</strong></td>
<td>Synapsin I</td>
<td>NM_001110780.1</td>
<td>Forward CAGCACAACATACCTGTTGG  &lt;br&gt; Reverse GGTCTCAGTTACCAGAAC</td>
</tr>
<tr>
<td><strong>Tnf-α</strong></td>
<td>Tumor necrosis factor</td>
<td>NM_001278601.1</td>
<td>Forward TGCTTATGGCTAGCTCCTC  &lt;br&gt; Reverse GAGGCCATTTGGGAACTTCT</td>
</tr>
<tr>
<td><strong>Tgf-β</strong></td>
<td>Transforming growth factor, beta 1</td>
<td>NM_011577.2</td>
<td>Forward TGCTTGGCTCAAGGGAGA  &lt;br&gt; Reverse TACTTGTGGCTCAGGCTC</td>
</tr>
</tbody>
</table>

Primers designed by PrimerDesing® are property of the company. The primers developed by PrimerDesign® were B3-Tub (Tubulin β 3 class III), Caspase-3, Nestin, Notch-1, and Pax6. Here, the qPCR analysis was performed on a CFX96 Real-Time System (BioRad, Hercules, CA), using SYBR-green as a dye-based fluorescent labelling method from SYBR-green 2x PrecisionPlus® MasterMix (Primerdesign UK). SYBR-green intercalates with every new copy of dsDNA molecule enables the quantification and collection of data. Therefore, fluorescent intensity is proportionate to the number of dsDNA copies formed. Considering that this method has a few drawbacks, since SYBR-green binds to any dsDNA present in the sample, careful RNA extraction, cDNA conversion and accurate selection of the reference housekeeping genes are necessary to minimize errors during the analysis and interpretation of the data.

Each lyophilised primer (sigma-Aldrich, Primerdesign UK) was reconstituted to a 100µM stock solution in nuclease-free water and were stored at -20°C until use. Subsequently the stock solutions were diluted in nuclease-free water to produce the working solutions, which had a concentration of 10µM. 2µl of forward and reverse primers (1.0µM) were used for each PCR reaction tube.
Reverse transcribed samples were thawed (on ice) and quick spun in a centrifuge at 2000rpm before added directly to the PCR plates. 10µl of 2x *Precisionplus®* qPCR Mastermix (PrimerDesign, UK), and 2µl of nuclease-free water were added to each reaction well of a low profile, non-skirted, clear 96-well plate (E1403-0200, Starlab, UK) (Table 2.7).

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primers</td>
<td>2µl each</td>
</tr>
<tr>
<td>2x PrecisionPlus® Mix</td>
<td>10µl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>2µl</td>
</tr>
<tr>
<td>cDNA sample</td>
<td>4µl</td>
</tr>
</tbody>
</table>

**Table 2.8 Components of each qPCR reaction mix.**

Each sample (20µl) was analysed in duplicate, and as controls few non-reverse transcribed RNA samples were used to confirm absence of genomic DNA, and water samples (no template controls) were used as negative controls. Plates were covered with microseal® B PCR plate sealing film (MSB1001, Bio-Rad, UK), and placed in a C1000 Thermal Cycler with a CFX96 module system (Bio-Rad, Hercules, UK).

Amplification program included enzyme activation at 95°C for 2 minutes, followed by denaturation at 95°C for 15 seconds, and annealing at 60°C for 45 seconds, steps 40 times, and a final extension step at 72°C for 10 minutes. Melting analysis was carried out to verify the amplification specificity, by fluorescent detection between 60°C and 95°C at 0.1°C steps, holding for 5 seconds, and maintained at 4°C until the plates were retrieved from the machine. CFX Manager® software version 1.6 (Bio-Rad, UK) was used to plot fluorescent intensity over time and C(t) values were calculated for each marker at the autocalculated threshold. Plates were stored in the fridge at 4°C until required for gel electrophoresis confirmation. Agarose gels were prepared in order to confirm the size of each RT-PCR products and verify unique bands. Amplified products were separated by gel electrophoresis with 1% agarose gel in 1X TBE buffer (50nM Tris, 100nM Borate, 10mM EDTA, pH8.0). 1.5 grams of agarose was added to 100ml TBE buffer and 7µl of nucleic acid gel stain, GelRed (Biotium, USA) to visualize the bands. Once the gel was set, 5µl of each sample mixed with 1µl of 6x DNA loading dye were loaded on to the different wells and run at 100volts for 45 min. all gels were visualized under ultraviolet light using a UV camera. A 50 base pair ladder (NEB B7025, New England BioLabs) was added to the first lane in order to confirm the fragment sizes (Figure 2.12).
2.9.4 Normalization of qPCR data.

Transcription gene differences are caused by biological variation or non-biological variation. Normalization helps to eliminate non-biological variation as much as possible. Non-biological variation can be introduced through several stages, and different factors contribute to this variation such as cDNA concentration template, cDNA quality template, differences in reverse transcription and qPCR efficiency. These factors could also contribute to flawed data, and cause misinterpretation of the derived gene expression data. Therefore, it is important to ensure that each step during the processing is controlled and standardized.

Therefore, it is essential to have a standard comparison or normalisation of the target gene levels in the samples to compensate sample to sample and run to run variations. The most common way to normalise data is to use endogenous reference genes as internal controls as they are the best markers to capture all of the non-biological variation. It is recommended to use multiple references genes for normalisation in order to reduce erroneous expression differences.

Reference or housekeeping genes are an invariable endogenous control whose expression is consistent in different experimental conditions. The reference genes are necessary for the basic functioning of the cell and must be expressed in the same way in all the cells of the organism under both normal and pathophysiological conditions.
Therefore, the selection of these reference genes must be done carefully, since variations of these genes in different experimental conditions will lead to changes in the data, thus reducing the accuracy and sensitivity of the assay. This was achieved by using the PrimerDesign geNorm® kit (PrimerDesign, UK) with 11-candidate reference genes for Mus musculus (Table 2.8). For each candidate reference gene, we use the three different diet groups (NFD, Emb and HFD), including male and female samples in a 96-well plate, in total 21 plates for brain samples, 11 for the cortex and 10 for this hippocampus were analysed. Liver samples were previously analysed by Dr Judith Eckert using the geNorm® kit (data not shown). We checked the specificity of the reference genes by analysing the melting curve, observing that all products generated a single peak.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>RNA, 18S Ribosomal</td>
</tr>
<tr>
<td>Actb</td>
<td>Actin Beta</td>
</tr>
<tr>
<td>Ap3d1</td>
<td>Adaptor Related Protein Complex 3 Subunit Delta 1</td>
</tr>
<tr>
<td>Cdc40</td>
<td>Cell Division Cycle 40</td>
</tr>
<tr>
<td>Csnk2a2</td>
<td>Casein Kinase 2 Alpha 2</td>
</tr>
<tr>
<td>Fbxw2</td>
<td>F-Box and WD Repeat Domain Containing 2</td>
</tr>
<tr>
<td>Gapdh</td>
<td>Glyceraldehyde-3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>Htatsf1</td>
<td>HIV-1 Tat Specific Factor 1</td>
</tr>
<tr>
<td>Mon2</td>
<td>MON2 Homolog, Regulator of Endosome-To-Golgi Trafficking</td>
</tr>
<tr>
<td>Pak1ip1</td>
<td>PAK1 Interacting Protein 1</td>
</tr>
<tr>
<td>Zfp91</td>
<td>ZFP91 Zinc Finger Protein</td>
</tr>
</tbody>
</table>

Table 2.9 List of reference genes included in the PrimerDesign geNorm® kit.
Primers sequences are property of PrimerDesign®.

Stability of reference genes was analysed using qBase+3.0 software (Biogazelle, Ghent, Belgium). The stability of the different candidate reference genes was achieved by calculating the average expression of all them, represented by M value in geNorm. Low M values indicate greater stability of the candidate reference gene in the different experimental conditions, while high M values (low
stability) indicate that candidate reference gene is not stably expressed, suggesting that the gene should not be used.

In addition, the minimum number of reference genes necessary to calculate a reliable normalization factor can be determined by geNorm V value. geNorm V is a normalisation factor that determines the optimal number of reference genes by averaging the Cq values obtained after analysing all the reference candidate genes on qBase+ (Biogazelle, Ghent, Belgium). This normalization factor is known as "pairwise variation" and uses the following formula V (n / n + 1). The optimal number of reference genes "n" is found when the value of V is less than 0.15, so a value of 0.25 is achieved where n=2 (two reference genes) and V is 2/3, but if we use a third reference gene (n=3), V will be 3/4 resulting in a value of 0.14. In this example the normalization factor geNorm V shows that the optimal number of reference genes is three for the given set of experiment.

For our maternal HFD model, we observed that in the brain cortex the optimal number of reference targets in this experimental situation is 2 (Figure 2.13) (geNorm M < 0.5, geNorm V < 0.15) when comparing a normalization factor based on the 2 or 3 most stable targets). As such, the optimal normalization factor can be calculated as the geometric mean of reference targets ACTB and FBXW2. In the hippocampus optimal number of reference targets in this experimental situation is 3 (Figure 2.14) (geNorm M < 0.5, geNorm V < 0.15), and the optimal normalization factor can be calculated as the geometric mean of reference targets Ap3d1, Pak1lp1, and FBXW2.

C(t) values of the gene of interest were normalized to the reference genes according to the region analysed (cortex: 2 reference genes, hippocampus 3 reference genes). Each sample was run in duplicate for RT-PCR analysis. Data analysis was performed using the comparative CT method (2−ΔΔCT) to determine relative expression levels.\textsuperscript{447}
Figure 2.13 geNorm M and geNorm V output for reference genes analysed in the cortical samples.

(Top image) geNorm analysis showing average gene expression stability (M value) after comparing 11 reference genes (X-axis), the most stable genes are shown on the right most side, and the less stable genes are shown on the left most side on the X-axis. (Bottom image) geNorm V shows the optimal number of reference genes selected for normalization, in this case the number is 2 since V2/3 is less than 0.15.
Figure 2.14 geNorm M and geNorm V output for reference genes analysed in the hippocampal samples.

(Top image) geNorm analysis showing average gene expression stability (M value) after comparing 10 reference genes (X-axis), and the most stable genes are shown on the right most side, and the less stable genes are shown on the left most side on the X-axis. (Bottom image) geNorm V shows the optimal number of reference genes selected for normalization, in this case the number is 3 since V3/4 is less than 0.15.
2.9.5 Generating a standard curve

A standard curve was generated for each gene of interest to quantify the PCR efficiency. A PCR efficiency greater than 95% indicates that the assay is robust and reproducible. Standard curves are usually made from a 10-fold serial dilution of cDNA, and ideally should be over five or six dilutions. The replicated Cq values for each dilution are plotted against the logarithm of the initial concentration, and a linear regression line is fitted to the data.

The standard curve should show curves that are equally spaced, but contamination or erroneous dilutions could generate inconsistent dilution curves. For each gene, a standard curve was generated, and the CFX manager software calculated the slope of the curve in order to determine the PCR efficiency. An optimal PCR efficiency should be 100%, which is characterized by a slope of -3.32. However, assays with amplification efficiencies between 90–110% (-3.6 ≥ slope ≥ -3.1)448, and it is calculated according to the following formula:

\[ \text{Efficiency} = 10^{\left(\frac{1}{\text{slope}}\right)} - 1. \]

Here we used a 10-fold serial dilution ranging from 300μg to 3000ng of cDNA for each gene. This dilution series was used to assess primer efficiency. Here are two examples of a standard curve generated from a dilution series for Synapsin gene in the cortex and the hippocampus, showing a PCR efficiency of 96% (Figure 2.15) in the cortex, and 107% (Figure 2.15) in the hippocampus (Figure 2.16).
Figure 2.15 An example of a standard curve generated from a dilution series for Synapsin gene using cDNA from the cortex.
(Top image) A linear regression line is fitted to the data (middle image) showing the slope value, the PCR efficiency (E) value, and the goodness of fit linear regression model. Melting curve indicating the specificity of the primers (bottom figure).
Figure 2.16 An example of a standard curve generated from a dilution series for *Synapsin* gene using cDNA from the hippocampus.
(Top image) A linear regression line is fitted to the data (middle image) showing the slope value, the PCR efficiency (E) value, and the goodness of fit linear regression model. Melting curve indicating the specificity of the primers (bottom figure).
2.10 **Statistical Analysis**

Data were first assessed for normal distribution. The Shapiro-Wilk test was used to evaluate whether each data set was normally distributed. Maternal body weight data were subjected to weighted one-way analysis of variance (ANOVA) (GraphPad Prism version 7.0). When the analysed data showed significant differences between the diet groups, Tukey’s multiple comparison test was applied.

The area under the curve test was carried out on the GraphPad Prism version 7.0 for Windows (GraphPad Software, La Jolla, CA, USA). The Pearson correlation test was used to assess the degree of association between two parameters of interest. All data for correlation analysis passed the normality test (SPSS version 24). For samples found to be non-normally distributed, data was first transformed using a Z-score transformation and assessed again (SPSS version 24). Data were z-transformed in order to standardise variables where necessary. The Z-score transformation generated a normal distribution of the data. It was calculated by the ratio of the difference each variable and its mean over the standard deviation of that variable. For all experiments, the z-transformed diet groups were compared using a multilevel random effects regression model accounting for different parameters (litter size, sex, and body weight) from individual animals, alongside fixed effects (such as maternal HFD during gestation and lactation and the preimplantation period) have been advocated by Professor Clive Osmond (SPSS version 24). The random-effects model and correlation syntaxes were developed in collaboration with Professor Clive Osmond, Senior Medical Statistician at the Medical Research Council Epidemiology Unit at Southampton General Hospital.

A $p$-value $< 0.05$ was considered significant. If a $p$-value of between 0.05 and 0.1 was observed, a trend was assumed to exist. All quantitative data were expressed as mean ± standard error of the mean (SEM). $p$-value is shown as * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. 
Chapter 3. MATERNAL HFD IMPAIRES METABOLISM IN THE MALE AND FEMALE OFFSPRING.

3.1 Introduction

The nutritional status during pregnancy and lactation is essential for proper programming and development of the neural circuitry that regulates behaviour and energy balance in the offspring. This implies that nutritional changes in the mother during pregnancy lead to physiological and behavioural alterations in the offspring later in life. This set of changes that occurred during development, caused by an insult or a stimulus, has been called metabolic imprinting. Metabolic imprinting allows the efficient utilization of metabolic fuels and a better energy balance regulation during intrauterine growth. Metabolic imprinting is essential during development, since it increases the survival rate of offspring, especially when the quality of maternal nutrition is not adequate.

Nowadays, the nutritional status of the mother during pregnancy in many countries does not depend on the lack of food, but on the energy-dense foods available in the market. These nutritional changes lead to a higher rate of maternal obesity, with the increase in high-fat food intake, one of the main causes. Different epidemiological studies have described how the increase in the rate of maternal obesity increases the incidence of metabolic syndrome and high BMI in children.

Metabolic syndrome is characterized by the presence of at least three of the following conditions: hypertension, impaired glucose tolerance or insulin resistance, systemic inflammation, central adiposity, decreased high-density lipoproteins and elevated triglycerides. This set of conditions leads to an increase in the individual's risk of suffering from different cardiovascular diseases and diabetes. Overweight during childhood has been correlated with a higher rate of morbidity and mortality in adulthood. Therefore, understanding how maternal obesity leads to the development of health disorders in the offspring is critical to developing effective interventions to prevent and treat this problem.

In murine models of maternal obesity, an increase in the risk of obesity and metabolic syndrome has been observed in the offspring. One possible explanation may be due to the change in levels of neuroendocrine factors during sensitive developmental periods. It has been found that during pregnancy in obese mothers, different factors can be transferred directly to the fetus, such as
leptin, glucose, and inflammatory cytokines. Increased levels of these factors can lead to the deregulation of neuroendocrine signals in the fetus, increasing the risk of obesity in the offspring. Additionally, in murine models fed with a high-fat diet (HFD), changes in birth weight, increase in fat mass, insulin resistance and increase in glucose levels have been observed in the offspring. They are indicating that maternal HFD increases the risk in the offspring of suffering from metabolic syndrome.

Murine models of maternal obesity have been created to mimic the nutritional trends of modern Western society. Most of these models commonly use HFD or cafeteria diet. During the cafeteria diet, animals receive, along with their regular diet, a selection of tasty foods that have high-fat content, which easily allows mothers to increase their body weight quickly. The most significant disadvantage of the cafeteria diet is the difficulty of normalizing the amount of fat or items consumed by each animal, which leads to the animals of the same dietary group being very variable among them. In the HFD, the concentration of fat is constant, in contrary to the cafeteria diet, and a percentage of carbohydrates is replaced by fat, with fat being the primary source of energy. The fat content in an HFD varies from 20% to 60%, which means an increase of up to three times the normal fat content in rodents. The normal content of fat in a control diet varies from 7 to 20%.

The offspring exposed to a maternal HFD, tend to be hyperphagic in adulthood. In the offspring hypothalamus, different orexigenic peptides are increased due to a more significant proliferation of neurons (during gestation) that will express more orexigenic peptides. There are different orexigenic peptides such as neuropeptide Y, agouti-related peptide (AGRP) in the arcuate nucleus, and galanin, enkephalin, and dynorphin in the paraventricular nucleus. Additionally, the reduced expression of leptin and melanocortin peptides was observed in the offspring of HFD mothers. Therefore, maternal HFD can modify the expression of orexigenic peptides and leptin during early stages of development in the hypothalamus that might induce hyperphagia later in life.

In the mouse brain leptin receptors are expressed around embryonic day 15, suggesting that leptin is crucial for an adequate foetal brain development. In the neonatal period, leptin levels increase between PND 4 and PND 15. This period of time is essential to establish connections between the hypothalamus and the hippocampus, enhancing dendritic and synapse morphology. The highest concentration of leptin receptors in the brain are found in the hypothalamus. However, these receptors are widely expressed and distributed in other areas such as the hippocampus and cortex.
Animal models of maternal HFD have shown an increase in leptin concentration that causes adverse effects on brain development. High leptin concentrations affect the proper development of the hippocampus and plasticity, possibly leading to problems in behaviour and memory in the offspring\textsuperscript{464}. High leptin concentrations may generate leptin resistance in the hippocampus, impairing leptin receptor signalling, similarly as described in the hypothalamus. However, the impact of high levels of leptin in other regions of the brain is not clear.

In the brain, insulin participates in the modulation of release and uptake of catecholamine, in the growth of neurites, and the modulation of synaptic plasticity. Insulin can cross the BBB barrier, playing an important role in energy regulation and glucose metabolism in the brain. Like leptin, insulin receptors are distributed in different regions of the brain such as the hypothalamus, the olfactory bulb and the hippocampus, mainly in the synapses. Insulin receptors regulate glucose metabolism through glucose transporters 4 and 8 (GLUT4 and GLUT8), especially in areas of the brain with high glucose metabolism, such as the cerebellum, cortex, hypothalamus and hippocampus\textsuperscript{465}.

The brain uses glucose as the primary source of energy, using about 50% to 75% of the daily consumption of glucose in humans. Glucose transporter 1 (GLUT1) allows the flow of glucose from the bloodstream to the brain. The major glucose transporters in the brain are GLUT1 and GLUT3 located in astrocytes and neurons, respectively, and GLUT5, which is situated in microglia cells\textsuperscript{383,466,467}. Cerebral glucose metabolism, in the context of maternal HFD, has not been investigated explicitly in other regions of the brain other than the hypothalamus.

The present chapter investigates whether it is maternal obesity or the HFD itself that causes metabolic changes in the offspring. These metabolic changes will be assessed in the offspring by measuring their body weights, their energy expenditure, activity and food intake.
Chapter 3

3.2 Methods

3.2.1 Indirect calorimetry,

Indirect calorimetry was measured using an Oxylet (Panlab, Cornella, Spain). Each mouse was placed in an individual metabolic chamber at 25°C, with free access to food and water. Oxygen (vO2) and carbon dioxide production (vCO2) were recorded at 10-min intervals using an Oxylet LE 405-gas analyser (PANLAB) and Metabolism software 2.1.02 a computer-assisted data acquisition program (Oxylet; Panlab, Cornella, Spain) over to 24-h period. Energy expenditure (EE) was calculated according to the following formula: EE (kcal / day / kg of body weight0.75) = vO2 X 1.44 X (3.815 + (1.232 X RQ). The RQ was calculated by dividing the vCO2 produced by the vO2 consumed (RQ = VCO2 / VO2). The system calculated the activity of each animal by detecting changes of potential and kinetic energy produced by the animal in its movements. The system measures the amount of force exerted by a particular activity which is detected by the weight transducer. The transducer is located below the cage to detect all activity variations (Oxylet LE 1335 Physiocage, PANLAB). These measurements were done by Oliver Hutton (Faculty of Medicine, University of Southampton).

3.2.2 Quantitative real-time PCR

Total RNA was isolated from frozen liver and frozen brain cortex and hippocampus using an RNeasy Lipid Tissue mini kit (Qiagen, UK) according to the manufacturer’s instructions. The isolated RNA was quantitated using the Nanodrop ND-1000 spectrophotometer (LabTech UK), and only samples with an adequate RNA concentration (A260/A280 \( \geq \) 1.8) and purity (A230/A260 \( \geq \) 2.0) were selected for reverse transcription. 1µg of RNA from each sample was reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad, UK) following the manufacturer’s protocol.

RNA levels of different markers were quantified using highly specific primers, table 3.1 (Sigma Aldrich, or Primer Design, UK). qPCR was then performed on a CFX96 Real-Time System (Bio-Rad, Hercules, CA). Melting analysis was carried out to verify the amplification specificity. Stability of 2–3 housekeeping genes was tested using GeNorm (gene stability value: 0.5; coefficients variation: 0.2) and used for normalization (qBase+3.0; Biogazelle, Ghent, Belgium). Data analysis was performed using the comparative CT method (2−ΔΔCT). All primer sequences are in chapter 2, table 2.6.
3.2.3 Statistical Analysis.

The area under the curve test was carried out on the GraphPad Prism version 7.0 for Windows (GraphPad Software, La Jolla, CA, USA). For all experiments, data from the different diet groups were first normalized using Z-score transformation. The Z-score was calculated by the ratio of the difference each variable and its mean over the standard deviation of that variable. We used the z-transformed data to compare the Emb or HFD group vs the NFD group using a multilevel random effects regression model accounting for different parameters (litter size, sex, and body weight) from individual animals (SPSS version 24). Normality of data was tested using the Shapiro–Wilk test. A probability of $p$ less than 0.05 was considered to be significant. All quantitative data were expressed as mean ± standard error of the mean (SEM). $P$-value is shown as * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p\leq0.0001$. 
3.3 Results

3.3.1 Maternal HFD does not induce obesity in the dams.

Previous data from our research group have demonstrated that there is not a significant difference in the total weight change between the three different diet groups NFD (Ch), Emb (emb-HF), and HFD (HF) during pregnancy (Figure 3.1 a). However, variations in weight gain were observed in particular days (days 3, 4, 7, 13, and 15), especially during the first days of gestation (Figure 3.1 b) (unpublish data generated by Dr Francesca Lock).

Figure 3.1 Maternal body weight during gestation.

a. Maternal diet does not affect the percentage of total weight gain throughout pregnancy in the different diet groups. b. Daily percentage of weight gain during gestation in the three different groups. Ch: NFD group. emb-HF: Emb group. HF: HFD group. Data belong to Dr Francesca Lock. Unpublish data.
Previous data from Dr Francesca Lock showed that there is not a significant difference in body weight in the dams during gestation (Figure 3.1). Therefore, the analysis of body weight in dams was done just before mating, after delivery and after weaning. Before mating, all dams were fed a standard diet, and after mating, the mothers were divided into three dietary groups: NFD, Emb and HFD as described in Chapter 2. HFD and Emb dams did not show significant changes in their body weight after birth and after weaning (Figure 3.1). The analysis of body weight did not show significant differences in body weight between Emb vs HFD dams before pregnancy (p=0.867), after birth (p=0.142), or after weaning (p=0.134). This data indicates that our maternal diet model was not able to induce significant weight gain after birth or after weaning.

<table>
<thead>
<tr>
<th></th>
<th>Dam pregravid bodyweight (g)</th>
<th>Dams at Birth bodyweight (g)</th>
<th>Dams at weaning bodyweight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFD</td>
<td>Mean 33.84</td>
<td>43.32</td>
<td>43.44</td>
</tr>
<tr>
<td></td>
<td>SEM 1.991</td>
<td>1.37</td>
<td>1.227</td>
</tr>
<tr>
<td>Emb</td>
<td>Mean 32.34</td>
<td>41.92</td>
<td>42.72</td>
</tr>
<tr>
<td></td>
<td>SEM 0.9329</td>
<td>1.508</td>
<td>1.572</td>
</tr>
<tr>
<td>Emb vs NFD</td>
<td>p=0.7386</td>
<td>p=0.8154</td>
<td>p=9257</td>
</tr>
<tr>
<td>HFD</td>
<td>Mean 32.64</td>
<td>45.82</td>
<td>45.99</td>
</tr>
<tr>
<td></td>
<td>SEM 1.018</td>
<td>2.213</td>
<td>1.421</td>
</tr>
<tr>
<td>HFD vs NFD</td>
<td>p=0.8485</td>
<td>p=0.5543</td>
<td>p=0.2798</td>
</tr>
</tbody>
</table>

Table 33.1 Maternal body weight. Data were collected before mating, after delivery and after weaning.
Data are expressed as mean ± SEM. n=6-8 in each group.
Figure 3.2. Maternal body weight before, after birth and after lactation. No significant differences were observed between the diet groups. NFD group in blue, Emb group in green and HFD in red. Data are expressed as mean ± SEM. n=6-8 in each group.

In this study, maternal HFD (during gestation or the preimplantation period) was shown to have no significant effect on the litter size (Figure 3.3). The litter size of males from the HFD group was not significantly different from the NFD-male group (p=0.978). Emb males are not significantly different from the males in the NFD group (p=0.418). There is not a significant difference between the Emb males and the HFD male groups in the litter size (p=0.530). Females from the HFD group were not significantly different from the NFD-female group (p=0.968). Emb females are not significantly different from the females in the NFD group (p=0.073). There is not a significant difference between the Emb females and the HFD female groups in body weight (p=0.112). There is not a significant sex effect (females vs males) in the body weight in the NFD (p=0.987), the Emb (p=0.972), and the HFD (p=0.945) groups.

Figure 3.3. Litter Size at birth. No significant differences in males (a.) and females (b.) were observed in litter sizes produced from HFD, Emb and NFD dams. Data are expressed as mean ± SEM. n=6-8 in each group.
Body weight of the offspring was analysed at the age of 26 weeks, and they never had direct exposure to the HFD. We selected six females and six males per dietary group, obtaining a total of 36 animals. There were no significant changes in body weight in males or females, in the different diet groups at age 26 weeks (Figure 3.4). The body weight of males from the HFD group was not significantly different from the NFD-male group (p=0.778). Emb males are not significantly different from the males in the NFD group (p=0.964). There is not a significant difference between the Emb males and the HFD male groups in the body weight (p=0.822). Females from the HFD group were not significantly different from the NFD-female group (p=0.790). Emb females are not significantly different from the females in the NFD group (p=0.828). There is not a significant difference between the Emb females and the HFD female groups in body weight (p=0.992). There is not a significant sex effect (females vs males) in the body weight in the NFD (p=0.531), the Emb (p=0.401), and the HFD (p=0.161) groups.

The brain to body weight ratio of males from the HFD group was not significantly different from the NFD-male group (p=0.622). Emb males are not significantly different from the males in the NFD group (p=0.707). There is not a significant difference between the Emb males and the HFD male groups in the brain to body weight ratio (p=0.918). Females from the HFD group were not significantly different from the NFD-female group (p=0.369). Emb females are not significantly different from the females in the NFD group (p=0.710). There is not a significant difference between the Emb females and the HFD female groups in the brain to body weight ratio (p=0.674). There is not a significant sex effect (females vs males) in the brain to body weight ratio in the NFD (p=0.245), the Emb (p=0.053), and the HFD (p=0.095) groups (Figure 3.4).

The liver to body weight ratio of males from the HFD group was not significantly different from the NFD-male group (p=0.600). Emb males are not different from the males in the NFD group (p=0.057). There is not a significant difference between the Emb males and the HFD male groups in the liver to body weight ratio (p=0.133). Females from the HFD group were not significantly different from the NFD-female group (p=0.362). Emb females are not different from the females in the NFD group (p=0.767). There is not a significant difference between the Emb females and the HFD female groups in the liver to body weight ratio (p=0.613). There is not a significant sex effect (females vs males) in the liver to body weight ratio in the NFD (p=0.207), the Emb (p=0.112), and the HFD (p=0.203) groups (Figure 3.4). These data indicate that the mice fed HFD did not have differences in body weight, neither their offspring compared with the NFD group.
Figure 3.4. Body, liver and brain weight in the offspring.

a) Body weight in males and b) body weight in females. c) Liver weight in males and d) liver weight in females, at 26 weeks of age. e) Brain weight in males and f) brain weight in females, at 26 weeks of age. NFD group in blue, Emb group in green and HFD in red. Data are expressed as mean ± SEM. n=6 in each group per sex.
3.3.2 Effects of maternal HFD on energy balance, respiratory quotient, physical activity and food intake.

Different studies in obese mouse models have shown that the consumption of HFD leads to metabolic disorders such as overweight, glucose intolerance and high levels of leptin\textsuperscript{468,469}. One of the leading causes of obesity is an energy imbalance since there are high energy intake and low energy expenditure\textsuperscript{470}. Since energy expenditure is not measured directly, the best approach to do it is by indirect calorimetry, in which O\textsubscript{2} consumption can be used to calculate energy expenditure, and CO\textsubscript{2} production to identify the metabolic subtract being used\textsuperscript{471}. Therefore, we want to assess whether the maternal diet leads to metabolic alterations in adult offspring.

3.3.2.1 Activity

Here we evaluated the effect of the maternal diet on spontaneous activity in the offspring in a period of 24 hours (Figure 3.5). The activity values were not significantly altered in the HFD or Emb males during the day (first part Emb p=0.784, HFD p=0.986; second part Emb p=0.643, HFD p=0.987) or the night (first part Emb p=0.653, HFD p=0.998; second part Emb p=0.820, HFD p=0.228) compared with NFD males. No changes between HFD males and Emb males were observed during the different parts of the day or night. In HFD females, we saw higher diurnal activity during the day periods (first $p=0.0174$, second $p=0.0266$ part of the day) compared with the NFD females. No changes were found in the Emb females during the day, and no significant differences between the HFD females or Emb with the NFD group were observed during the whole night (Figure 3.6). No changes between HFD females and Emb females were observed during the first part of the day ($p=0.083$), but it was significantly different in the second part of the day ($p=0.021$). No changes between HFD females and Emb females were observed during the different parts of the night.

Then we compared whether the sex was influencing the activity in the animals. In the first part of the day, no differences were observed between the Emb male vs females ($p=0.067$) or HFD males vs HFD females ($p=0.889$). Still, there was a significant difference between the NFD males and the NFD females ($p=0.0015$), having the males higher activity values. In the second part of the day, no differences were observed between the Emb male vs females ($p=0.362$) or HFD males vs HFD females ($p=0.998$). Still, there was a significant difference between the NFD males and the NFD females ($p=0.049$), having the males higher activity values.
In the first part of the night, the males in all diet groups had higher activity values than females (NFD $p=0.0021$, Emb $p=0.0089$, and HFD $p=0.034$). In the second part of the night, no differences were observed between the Emb males vs females ($p=0.575$) or HFD males vs HFD females ($p=0.998$). Still, there was a significant difference between the NFD males and the NFD females ($p=0.045$), having the males higher activity values. This data suggests that NFD males were more active than NFD females, but in general, no differences in activity were observed between males and females in the Emb or HFD (only in the first part of the night).

**Figure 3.5. The mean average locomotor activity.**

Activity analysis was performed over a 24 h period with a 12 h light and 12 h dark for NFD group (blue), Emb group (green) and HFD (red) at 26 weeks age. The shaded areas demarcate the dark phases (from 7:00 pm to 7:00 am). All points represent means ± SEM averaged over 2 h intervals.
Figure 3.6. AUC activity.

a. Activity values were similar between groups in males throughout the 24 h period. b. Significant changes during the day were observed between HFD vs NFD females. α: the difference between NFD males and NFD females first part of the day (p=0.0015). β: difference between NFD males and NFD females second part of the day (p=0.049). γδε: difference between males and females first part of the night (γ NFD p=0.0021, δ Emb p=0.0089, and ε HFD p=0.034). ζ: difference between NFD males and NFD females second part of the night (p=0.045). NFD group (blue), Emb group (green) and HFD (red) at 26 weeks age. The shaded areas demarcate the dark phases (from 7:00 pm to 7:00 am). All bars represent means ± SEM. n=4-6 in each group. * p<0.05.
3.3.2.2 Oxygen consumption (vO$_2$)

The analysis of oxygen consumption (vO$_2$) in the offspring showed a clear difference between the amount of oxygen during the day and night (Figure 3.7). As expected, the pattern showed an increase in the volume of oxygen at night concerning the day, since the animals are more active at night compared to the day. After analysing the data for a period of 24 hours, the males of the HFD group showed a smaller area under the curve (AUC) during the first ($p=0.0043$) and second ($p=0.0005$) part of the day compared with the NFD group. Similarly, we observed a lower AUC during the first ($p=0.0010$) and second ($p=0.0001$) part of the night in the males of the HFD group to the NFD group. In the Emb group male mice, we observed a significant decrease in the AUC in the second part of the day ($p=0.0012$) when compared with the NFD group. Although in general, the males of the Emb group showed a slight reduction in the consumption of vO$_2$, this difference did not reach significance (Figure 3.8). No changes between HFD males and Emb males were observed during the different parts of the day. However, there is a significant difference between Emb and HFD males in the first part ($p=0.039$), and the second part ($p=0.002$) of the night.

The analysis of vO$_2$ in females did not show significant differences during the first or second part of the day, nor in the first part of the night, between the diet groups. There was a significant difference between the HFD group ($p=0.0089$) and the NFD group, due to an increase in the AUC in the second part of the night (Figure 3.8). No changes between HFD females and Emb females were observed during the different parts of the day. However, there is a significant difference between Emb and HFD females in the first part ($p=0.008$), and the second part ($p=0.004$) of the night.

Then we compared whether the sex was influencing the vO$_2$ in the animals. In the first part of the day, no differences were observed between the Emb males vs females ($p=0.561$) or HFD males vs HFD females ($p=0.819$). Still, there was a significant difference between the NFD males and the NFD females ($p=0.0002$), having the males higher vO$_2$ values. In the second part of the day, no differences were observed between the Emb males vs females ($p=0.829$) or HFD males vs HFD females ($p=0.998$). Still, there was a significant difference between the NFD males and the NFD females ($p=0.0007$), having the males higher vO$_2$ values. In the first part of the night, the males in the Emb and NFD groups had higher vO$_2$ values than females (NFD $p=0.0007$, and Emb $p=0.0001$), but not in the HFD group ($p=0.879$). In the second part of the night, the males in all diet groups had higher vO$_2$ values than females (NFD $p=0.003$, Emb $p=0.043$, and HFD $p=0.0004$). Here, we observed in HFD male offspring a reduction in oxygen consumption, during the day and night.
Figure 3.7. The mean average in oxygen (\(vO_2\)) consumption.
\(vO_2\) analysis was performed over a 24 h period with a 12 h light and 12 h dark for NFD group (blue), Emb group (green) and HFD (red) at 26 weeks age. The shaded areas demarcate the dark phases (from 7:00 pm to 7:00 am). All points represent means ± SEM averaged over 2 h intervals.
Figure 3.8. AUC oxygen consumption (vO$_2$).

a. Significant changes in oxygen consumption rate were observed between HFD vs NFD in males. Emb males were different only during the second part of the day. b. In contrast, oxygen consumption was similar between groups in females throughout the 24 h period, only during the second part of the night, it was a significant difference between HFD vs NFD. α: difference between NFD males and NFD females first part of the day (p=0.0002). β: difference between NFD males and NFD females second part of the day (p=0.0007). γ,δ: difference between males and females first part of the night (γ NFD p=0.0007, and δ Emb p=0.043). ε,ζ,η: difference between males and females second part of the night (ε NFD p=0.003, ζ Emb p=0.043, and η HFD p=0.0004). NFD group (blue), Emb group (green) and HFD (red) at 26 weeks age. The shaded areas demarcate the dark phases (from 7:00 pm to 7:00 am). All bars represent means ± SEM. n=4-6 in each group. * p<0.05, ** p<0.01, *** p<0.001, **** p≤0.0001.
3.3.2.3 Carbon dioxide production (vCO₂)

In addition to the analysis of vO₂, we evaluated the amount of vCO₂ produced by the mice in the same 24-hour period (Figure 3.9). After analysing the males of the HFD group, the data showed a similar trend to the data obtained during the analysis of vO₂. During the first part of the day, the HFD males had a slight decrease in the AUC, although this difference was not significant (p=0.1007). However, HFD males showed a lower AUC during the second part (p=0.0446) of the day, the first (p=0.0259) and second (p=0.0002) part of the night compared to the NFD group. In the male mice of the Emb group, there was no significant difference in the AUC analysis during the different parts of the day or night (Figure 3.10). No changes between HFD males and Emb males were observed during the different parts of the day. However, there is a significant difference between Emb and HFD males in the first part (p=0.020), and the second part (p=0.008) of the night.

In the females of the HFD group, the vCO₂ analysis did not show significant differences during the first or second part of the day. There was a significant difference between the HFD females and the NFD group during the first and second part of the night (p=0.0306 and p=0.0042 respectively) due to an increase in the AUC. However, there were no significant differences between the Emb group and the NFD during the day and night in general (Figure 3.10). No changes between HFD females and Emb females were observed during the different parts of the day. However, there is a significant difference between Emb and HFD females in the first part (p=0.004), and the second part (p=0.001) of the night.

Then we compared whether the sex was influencing the vCO₂ in the animals. In the first part of the day, the males in all diet groups had higher vCO₂ values than females (NFD p=0.0001, Emb p=0.008, and HFD p=0.018). In the second part of the day, no differences were observed between the Emb males vs females (p=0.098) or HFD males vs HFD females (p=0.945). Still, there was a significant difference between the NFD males and the NFD females (p=0.002), having the males higher vCO₂ values. In the first part of the night, the males in the Emb and NFD groups had higher vCO2 values than females (NFD p=0.0011, and Emb p=0.0002), but not in the HFD group (p=0.732). In the second part of the night, the males in the HFD and NFD groups had higher vCO₂ values than females (NFD p=0.002, and HFD p=0.0001), but not in the Emb group (p=0.094).
Figure 3.9. The mean average of carbon dioxide (vCO₂) production.

vCO₂ analysis was performed over a 24 h period with a 12 h light and 12 h dark for NFD group (blue), Emb group (green) and HFD (red) at 26 weeks age. The shaded areas demarcate the dark phases (from 7:00 pm to 7:00 am). All points represent means ± SEM averaged over 2 h intervals.
Figure 3.10. AUC Carbon dioxide production (vCO₂).

a. Significant changes in vCO₂ production rate were observed between HF vs NFD males. b. In contrast, oxygen consumption was similar between groups in females throughout the first 12 h of the day. Significant changes were observed during the night between HFD vs NFD females. α, β, γ: difference between males and females first part of the day (α NFD p=0.0001, β Emb p=0.008, γ HFD p=0.018). δ: difference between NFD males and NFD females second part of the day (p=0.002). ε, ζ: difference between males and females first part of the night (ε NFD p=0.0011, and ζ Emb p=0.0002). η, θ: difference between males and females second part of the night (η NFD p=0.002, and θ HFD p=0.0001). NFD group (blue), Emb group (green) and HFD (red) at 26 weeks age. The shaded areas demarcate the dark phases (from 7:00 pm to 7:00 am). All bars represent means ± SEM. n=4-6 in each group. * p<0.05, ** p<0.01, *** p<0.001.
3.3.2.4 Respiration Quotient (RQ) values

We also measured the RQ that corresponds to the volume of carbon dioxide produced in a given time, divided by the oxygen that was consumed simultaneously (RQ = VCO2 / VO2). RQ indicates which substrates are oxidized, a ratio close to 1 indicates oxidation of carbohydrates, while a value of 0.7 is an indication of lipid oxidation, and Intermediate values indicates a mixture of fat and carbohydrates\(^472\). All the mice showed a higher RQ during the night compared to the RQ during the day as expected, since the feeding pattern of these animals is nocturnal, also they are more active in this period (Figure 3.11).

The data obtained show that basal metabolism was impaired in HFD males and females, since there was an increase in AUC values during the first (male \(p=0.0001\), female \(p=0.0009\)) and second (male \(p=0.0327\), female \(p=0.0003\)) part of the day compared to the NFD group. Similarly, an increased AUC was observed in these animals in the first part (male \(p=0.0001\), female \(p=0.0015\)) and second part (male \(p=0.0062\), female \(p=0.0091\)) of the night when compared to the NFD group. (Figure 3.12). Male Emb mice had a significantly higher RQ compared to their NFD group during the first (\(p=0.0001\)) and second (\(p=0.0032\)) part of the day, and the first part of the night; however, no statistically significant differences were observed in the RQ in the second part of the night. Female Emb mice also showed a greater area under the curve in the first part of the day (\(p=0.0013\)), but not in the second part (\(p=0.0819\)) compared to their NFD group and did not show significant differences during the night (Figure 3.12). No changes between HFD males and Emb males were observed during the different parts of the day or night. No changes between HFD females and Emb females were observed during the first part of the day (\(p=0.636\)). However, it was significantly different in the second part of the day (\(p=0.004\)), and the first part of the night (\(p=0.007\)). No changes between HFD females and Emb females were observed during the second part of the night (\(p=0.447\)).

Then we compared whether the sex was influencing the RQ in the animals. In the first part of the day, no differences were observed between NFD males vs females (\(p=0.052\), Emb males vs females (\(p=0.067\)) or HFD males vs HFD females (\(p=0.061\)). In the second part of the day, no differences were observed between the NFD males vs females (\(p=0.263\), Emb males vs females (\(p=0.064\)) or HFD males vs HFD females (\(p=0.378\)). In the first part of the night, the males in the Emb group had higher RQ values than females (Emb \(p=0.006\)), but not in the NFD (\(p=0.388\)) or HFD group (\(p=0.932\)). In the second part of the night, no differences were observed in the different diet groups between males and females (NFD \(p=0.063\), Emb \(p=0.998\), and HFD \(p=0.998\)). RQ was found to be increased in HFD male and female animals during day and night.
Figure 3.11. The mean average respiratory exchange quotient (RQ).

RQ analysis was performed over a 24 h period with a 12 h light and 12 h dark for NFD group (blue), Emb group (green) and HFD (red) at 26 weeks age. The shaded areas demarcate the dark phases (from 7:00 pm to 7:00 am). All points represent means ± SEM averaged over 2 h intervals.
Figure 3.12. AUC respiratory exchange quotient (RQ).

a. Significant changes in RQ were observed between HFD vs NFD males. Emb males were different during the day and first part of the night. B. In females, RQ values were different between HFD vs NFD group throughout the 24 h period. Significant changes were observed during the first part of the day between Emb vs NFD females. α: difference between Emb males and Emb females first part of the night (p=0.006). NFD group (blue), Emb group (green) and HFD (red) at 26 weeks age. The shaded areas demarcate the dark phases (from 7:00 pm to 7:00 am). All bars represent means ± SEM. n=4-6 in each group. * p<0.05, ** p<0.01, *** p<0.001, **** p≤0.0001.
3.3.2.5 Energy Expenditure

Energy expenditure is the measure of the oxidation of substrates that contain high energy, generating heat, carbon dioxide, urea and water. Energy expenditure was assessed by indirect calorimetry, quantifying oxygen consumption and carbon dioxide production (Figure 3.13). The AUC was lower in the males of the HFD group during the first \( p=0.0109 \) and second \( p=0.0021 \) part of the day, and the first \( p=0.0023 \) and second \( p=0.0001 \) part of the night, whereas, in the Emb group, it was only reduced in the second part of the day \( p=0.0099 \), in comparison with the NFD group (Figure 3.14). No changes between HFD males and Emb males were observed during the different parts of the day. However, there is a significant difference between Emb and HFD males in the first part \( p=0.032 \), and the second part \( p=0.0003 \) of the night.

The AUC did not show significant differences during the day in the HFD females, while in the Emb females we observed a significant increase only in the first part of the day \( p=0.0234 \), respect to the NFD group. During the night period, the HFD females showed an increase only in the second part of the night \( p=0.0066 \), but the Emb females did not show significant differences when compared with the NFD group (Figure 3.12). No changes between HFD females and Emb females were observed during the different parts of the day. However, there is a significant difference between Emb and HFD males in the first part \( p=0.006 \), and the second part \( p=0.0003 \) of the night.

Then we compared whether the sex was influencing the energy expenditure in the animals. In the first part of the day, no differences were observed between the Emb males vs females \( p=0.190 \) or HFD males vs HFD females \( p=0.583 \). Still, there was a significant difference between the NFD males and the NFD females \( p=0.0012 \). In the second part of the day, no differences were observed between the Emb males vs females \( p=0.574 \) or HFD males vs HFD females \( p=0.998 \). Still, there was a significant difference between the NFD males and the NFD females \( p=0.0010 \). In the first part of the night, the males in the Emb and NFD groups had higher energy expenditure values than females (NFD \( p=0.0008 \), and Emb \( p=0.002 \)), but not in the HFD group \( p=0.841 \). In the second part of the night, the males in all diet groups had higher energy expenditure values than females (NFD \( p=0.0031 \), Emb \( p=0.049 \), and HFD \( p=0.0001 \)). These results show how males display changes in energy expenditure in the absence of obesity or changes in activity, suggesting that other factors such as insulin and leptin might be associated with low energy expenditure\(^{30,473}\).
Figure 3.13. The mean average energy expenditure (EE).

EE analysis was performed over a 24 h period with a 12 h light and 12 h dark for NFD group (blue), Emb group (green) and HFD (red) at 26 weeks age. The shaded areas demarcate the dark phases (from 7:00 pm to 7:00 am). All points represent means ± SEM averaged over 2 h intervals.
a. Significant changes in EE were observed between HF vs NFD males. Emb males were different during the second part of the day. b. In females, EE values were different between HF vs NFD group only during the second part of the night. Significant changes were observed during the first part of the day between Emb vs NFD females. α: difference between NFD males and NFD females first part of the day (p=0.0012). β: difference between NFD males and NFD females second part of the day (p=0.0010). γ,δ: difference between males and females first part of the night (γ NFD p=0.0008, and δ Emb p=0.002). ε,ζ,η: difference between males and females second part of the night (ε NFD p=0.0031, ζ Emb p=0.049, and η HFD p=0.0001). NFD group (blue), Emb group (green) and HFD (red) at 26 weeks age. The shaded areas demarcate the dark phases (from 7:00 pm to 7:00 am). All bars represent means ± SEM. n=4-6 in each group. * p<0.05, ** p<0.01, *** p<0.001, **** p≤0.0001.

Figure 3.14. AUC respiratory energy expenditure (EE).
3.3.2.6 Food Intake

Food intake is associated with the sleep-wake pattern in mice (nocturnal pattern), indicating that mice consume more food during the dark period (night). It is known that mice consume approximately two-thirds of the food during the night phase\(^{474}\), we analysed the food intake for 24 hours, weighing the food at the beginning of the experiment, and again at the end of the it.

Considering that the HFD males presented a lower AUC in energy expenditure during the day and night, we evaluated the food intake during the time the animals were analysed in the metabolic cage. Changes in energy expenditure may reflect differences in diurnal diet rhythms, reducing or increasing food intake during the 24 hours cycle. Food intake in males was not significantly different between the diet groups, HFD vs NFD (p=0.604), and Emb vs NFD (p=0.483). There was not a significant difference between HFD and Emb males (p=0.870). No significant changes were observed between HFD vs NFD females (p=0.646), or Emb vs NFD (p=0.482). There was not a significant difference between HFD and Emb females (p=0.659). However, there was no difference in food intake between groups in either male or female offspring groups (Figure 3.15). Then we compared whether the sex was influencing the food intake in the animals. No differences were observed between the NFD males vs females (p=0.200), the Emb males vs females (p=0.675) or HFD males vs HFD females (p=0.541).

![Graph](image)

**Figure 3.15.** Food intake across the entire 24 hr period.

a. Food intake in males was not significantly different between the diet groups b. No significant changes were observed between the females. NFD group (blue), Emb group (green) and HFD (red) at 26 weeks age. All bars represent means ± SEM. n=4-6 in each group.
3.3.3 Activity and Energy Expenditure

Taking into account the significant findings observed on activity, especially in HFD females, we decided to explore if there is a relationship between activity and energy expenditure in the different groups that could explain the differences observed. In the NFD groups, there was a significant positive correlation between activity and energy expenditure in male and female offspring during the first and second part of the day and night, which was reduced to a trend in the males for the first part of the night (Table 3.3, Figures 3.16 & 3.16). However, during the first part of the night, in males and females HFD, we did not observe a significant correlation between EE and Activity ($p=0.1612$ and $p=0.0504$ respectively), with a trend in females. In the second part of the night, we did not observe the correlation between energy expenditure and Activity in the Emb females ($p=0.0902$), despite the tendency towards positive correlation. In general, these data suggest that as activity increases, there is a corresponding increase in energy expenditure (Table 3.2, Figure 3.16 & 3.17).

<table>
<thead>
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<th>2nd part Day</th>
<th>1st part Night</th>
<th>2nd part Night</th>
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<td>p</td>
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<td>0.0107</td>
<td>0.8250</td>
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Table 3.3.2 Correlation Energy expenditure (EE) and Activity in males and females.
A positive correlation between activity and energy expenditure in males and females in the HFD and Emb groups was observed. Non-significant values are bold ($p>0.05$).
Figure 3.16. Correlation Energy expenditure (EE) – Activity during the day.
NFD group (blue), Emb group (green) and HFD (red).
In males, we observe a positive correlation between EE and Activity in the first and second part of the night in the Emb group. In females, in the second part of the night was positively correlated. Emb females showed a positive correlation in the first part of the night, but not during the second part of the night. NFD group (blue), Emb group (green) and HFD (red).
3.3.4 Activity and respiration quotient (RQ)

RQ is the ratio between vCO2 and vO2, which will allow us to observe how the changes in respiration could be correlated with activity (Table 3.3). NFD males showed a significant positive correlation between activity and RQ only in the first part (p=0.0001) and second part (p=0.0178) of the day, not at night. HFD males showed a significant positive correlation between activity and RQ during the second part of the night (p=0.0007). The Emb males exhibited a significant correlation during the second part of the day (p=0.0185) and the first part of the night (p=0.0077) (Table 3.3, Figure 3.18 & 3.19).

In NFD females it was a significant positive correlation only during the second part of the day (p=0.0428), and the first part of the night (p=0.0172). There was a significant correlation in the HFD females during the first (p=0.0111) and second (0.0440) part of the day, but not during the night. This is interesting, since the HFD females showed greater activity during the day, compared to the NFD group. The Emb females exhibited a significant correlation during the first part of the day (p=0.0106), the first part (p=0.0032) and the second part (p=0.0174) of the night (Table 3.4, Figure 3.18 & 3.19).

<table>
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<th>1st part Night</th>
<th>2nd part Night</th>
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<td>0.7700</td>
</tr>
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<td>0.5279</td>
</tr>
<tr>
<td></td>
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</table>

Table 3.3. Correlation Respiratory Quotient (RQ) – Activity.

Correlation between activity and RQ in males and females in the HFD and Emb groups was not clear at the different periods of the day and night. Non-significant values are bold (p>0.05).
In males, we did not observe a correlation between RQ and Activity in the first part of the day, but it was positively correlated in the second part of the day Emb, HFD. In females, we also observe a positive correlation between RQ and Activity in the first part of the day (Emb and HFD), and in the second part of the day only in the HFD. NFD group (blue), Emb group (green) and HF (red).

Figure 3.18. Correlation Respiratory Quotient (RQ) – Activity during the day.
Figure 3.19. Correlation Respiratory Quotient (RQ) – Activity during the night.

In males, we observe a positive correlation between EE and Activity in the first and of the night in the Emb group. Emb females were positively correlated in the first part ($r=0.9213$, $p=0.0032$), and the second part ($r=0.8424$, $p=0.0174$) of the night. NFD group (blue), Emb group (green) and HF (red).
3.3.5  Expression profile of genes related to energy metabolism in the liver in the offspring exposed to a maternal HFD.

The liver is a vital organ, which plays a critical role in regulating energy balance, and glucose and lipids metabolism\textsuperscript{475}. One of the main functions in the liver is to maintain an adequate level of plasma glucose, providing sufficient glucose to glucose-dependent tissues, such as the central nervous system. To analyse the effect of maternal HFD on glucose transport in the liver, we evaluated the mRNA expression of different glucose transporters such as Glut1, Glut3, Glut4, and Glut5.

The mRNA levels of the genes of interest in the 6-month-old animals’ liver are shown in Figure 3.20. The analysis of the relative mRNA expression of glucose transporter genes between Emb vs NFD, and HFD vs NFD, in males and females did not show significantly differences. Glut1 (males: Emb p=0.142, HFD p=0.415; females: Emb p=0.994, HFD p=0.562), Glut3 (males: Emb p=0.369, HFD p=0.496; females: Emb p=0.128, HFD p=0.328), Glut4 (males: Emb p=0.821, HFD p=0.610; females: Emb p=0.686, HFD p=0.895), and Glut5 (males: Emb p=0.252, HFD p=0.093; females: Emb p=0.617, HFD p=0.286).

There were no differences between the Emb and the HFD groups when we analysed the mRNA levels of Glut1 (males p=0.415, females p=0.562), Glut3 (males p=0.496, females p=0.328), Glut4 (males p=0.610, females p=0.895), and Glut5 (males p=0.093, females p=0.286). Then we compared whether the sex was influencing the mRNA expression of glucose transporter genes in the animals. The data showed no differences between males vs females when we analysed the mRNA levels of Glut1 (Emb: p=0.053, HFD p=0.525), Glut3 (Emb: p=0.155, HFD p=0.516), Glut4 (Emb: p=0.585, HFD p=0.796), and Glut5 (Emb: p=0.080, HFD p=0.059).

Leptin is known to regulate energy homeostasis not only in the central nervous system, but also in peripheral tissues such as the liver, and its role in metabolism is mediated via leptin receptors (ObRa and ObRb)\textsuperscript{476,477}. To determine whether hepatic expression of leptin receptors is affected by the maternal HFD, leptin receptors mRNA was analysed in the different diet groups. The expression levels of genes related to the signalling of leptin ObRa (males: Emb p=0.198, HFD p=0.032; females: Emb p=0.568, HFD p=0.672), and ObRb (males: Emb p=0.222, HFD p=0.812; females: Emb p=0.291, HFD p=0.492), in general did not show significant differences between the groups. However, in HFD males, we observed an increase in ObRa (p=0.032) mRNA levels, when compared with the NFD diet (Figure 3.20). There were no differences between the Emb and the HFD groups when we analysed the mRNA levels of ObRa (females p=0.672), and ObRb (males p=0.812, females p=0.492). However,
we observed a significant difference in Emb vs HFD males in the mRNA levels of ObRa ($p=0.032$).
Then we compared whether the sex was influencing the mRNA expression of leptin receptors genes in the animals. The data showed no differences between males vs females when we analysed the mRNA levels of ObRa (Emb: $p=0.239$, HFD $p=0.555$), and ObRb (Emb: $p=0.598$, HFD $p=0.099$).

**Figure 3.20.** Effect of maternal HFD on the regulation of metabolic markers in the liver.

The mRNA levels of Glu1, Glut3, Glut4, Glut5, ObRa, and ObRb in males (a.) and females (b.) were assessed using quantitative RT-PCR. The mRNA levels of the selected markers were normalized to Pgk1 and Tbp transcripts. NFD group (blue), Emb group (green) and HFD (red) at 26 weeks of age. All bars represent means ± SEM. n=6 mice from 6 litters in each group. * $p<0.05$
3.3.6 Expression of genes related to energy metabolism in the brain.

At this point in the project, we decided to study two different regions of the brain: the somatosensory cortex and the hippocampus. The cortex is responsible for processing sensations and sensory information. The hippocampus regulates different functions such as memory, spatial orientation and neurogenesis. Therefore, modifications in terms of metabolism could reflect changes in behaviour (anxiety, locomotive activity) in these animals.

3.3.6.1 Glucose transporters in the cortex and hippocampus.

The brain uses glucose as the primary source of energy, and glucose transporters (Glut) facilitate glucose transport across the BBB and the uptake of glucose into neurons and glial cells. Each one of this Glut has a specific function and is present in different cells in the brain. Glut1, which is expressed in glial cells, allows the flow of glucose from the bloodstream to the brain. Once the glucose is in the extracellular space in the brain, is rapidly taken by Glut3 (expressed in neurons). Glut4 and Glut8 are regulated by insulin receptors, in areas of high glucose demand such as the cortex and the hippocampus, and Glut5 is expressed in microglia and is a fructose transporter.

Due to the critical role glucose transporters have in glucose homeostasis, we analysed the mRNA expression of different glucose transporter genes Glut1, Glut3, Glut4, Glut5, and Glut8 in the adult mouse brain.

**Cortex:** When analysing the HFD males, we observed that the levels of Glut1 (p=0.025) and Glut5 (p=0.039) were lower compared to the NFD group, no differences in Glut3 (p=0.689), Glut4 (p=0.149) and Glut8 (p=0.687) genes. No differences in expression levels of mRNA were observed in the Emb males in Glut1 (p=0.207), Glut3 (p=0.134), Glut4 (p=0.465), Glut5 (p=0.099) and Glut8 (p=0.100). In females, no significant differences between groups (HFD or Emb vs NFD) were observed regarding the expression levels of the different glucose transporters genes. Glut1 (Emb p=0.304, HFD p=0.104), Glut3 (Emb p=0.204, HFD p=0.245), Glut4 (Emb p=0.509, HFD p=0.857), Glut5 (Emb p=0.723, HFD p=0.428), and Glut8 (Emb p=0.463, HFD p=0.662).

We compared Emb vs HFD groups in males and females and observed that the levels of mRNA of Glut1 in males were significantly different (p=0.001), but not in females (p=0.661). We did not
observed changes in the mRNA level of *Glut3* (males p=0.248, females p=0.762), *Glut4* (males p=0.449, females p=0.592), *Glut5* (males p=0.053, females p=0.735), and *Glut8* (males p=0.603, females p=0.710), when Emb and HFD were compared. Then we compared whether the sex was influencing the mRNA expression of glucose transporter genes in the animals. The data showed no differences between males vs females when we analysed the mRNA levels of *Glut1* (Emb: p=0.119, HFD p=0.052), *Glut3* (Emb: p=0.073, HFD p=0.241), *Glut4* (Emb: p=0.521, HFD p=0.065), and *Glut5* (Emb: p=0.220, HFD p=0.815).

**Figure 3.21. Effect of maternal HFD on the regulation of glucose transporters in the cortex.**
The mRNA levels of *Glut1, Glut3, Glut 4, Glut 5, and Glut8* in males (A.) and females (B.) were assessed using quantitative RT-PCR. α: difference between NFD males and NFD females (p=0.001). The mRNA levels of the selected markers were normalized to *Fbxw2* and *Htatsf1* transcripts. NFD group (blue), Emb group (green) and HFD (red) at 26 weeks of age. All bars represent means ± SEM. n=6 mice from 6 litters in each group. * p<0.05. ** p<0.01.
**Hippocampus:** In HFD males the relative mRNA expression of *Glut1* (*p*=0.006), *Glut3* (*p*=0.0001), *Glut4* (*p*=0.0001), and *Glut8* (*p*=0.002), was reduced compared to the NFD males. However, in HFD males, the relative mRNA expression of *Glut5* (*p*=0.152) was not different compared to the NFD males. The Emb males showed lower expression levels than NFD group of *Glut3* (*p*=0.001), *Glut4* (*p*=0.0002), and *Glut8* (*p*=0.013) genes compared to the NFD males, no differences in expression levels of mRNA were observed in *Glut1* (*p*=0.885), and *Glut5* (*p*=0.200) when compared to the NFD group (Figure 3.22).

HFD females showed lower expression levels than NFD group of *Glut1* (*p*=0.039), *Glut3* (*p*=0.002), *Glut4* (*p*=0.0003), and *Glut5* (*p*=0.0003) genes, but no differences in expression levels of *Glut8* mRNA (*p*=0.681). The relative mRNA expression of *Glut3* (*p*=0.006), *Glut4* (*p*=0.010), and *Glut5* (*p*=0.018) genes in Emb females was significantly reduced, no changes in *Glut1* (*p*=0.852), and *Glut8* (*p*=0.795) (Figure 3.22).

We compared Emb vs HFD groups in males and females and observed that the levels of mRNA of *Glut1* in males were significantly different (*p*=0.005), but not in females (*p*=0.064). We did not observed changes in the mRNA level of *Glut3* (males *p*=0.335, females *p*=0.927), *Glut4* (males *p*=0.103, females *p*=0.269), *Glut5* (males *p*=0.387, females *p*=0.181), and Glut8 (males *p*=0.264, females *p*=0.547), when Emb and HFD were compared.

Then we compared whether the sex was influencing the mRNA expression of glucose transporter genes in the animals. The data showed no differences between males vs females when we analysed the mRNA levels of *Glut1* (Emb: *p*=0.462, HFD *p*=0.846), *Glut3* (Emb: *p*=0.149, HFD *p*=0.114), *Glut4* (Emb: *p*=0.453, HFD *p*=0.116), *Glut5* (Emb: *p*=0.287, HFD *p*=0.337), and *Glut8* (Emb: *p*=0.052, HFD *p*=0.213).
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Figure 3.22. Effect of maternal HFD on the regulation of glucose transporters in the hippocampus. The mRNA levels of Glu1, Glut3, Glut 4, Glut 5, and Glut8 in males (a.) and females (b.) were assessed using quantitative RT-PCR. The mRNA levels of the selected markers were normalized to Fbxw2, Pak1lp1, and Ap3d1 transcripts. NFD group (blue), Emb group (green) and HFD (red) at 26 weeks of age. All bars represent means ± SEM. n=6 mice from 6 litters in each group. * p<0.05, ** p<0.01, *** p<0.001.
3.3.6.2 Receptors of hormones that regulate metabolism in the cortex and hippocampus

In terms of regulation of metabolism, we decided to evaluate the receptors of different critical hormones for the control of energy metabolism, such as adiponectin (AdipoR1 and AdipoR2), insulin (InsR) and leptin (ObRa and ObRb) receptors, and the insulin-like growth factor 1 receptor (Igf1R).

Cortex: When analysing the HFD males we observed that the levels of AdipoR2 \( (p=0.026) \), ObRb \( (p=0.007) \), and InsR \( (p=0.022) \) were lower than the NFD group, no differences in AdipoR1 \( (p=0.588) \), Igf1R \( (p=0.141) \) and ObRa \( (p=0.408) \) genes (Figure 3.23). In Emb males, we observed decrease expression levels of InsR \( (p=0.035) \) mRNA, but no differences in AdipoR1 \( (p=0.089) \), AdipoR2 \( (p=0.660) \), Igf1R \( (p=0.163) \), ObRa \( (p=0.249) \), and ObRb \( (p=0.312) \) genes (Figure 3.23).

Females Emb displayed higher expression levels of ObRa \( (p=0.003) \), and ObRb \( (p=0.047) \), than the NFD group, but no significant changes in AdipoR1 \( (p=0.470) \), AdipoR2 \( (p=0.124) \), Igf1R \( (p=0.536) \), and InsR \( (p=0.198) \) genes (Figure 3.23). In HFD females we did not observe differences in AdipoR1 \( (p=0.998) \), AdipoR2 \( (p=0.063) \), Igf1R \( (p=0.396) \), ObRa \( (p=0.412) \), ObRb \( (p=0.420) \) and InsR \( (p=0.763) \) genes (Figure 3.23). We compared Emb vs HFD groups in males and females and observed that the levels of mRNA of AdipoR1, AdipoR2, and ObRb in males were significantly different \( (p=0.001, p=0.009, and p=0.017 \) respectively), but not in females \( (p=0.824, p=0.142, p=0.059 \) respectively).

We did not observed changes in the mRNA level of ObRa (males \( p=0.370 \), females \( p=0.451 \)), InsR (males \( p=0.205 \), females \( p=0.081 \)), and Igf1R (males \( p=0.053 \), females \( p=0.171 \)), when Emb and HFD were compared.

Then we compared whether the sex was influencing the mRNA expression of these genes in the animals. The data showed no differences between males vs females when we analysed the mRNA levels of AdipoR1 (Emb: \( p=0.286 \), HFD \( p=0.061 \)), AdipoR2 (Emb: \( p=0.472 \), HFD \( p=0.419 \)), ObRa (Emb: \( p=0.053 \), HFD \( p=0.535 \)), ObRb (Emb: \( p=0.287 \), HFD \( p=0.337 \)), InsR (Emb: \( p=0.116 \), HFD \( p=0.241 \)), and Igf1R (Emb: \( p=0.061 \), HFD \( p=0.126 \)).
Figure 3.23. Effect of maternal HFD on metabolic hormone receptors in the cortex.
The mRNA levels of AdipoR1, AdipoR2, ObRa, ObRb, InsR, and Igf1R in males (a.) and females (b.) were assessed using quantitative RT-PCR. The mRNA levels of the selected markers were normalized to Fbxw2 and Htatsf1 transcripts. NFD group (blue), Emb group (green) and HFD (red) at 26 weeks of age. All bars represent means ± SEM. n=6 mice from 6 litters in each group. * p<0.05, ** p<0.01, *** p<0.001.
**Hippocampus:** In HFD males the relative mRNA expression of *AdipoR1* \( (p=0.0001) \), *AdipoR2* \( (p=0.002) \), *InsR* \( (p=0.001) \), and *Igf1R* \( (p=0.004) \), was reduced. However, the expression of *ObRa* \( (p=0.013) \), and *ObRb* \( (p=0.002) \) was increased (Figure 3.24). Males Emb showed lower expression levels of *insR* \( (p=0.006) \) gene, and higher expression levels of *ObRb* \( (p=0.0002) \) than NFD, but no differences in *AdipoR1* \( (p=0.689) \), *AdipoR2* \( (p=0.348) \), *Igf1R* \( (p=0.187) \), and *ObRa* \( (p=0.090) \) genes (Figure 3.24).

The females HFD showed lower expression levels of *AdipoR2* \( (p=0.017) \), and higher expression levels of *ObRa* \( (p=0.049) \), and *ObRb* \( (p=0.008) \) genes, no changes in *AdipoR1* \( (p=0.472) \), *InsR* \( (p=0.184) \), and *Igf1R* \( (p=0.826) \). In Emb females the relative mRNA expression of *ObRa* \( (p=0.023) \), *ObRb* \( (p=0.0002) \), genes was significantly increased, no differences were observed in *AdipoR1* \( (p=0.404) \), *AdipoR2* \( (p=0.276) \) *InsR* \( (p=0.223) \), and *Igf1R* \( (p=0.145) \) (Figure 3.24).

We compared Emb vs HFD groups in males and females and observed that the levels of mRNA of *AdipoR1*, *AdipoR2*, and *ObRb* in males were significantly different \( (p=0.001, p=0.009, \text{and } p=0.017) \) respectively), but not in females \( (p=0.824, p=0.142, p=0.059) \) respectively). We did not observed changes in the mRNA level of *ObRa* (males \( p=0.370 \), females \( p=0.451 \)), *InsR* (males \( p=0.205 \), females \( p=0.081 \)), and *Igf1R* (males \( p=0.053 \), females \( p=0.171 \)), when Emb and HFD were compared.

Then we compared whether the sex was influencing the mRNA expression of these genes in the animals. The data showed no differences between males vs females when we analysed the mRNA levels of *AdipoR1* (Emb: \( p=0.286 \), HFD \( p=0.061 \)), *AdipoR2* (Emb: \( p=0.472 \), HFD \( p=0.419 \)), *ObRa* (Emb: \( p=0.053 \), HFD \( p=0.535 \)), *ObRb* (Emb: \( p=0.287 \), HFD \( p=0.337 \)), *InsR* (Emb: \( p=0.116 \), HFD \( p=0.241 \)), and *Igf1R* (Emb: \( p=0.061 \), HFD \( p=0.126 \)).
Figure 3.24. Effect of maternal HFD on metabolic hormone receptors in the hippocampus.

The mRNA levels of *AdipoR1*, *AdipoR2*, *ObRa*, *ObRb*, *InsR*, and *Igf1R* in males (a.) and females (b.) were assessed using quantitative RT-PCR. The mRNA levels of the selected markers were normalized to *Fbxw2*, *Pak1lp1*, and *Ap3d1* transcripts. NFD group (blue), Emb group (green) and HFD (red) at 26 weeks of age. All bars represent means ± SEM. n=6 mice from 6 litters in each group. * p<0.05, ** p<0.01, *** p<0.001.
3.4 Discussion

The thrifty phenotype hypothesis describes how poor nutrition during the early stages of development leads to an increased risk of type 2 diabetes later in life\(^{479,480}\). Different studies have described how maternal under-nutrition leads to a higher predisposition to metabolic disease in the offspring\(^{481,482}\). Recently the number of animal models that evaluate over-nutrition, using an HFD (reflecting dietary habits in western society) have increased\(^{453}\). Maternal HFD in these animals has made it possible to show how the exposure to this diet generates alterations in the development of different organs, thus triggering a higher susceptibility to various diseases in the offspring. In this study, we proposed to evaluate not only the impact of maternal HFD during pregnancy but also the time of exposure to it. Considering that the development of the fetus is based on rapid and constant cell divisions, different windows of exposure to maternal HFD may lead to various changes in different tissues or organs in the offspring.

We found that dams fed with an HFD (during the preimplantation period, or gestation and lactation) did not show increase body weight, after birth or after weaning. These data confirmed that our model did not produce obesity, and the maternal HFD caused all the effects observed in the offspring phenotype. This result is similar to studies in rats where an HFD (45% kcal of fat) was given during gestation and lactation, without causing obesity in the dams\(^{162}\). Also, different studies have observed a similar effect, concerning maternal body weight, when the introduction of HFD to mothers occurs for a short period before mating\(^{453,483}\). Then we compared the body weight of the offspring at 26 weeks of age. Here we have seen that the body weight and the ratio of brain and liver to body weight in males and females that belong to either the HFD or Emb groups, were not different from their respective NFD groups. These results are suggesting that at this point in the study, maternal HFD does not affect the body weight in the offspring at 26 weeks, similarly to previous studies of maternal HFD during gestation and lactation in rats\(^{162}\).

Different studies have shown that the consumption of HFD leads to metabolic disorders such as for overweight, glucose intolerance, high levels of leptin and imbalance between activity and energy expenditure\(^{468,469}\). Here, the effect of maternal HFD on activity in the offspring at 26 weeks of age was observed in HFD females. There was increased activity in HFD females during the day, but not in the Emb females, and no changes were observed in HFD or Emb males. Interestingly Emb and HFD males had higher activity values during the first part of the night compared to the Emb and HFD females. NFD males had higher activity values during the day and night compare to the NFD females, showing a clear difference between males and females fed a standard control diet. These findings are in line with different models of maternal obesity led to an increase in locomotor activity.
in the offspring\textsuperscript{484-487}. Some of these studies have shown females were more active compared to their control groups\textsuperscript{484,485,487}. Here, our results are suggesting that maternal HFD in the absence of obesity induces a higher activity in females during the day. However, we did not test whether this increase in activity is a result of an anxiety-provoking environment or just increase in hyperactivity\textsuperscript{488,489}. Further experiments should be done to identify the cause of this increase in activity in the HFD females.

Other factors that can be modified with the increase in activity of the HFD females might be oxygen consumption and carbon dioxide production\textsuperscript{490}. However, measurements of \( \text{vO}_2 \) and \( \text{vCO}_2 \) showed lower levels of oxygen consumption during the day and night in the male offspring of the HFD group, but no in females HFD during the day or the first part of the night. The Emb groups (males and females) did not show changes in terms of \( \text{vO}_2 \) and \( \text{vCO}_2 \). NFD males had higher \( \text{vO}_2 \) and \( \text{vCO}_2 \) values during the day and night compare to the NFD females, showing a clear difference between males and females fed a standard control diet. This data indicates that maternal exposure to an HFD during pregnancy and lactation can modify respiration in the adult male offspring, but not during the preimplantation period.

Lower oxygen consumption has been observed in animal models of mitochondrial mutations\textsuperscript{491-493}. Male mice with a single copy of \( Mclk1 \) gene (a gene encoding a mitochondrial enzyme implicated in the biosynthetic pathway of ubiquinone) showed a decrease in \( \text{vO}_2 \) consumption, and ATP production, but not in females\textsuperscript{493}. These findings obtained by Lapointe et al\textsuperscript{493} illustrate a high similarity to the data collected in our experiments. Although our model refers to a dietary intervention model and not a genetic model, we could extrapolate these data and suggest that maternal HFD could modify processes such as the electron transport rate and ATP production in offspring.

Measurements of RQ were showing how male and female HFD were displaying higher values compared to the NFD groups. Emb males (first and second part) and females (first part) showed significant differences during the day compared to their NFD groups. RQ (\( \text{vCO}_2 / \text{vO}_2 \)) is used to indirectly determine whether lipids or carbohydrates were used as the primary fuel source. Low values of RQ (0.7) reflect more significant use of lipids, while high values of RQ (1.0) indicate more significant use of carbohydrates\textsuperscript{472}. Intermediate values indicate a mixture of fat and carbohydrates as a fuel source, like our NFD group. We could observe in our data that HFD and Emb animals a tendency towards carbohydrate oxidation since the RQ ratio (≥1.0) was higher in these animals compared with the NFD group (RQ <1.0). In general, high RQ (≥1.0) values can be associated with a sedentary lifestyle, resulting in a low-fat oxidation rate. Physically active individuals tend to have
low RQ values (≈0.7) compared with inactive individuals (≥1.0)\textsuperscript{494}. We evaluated the relationship between RQ and activity in the different diet groups. In general terms, there was a significant correlation in the HFD females during the first and second part of the day, but not during the night. This is interesting, since the HFD females showed higher activity during the day, compared to the NFD group. The Emb females exhibited a significant correlation during the first part of the day and the first and second part of the night. This data suggest that these correlations are dependent on the period analysed, reflecting that the first and second part of the day and night have different patterns. Our observations of 26 weeks old HFD animals show that maternal HFD can lead to high RQ ratio later in life, without an increase in body weight or decrease in spontaneous activity.

Energy expenditure is the measure of the oxidation of substrates that contain high energy, generating heat, carbon dioxide, urea and water\textsuperscript{471}. Here we have seen that the male offspring of HFD fed dams during gestation and lactation, show lower energy expenditure during day and night, but no in female or the offspring of HFD fed dams during the preimplantation period. This data suggested that more prolonged exposure to an HFD during gestation, the offspring is more susceptible to alterations in energy expenditure. Interestingly, our data show that HFD males in the absence of obesity display low levels of energy expenditure, but not females. However, NFD males had increased values compared to the NFD females, suggesting a sex effect on energy expenditure\textsuperscript{495}.

In order to observe the effect of activity on energy expenditure, we correlate these two factors, showing a positive correlation between them in all the diet groups. It is common that energy expenditure declines with less physical activity, and to increase with more physical activity\textsuperscript{30}. However, activity was increased in the HFD females during the day, but no changes were observed in Emb groups (males and females) or HFD males. This observation suggests that different factors, other than activity, may be involved in the reduced energy expenditure in males from dams fed to HFD. One of these factors is thermogenesis since low diet-induced thermogenesis is associated with low energy expenditure\textsuperscript{30,473}. Although the objective of this thesis was not to evaluate how thermogenesis varies in these animals, it would be interesting to examine whether the effect of maternal HFD could alter thermogenesis leading to a decreased energy expenditure. Another factor associated with low energy expenditure is impaired insulin and leptin sensitivity\textsuperscript{30,473}. Leptin and insulin main action is to control energy homeostasis and energy expenditure, mediated by leptin receptor (ObRb) and insulin receptor (InsR) respectively\textsuperscript{496}. Therefore, we will discuss next the impact of maternal HFD on leptin and insulin receptors in different brain regions.
3.4.1 Maternal HFD alters energy metabolism in the mouse liver and brain

Our data indicate metabolic alterations in males from HFD fed dams; however, it is not clear which mechanisms are related to these alterations. For this reason, we decided to evaluate two crucial organs for energy balance and body metabolism, liver and brain. The liver maintains an adequate level of plasma glucose by breaking down glucose (glycogenolysis), or storing glycogen and producing glucose (gluconeogenesis) from non-carbohydrate precursors, such as lactate, amino acids and glycerol\(^{475}\). Glucose transport in the liver depends on a set of transporters called glucose transporters. These transporters obtain glucose from the blood to be an uptake in hepatocytes to be converted into glycogen\(^{497}\). To analyse the effect of maternal HFD-induced glucose uptake in hepatocytes, we investigated the liver mRNA expression of different glucose transporters such as Glut1, Glut3, Glut4 and Glut5. We did not observe changes in the liver mRNA expression of the glucose transporters in the offspring undergoing maternal high-fat feeding during pregnancy and lactation or the preimplantation period.

An essential hormone in the regulation of metabolism is leptin. Although leptin has its primary function in the central nervous system, leptin extends its functions to the liver, suppressing the production of hepatic glucose and hepatic lipogenesis, thereby providing an insulin-sensitizing and anti-steatotic effect\(^{498}\). The leptin receptor (ObR) has six isoforms generated by alternative mRNA splicing, ObR\(^b\) known as long form is critical for most of the biologic effects of leptin. The short-form ObRa is abundantly expressed in most tissues, taking part in signal transduction mediating the biological effects of leptin\(^{499-501}\). Our present data from the male offspring of HFD mothers show increased levels of ObRa mRNA but not ObR\(^b\). There were no changes in Emb males or females compared to the NFD groups. These results are in line with previous studies, where sexual dimorphism of plasma leptin concentrations has been observed\(^{502-503}\). These reports have described that male rodents exhibit higher plasma concentrations than females\(^{502-503}\). There are different factors involved in this sexual dimorphism, such as the amount of adipose tissue and the estrous cycle in females\(^{502-504,505}\). This could be one of the reasons we observed higher leptin mRNA levels in males rather than females since one of the limitations in our study was not know the estrous cycle in females. In the hepatic cell, leptin binds ObR\(^b\) resulting in the activation of the Jak2/Stat3 pathway, and the activation of the PI3K/Akt pathway\(^{501}\). However, ObRa is unable to activate the Jak-Stat pathway, but in the liver mediates leptin-regulated gluconeogenesis\(^{506,507}\). Our data raised the possibility of compensatory upregulation of ObRa expression in hepatic gluconeogenesis, associated possibly with an altered leptin or insulin signal.
The central nervous system (CNS) regulates the energy fluxes across different organs, and its role in glucose homeostasis, and insulin sensitivity has been described recently. The brain uses glucose as the primary source of energy, although in periods of starvation, it can use ketone bodies as a source of energy. Glucose is not only an energy substrate but also a signalling molecule involved in the glucoregulatory mechanism. Glucose transporters (GLUT) facilitate glucose transport across the blood-brain-barrier, and the uptake of glucose into neurons and glial cells. Knowing the fundamental role that GLUTs have in the brain, the present study examined the Glut1, Glut3, Glut4, Glut5, and Glut8 mRNA expression in the cortex and the hippocampus.

In the mouse brain cortex, Glut1 and Glut8 expression decreased in HFD males but no in HFD females. No changes were observed in the offspring of dams fed an HFD during the preimplantation period. Furthermore, sex and the time of exposure to the maternal HFD are important variables in assessing the impact of maternal diet on GLUT transcript expression in the offspring cortex. Glut1 is the most abundant glucose transporter in the brain and is present in astrocytes, and in endothelial cells and astrocytes in the blood-brain barrier. GLUT1 is essential for the normal development of the brain since mutations in the GLUT1 gene in humans generates seizures, intellectual disability, ataxia and dystonia starting in childhood due to low brain glucose levels. However, our mice did not display any of the human features caused by mutations in the GLUT1 gene. Here we demonstrate that Glut1 expression was reduced in the cortex of HFD males. However, we did not evaluate the protein in adults or mRNA expression in neonates, which could explain why we did not observe a human-like phenotype.

The role of Glut8 in the cortex is less known. Glut8 mice have been associated with neurogenesis (increasing cell proliferation in the dentate gyrus), and hyperactivity showed Glut8 was strongly expressed in hippocampus and cortex, compared to other brain regions, highlighting the importance of Glut8 in this area. Here, for the first time, we are showing how maternal HFD decreases Glut8 expression in the offspring male hippocampus. To date, the role of Glut8 in the cortex is not clear; future analyses should focus on understanding the role of Glut8 in the cerebral cortex.

In the hippocampus, we observed decrease expression of Glut1, Glut3, Glut4, and Glut8 in HFD males and Glut1, Glut3, Glut4, and Glut5 in HFD females. Emb males and females had lower values of Glut3, Glut4, and Glut5. Sex and maternal HFD modify regional and specific GLUT expression in the brain. Glut3 is mainly found in neurons, is the major glucose transporter in the cortex and hippocampus. Heterozygous Glut3 mutant mice show normal body development and brain size but display cognitive abnormalities. Glut4 and Glut8 are expressed in neurons and are insulin-
sensitive glucose transporters, located in selective areas of the brain, such as hippocampus, hypothalamus, and cortex, and Glut5 is expressed explicitly in microglia\textsuperscript{465}. These discrete localizations of these GLUTs could indicate that they may be involved in specialized activities in the CNS. Glucose transporters in the hippocampus seem to be more sensitive to maternal HFD than in other brain regions (cortex). Another reason is that neurons in the hippocampus constantly undergo dendritic pruning and reorganization, since the rate of pruning in the cortex is lower\textsuperscript{516,517}. Reduction in the levels of mRNA GLUTs in the hippocampus may reflect an increased neuronal pruning, possibly affecting neuronal-glial interactions for nutrient transport. These results obtained in the hippocampus of the offspring are like the one obtained in the offspring of dams fed an HFD during gestation and lactation (diet 60% fat)\textsuperscript{165}. In general, the reduction of these transporters in the hippocampus can modify behaviour, and explicitly learning\textsuperscript{518,519}. Maternal HFD models suggest that maternal diet leads to an increased risk of cognitive impairment and behavioural dysfunctions in the offspring\textsuperscript{128}. However, to date, no model correlates reduced expression of glucose transporters (caused by a maternal HFD), and cognitive deficits, or memory impairment in adulthood. In the following chapters, we will talk about the possible association between Glut3 and Glut8 mRNA levels and neurogenesis.

Adiponectin is made exclusively by adipose tissue, and its primary function is to increase insulin sensitivity. There is an inverse correlation between adiponectin levels and obesity\textsuperscript{520,521}. Adiponectin binds to two membrane receptors, AdipoR1 and AdipoR2 that stimulate Glut4 transcription, increasing glucose uptake\textsuperscript{522}. In the present study, the expression of AdipoR2, and AdipoR1, AdipoR2 and Glut4 mRNA decreased in the cortex and hippocampus respectively in the adult HFD offspring. These results may indicate a possible adiponectin signalling alteration that may correlate with insulin resistance; therefore, a future analysis that allows quantifying serum insulin levels is necessary. In an animal study of HFD decreased expression of AdipoR1 and AdipoR2 was observed in the cortex, and altered neurogenesis in the hippocampus, suggesting that AdipoR1 signalling might be neuroprotective against metabolic insults\textsuperscript{523}.

Other hormones controlling energy homeostasis, feeding behaviour and energy expenditure are leptin and insulin, mediating their actions though the leptin and insulin receptors, respectively\textsuperscript{496,524}. By binding to ObRb, leptin activates JAK2-STAT3 and PI3K/Akt pathway\textsuperscript{501}, and binding to InsR, insulin promotes the activation of the PI3K-Akt pathway\textsuperscript{525}. Leptin and insulin can mediate the anorexigenic effect suppressing the activity of the orexigenic NPY and AgRP neurons, and stimulating POMC and cocaine- and amphetamine-related transcript (CART) neurons\textsuperscript{526}. ObRb and InsR have PI3K as an element in common on their signalling pathways, suggesting that leptin and insulin may interact through PI3K to regulate reduction of food intake controlling the activation of
arcuate nucleus neurons\textsuperscript{527}. Therefore, it indicates a possible compensatory mechanism that would explain lower expression levels of $\text{InsR}$ and higher expression levels of $\text{ObRa}$ and $\text{ObRb}$ in the cortex and the hippocampus.

### 3.5 Conclusions

In conclusion, maternal HFD (without obesity) either confined to the preimplantation period (Emb group) or throughout gestation and lactation (HFD group) leads to adverse metabolic phenotypes without changing body weight or food intake. These results indicate a reduction in $\text{vO}_2$ consumption, $\text{vCO}_2$ production, and energy expenditure in HFD male offspring, and increase diurnal activity in HFD female offspring, as a result of maternal HFD in the absence of obesity (Figure 3.25). Different genes related to energy metabolism (glucose and leptin signalling markers) in the brain and liver were altered. However, more studies are needed to evaluate the different mechanisms that lead to the observed expression changes. Gender has been apparent throughout this chapter, showing males more sensitive to metabolic changes as a result of maternal HFD exposure. For the first time, we are showing how a maternal HFD, in the absence of obesity, can alter some parameters of the metabolic cage in the offspring at 26 weeks of age.

### 3.6 Future Work

Here we have seen mRNA expression changes in different markers in the cortex and the hippocampus in the brain. However, we did not analyse the protein expression of these markers, which would allow us to understand the role of each of these markers on energy metabolism.

The hypothalamus senses different metabolic signals to control body energy homeostasis\textsuperscript{528}. Therefore, to analyse in the hypothalamus, the same markers used in the cortex and the hippocampus would be essential to identify common pathways that help elucidate a possible mechanism that explains the different findings in the Emb and HFD groups.
Figure 3.25. Schematic diagram of overarching results in the offspring from Chapter 3. Overall effects of maternal HFD on the offspring in terms of activity, \( \text{vO}_2 \), \( \text{vCO}_2 \), RQ and energy expenditure (EE) in the offspring at 26 weeks of age. Only the significant changes observed in both parts of the day or night are shown as day or night. Significantly changes in mRNA levels of glucose transporters and metabolic genes in the cortex (Ctx) and hippocampus (hippo).
Chapter 4. The effect of maternal HFD on cell composition in the offspring new-born (PND01) and adult brain. A cortex and hippocampus story.

4.1 Introduction

Cortex development or corticogenesis consists of different stages, such as cell proliferation, differentiation, and migration. The development of the cerebral cortex in mice begins around the 11.5 embryonic day, where progenitor cells initiate a series of symmetric and asymmetric divisions\(^{529}\). These divisions will produce two different main classes of neurons, intermediate progenitors or projection neurons. Interneurons are inhibitory cells and connect locally within the neocortex, whereas projection neurons, which are mostly inhibitory, connect to different targets by sending their axons to distant areas\(^{529}\). Finally, corticogenesis ends when differentiated neurons migrate and form the different layers of the cortex in an inside-out manner.

The cortex consists of six distinct layers (layers I, II, III, IV, V, and VI), each one with different cell densities\(^{529}\). Cells in these layers generate connections with each other and with other regions of the brain, creating a series of microcircuits that can cover the entire cortex\(^{281,284,530}\). Due to this connectivity, the cerebral cortex is a vital component of the brain, involved in different processes such as cognition, motivation, and emotion\(^{274}\). Therefore, normal development steps must be preserved.

Hippocampus is another area in the mouse brain. The hippocampus is located dorsally with the neocortex and is around the curved convex medial lobe of the lateral cortex. The hippocampus is constituted of different regions, the subiculum, presubiculum, parasubiculum, and entorhinal cortex, the cornu Ammonis, and the dentate gyrus\(^{285,286,531}\). The cornu Ammonis (CA) region is compartmentalized into three subregions CA1, CA2, and CA3, which communicates with the entorhinal cortex, and the dentate gyrus (DG) creating the classic hippocampal circuitry called the trisynaptic loop (Figure 4.1). The information flows through fibres from layer II and III of the entorhinal cortex, to granule cells in the DG, which in turn send the information to CA3 through mossy fibres. CA3 pyramidal neurons send projections through the Schaffer collateral pathway to the CA1 pyramidal neurons. Finally, CA1 neurons project back to layers V and VI of the EC completing the loop\(^{532}\).
Chapter 4

The integrity of the hippocampus is critical to regulating processes such as learning and memory, anxiety, and spatial navigation. The nutritional imbalance suggests causing cognitive deficits\textsuperscript{104,105}. Several studies have evidenced that high-fat diets impair the structure and function of the hippocampus, affecting neurogenesis and hippocampal brain-derived neurotrophic factor (BDNF) levels\textsuperscript{107,109,110,533,534}. Additionally, the consumption of HFD (high in saturated fatty acids and omega-6) in humans, was enough to impair performance in a cognitive task, whereas a low-fat and omega-3 rich diet seems to play a protective role against cognitive impairment\textsuperscript{107,109,110}. Different mechanisms have been proposed to understand the relationship between HFD and cognitive effects, such as oxidative stress, insulin resistance, inflammation, among others\textsuperscript{107,109,110}. Interestingly, different studies have shown how maternal obesity caused by an HFD leads to long-term detrimental effects on learning and memory in the offspring\textsuperscript{158,149,126}. This evidence suggests that perinatal and neonatal nutrition imbalance might be responsible for hippocampal impairment.

In this chapter, we have investigated the influence of maternal HFD on the offspring brain cortex and hippocampus morphology. In order to explore eventual changes in different cell populations, we have analysed the cell density of neurons, astrocytes, and microglia in the cortex and hippocampus.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.1}
\caption{A schematic map of the trisynaptic pathway (entorhinal cortex (EC)\textendash dentate gyrus\textendash CA3\textendash CA1\textendash EC) in the hippocampus.}
\end{figure}

Cornu Ammonis (CA); Dentate Gyrus (DG); Entorhinal Cortex (EC); Lateral Perforant Path (LPP); Medial Perforant Path (LPP); Mossy Fibres (MF); Schaffer Collaterals (SC); Subiculum (S). Taken from Patten et al., 2015\textsuperscript{289}. 

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4.2 Materials and Methods

4.2.1 Determination of thickness of the adult cortex and cortical layers

Coronal DAPI-stained adult brain sections from six males and six female adult mice per group were used for this analysis. Cortical thickness was evaluated from different coronal brain sections considering three defined points in the right cortical hemisphere. Images were acquired at the rostral and the central level of the somatosensory cortex, with a Plan semi-Apochromat x10/0.3 objective. Cortex thickness measurements were done using the ImageJ software.

4.2.2 Immunofluorescence

PND1 animal bodies were fixed with 4% formaldehyde solution and stored at room temperature in a 70% ethanol solution for two years. Brains were flushed with 1XPBS, removed from the skull, and then slowly rehydrated in 50%, and 30%, 1X PBS, 24 hours each step. Then brains were cryoprotected by transferring them to 15% sucrose and then 30% sucrose solution in PBS until they sank at 4°C. The next day, tissues were equilibrated in 50:50 30% sucrose in PBS: OCT (Tissue Tek) for 1 hour and subsequently embedded in OCT. Tissue blocks were stored at -80°C. Tissue was sectioned at 14 µm thickness onto Superfrost Plus slides (Fisher Scientific) on a Leica cryostat and stored at -20°C. Coronal brain sections were made between bregma 3.39mm and bregma 3.63 mm for hippocampal samples, according to the stereotaxic coordinates of the mouse brain atlas\textsuperscript{436}.

Adult brains were dissected before 4% PFA fixation for 12-24 hours and then placed in 30% sucrose for 24-48 hours. Then, they were embedded in Optimum Cutting Temperature (OCT, Tissue Tek) and stored at -80°C until sectioning. For cortical analysis, coronal brain cryostat sections (14 µm thick) were made between bregma 1.42mm and bregma 0.26 mm, according to the stereotaxic coordinates of the mouse brain atlas\textsuperscript{235}. For hippocampal analysis, coronal brain sections (14 µm thick) were made between bregma -1.22mm and bregma -2.70 mm for hippocampal samples, and from bregma 1.10mm to bregma 0.14 mm for lateral ventricle.

Primary antibodies used include NeuN (Merck Millipore, MAB377, 1:200), mouse anti-S100b (Abcam, Ab52642, 1:200), rabbit anti-GFAP (Dako, Z0334, 1:500), and rabbit anti-Iba1 (Wako, 019-19741, 1:500). The secondary antibodies were Alexa Fluor 488, or 568-conjugated donkey anti-mouse, anti-rabbit IgG (1:200), and anti-goat. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Then Mowiol®4-88 Reagent (Calbiochem, Darmstadt, Germany) was used as mounting media. The detailed protocol is described in the materials and methods chapter (Chapter
Primary antibodies were not used in the negative controls, and stained sections were examined under a Leica fluorescence microscope at x10, x20, and x40 magnification (Leica, DM 5000B Germany).

### 4.2.3 Imaging analysis

Postnatal day 1 (PND1) brains were analysed by counting positive cells in the different regions of interest in the hippocampus and the cortex. A part of this assay was done in collaboration with Callum Robins (BM student, Faculty of Medicine, University of Southampton).

In the adult brain, the quantification for NeuN, GFAP, S100b, and Iba1 markers was performed by counting positive cells with Image J. Three slices per animal, and three non-overlapping fields per layer were analysed in the somatosensory cortex and the hippocampus.

### 4.2.4 Quantitative real-time PCR

Total RNA was isolated from frozen liver and frozen brain cortex and hippocampus using a RNeasy Lipid Tissue Mini kit (Qiagen, UK) according to the manufacturer’s instructions. The isolated RNA was quantitated using the Nanodrop ND-1000 spectrophotometer (LabTech UK), and only samples with an adequate RNA concentration (A260/A280 ≥1.8) and purity (A230/A260 ≥2.0) were selected for reverse transcription. 300ng of RNA from each sample was reverse transcribed using a Precision Reverse Transcription Premix kit (Primer Design, UK) following the manufacturer’s protocol. Gene expression of different markers was quantified using highly specific primers as listed in Table 5.1 (Sigma Aldrich, or Primer Design, UK). qPCR was then performed on a CFX96 Real-Time System (Bio-Rad, Hercules, CA). Melting curve analysis was carried out to verify the amplification specificity. Stability of 2–3 housekeeping genes were validated using GeNorm (gene stability value: 0.5; coefficients variation: 0.2) and used for normalization (qBase+3.0; Biogazelle, Ghent, Belgium). Finally, data analysis was performed using the comparative CT method (2−ΔΔCT). All primer sequences are in chapter 2, table 2.6.

### 4.2.5 Western blot analysis

For Western blot, we followed the procedures according to the protocol described in materials and methods (Chapter 2). The proteins were separated by SDS-PAGE and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% fat-free milk in PBST for one hour at 4°C, and then, incubated overnight with anti-NeuN (mouse, 1:5000; Millipore),
anti-GFAP (rabbit, 1:10000; Dako), anti-S100b (mouse, 1:1000; Abcam), anti-GAPDH (mouse or rabbit depending on the marker of interest, 1:10000) at 4°C overnight. After washing, the membranes were incubated 1 hour with secondary antibodies IRDye®680RD Goat anti-mouse red (1:10000) and IRDye®800CW Goat anti-rabbit green (1:10000). The detection and quantification of the blots were performed using the Odyssey imaging system (LI-COR). Densitometric quantification of protein bands was performed with Image Studio Lite, version 5.2 (Li-Cor, Lincoln, NE, USA).

4.2.6 Statistical analysis

For all experiments, data from the different diet groups were first normalized using the Z-score transformation. The Z-score was calculated by the ratio of the difference of each variable and its mean over the standard deviation of that variable. We used the Z-transformed data to compare the Emb, HFD, and the NFD groups using a multilevel random effects regression model accounting for different parameters (litter size, sex, and body weight) from individual animals (SPSS version 24). For gender comparison, we used the Z-transformed data using a multilevel random effects regression model accounting for litter size, diet group, and body weight. The normality of data was tested using the Shapiro–Wilk test. A probability of $p$ less than 0.05 was significant. All data are expressed as the mean ± standard error of the mean (SEM). $P$-value is shown as * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p\leq 0.0001$. 
4.3 Results

4.3.1 Maternal HFD impairs cortical thickness and cell density of the adult offspring brain.

Maternal HFD diets have been described to produce changes at hormonal and metabolic levels, affecting the early development of the brain. This maternal consumption of HFD can influence the offspring development creating a stressful environment in utero. Therefore, we assessed the possible impact of maternal HFD on structure, and cell densities in the adult cortex, as a result of changes in neurogenesis in the early stages of brain development.

4.3.1.1 Cortex layer thickness

The mean thickness of each layer in the cortex was analysed for all animals (Tables 4.1 and 4.2). In the male HFD group, there was no significant difference between the HFD diet group compared to the NFD group for layers 1 (p=0.996), 2/3 (p=0.111), 4 (p=0.692), 5 (p=0.262) and 6 (p=0.390). No significant differences were found between the Emb males and the NFD males in regard to their cortex thickness in any layer analysed: layers 1 (p=0.183), 2/3 (p=0.246), 4 (p=0.385), 5 (p=0.730) and 6 (p=0.477). There is not a significant difference between the Emb-HFD and the HFD group for layers 1 (p=0.177), 2/3 (p=0.683), 4 (p=0.216), 5 (p=0.452) and 6 (p=0.909).

In females the HFD did not show significant differences compared to the NFD group for layers 1 (p=0.394), 2/3 (p=0.640), 4 (p=0.130), 5 (p=0.897) and 6 (p=0.794). The different layers in the Emb females were not different across the layers when compared to the NFD layers 1 (p=0.788), 2/3 (p=0.836), 4 (p=0.121), 5 (p=0.743) and 6 (p=0.742). There is not a significant difference between the Emb and the HFD group for layers 1 (p=0.310), 2/3 (p=0.535), 4 (p=0.777), 5 (p=0.653) and 6 (p=0.913).

There is not a significant sex effect (females vs. males) on the different layer in the adult brain cortex: layers 1 (NFD p=0.806; Emb p=0.145; HFD p=0.470), 2/3 (NFD p=0.636; Emb p=0.555; HFD p=0.772), 4 (NFD p=0.181; Emb p=0.840; HFD p=0.680), 5 (NFD p=0.749; Emb p=0.448; HFD p=0.348) and 6 (NFD p=0.627; Emb p=0.729; HFD p=0.636).

The total thickness of the cortex across the mice born from mothers fed on either the HFD, Emb, or NFD diets was measured. There was no significant increase in total cortex thickness in the HFD (males p=0.438, females p=0.427) and Emb (males p=0.187, females p=0.968) groups compared to the NFD group. There was not a significant increase in total cortex thickness in the HFD group.
compared to the Emb group (males p=0.533, females p=0.460). No significant effects were found between females vs males on the total cortex thickness in the adult brain cortex (NFD p=0.244; Emb p=0.492; HFD p=0.736). In conclusion, we observed that maternal HFD does not cause a change in the thickness in the offspring mouse cortex in the Emb or HFD (Figure 4.2).

Table 4.1 The thickness of the somatosensory cortex (CTX) and each cortical layer in males and females.

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<td>Mean (µm)</td>
<td>SEM</td>
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<td>55.79</td>
<td>1783.49</td>
</tr>
</tbody>
</table>

Table 4.2 The thickness of the somatosensory cortex (CTX) and each cortical layer in males and females.

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<td>SEM</td>
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<td>Whole CTX</td>
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Figure 4.2. The thickness of the cortex measured from DAPI stained cortex sections;

Each chart represents the thickness of the different layers and the whole cortex by gender. NFD group (blue), Emb group (green), and HFD (red). All bars represent means ± SEM. n=6 mice from 6 litters in each group. a. There were no significant differences in HFD or Emb males compared to the NFD male group, or Emb males compared to HFD males. b. No differences were observed in females neither in HFD or Emb groups compared to the female NFD group, or Emb males compared to HFD males.
4.3.1.2 Total cell density in the cortex

After analysing the thickness of the cortex, we wondered whether exposure to a maternal HFD is enough to cause a change in the number of cells residing in the cortex of the adult offspring. Therefore, we analysed the number of positive DAPI stained cells present in the regions of interest (Figure 4.3), to evaluate the total cell density, since DAPI is a nuclear marker that binds strongly to Adenine and Thymine rich sequence of DNA in the cell538.

Our results revealed a significant increase in cell density of DAPI-positive cells/mm2 in some layers of the offspring cortex in the HFD groups (males and females) compared to the NFD groups (Figure 4.4). The number of cells (DAPI positive cells) within the different regions of interest in each layer in the cortex, were analysed across the three diet conditions.

![Figure 4.3. Cell density measured from DAPI stained cortex sections; blue DAPI cells. Scale bar 100µm.](image)

Layer 1 showed a significant difference between the mean of the three diet groups in males. The HFD males showed increased cell density ($p=0.00019$), the Emb group ($p=0.006$) as well when compared to the NFD group. There is not a significant difference between the Emb males and the
HFD males (p=0.128). In females, there are no significant differences between the HFD (p=0.171) or Emb (p=0.507) and the NFD group. There is not a significant difference between the Emb females and the HFD females (p=0.070). There was an effect of sex on Layer 1, with Emb males showing a higher cell density than females (p=0.008), but no differences in NFD (p=0.114) or HFD groups (p=0.110).

Layer 2/3 showed an increase in cell density between HFD males and the NFD group (p=0.035), but, we did not observe a significant difference in the Emb males (p=0.967). There is a significant difference between the Emb males and the HFD males (p=0.038). In females, we observed an increased cell density in the Emb (p=0.000356) and the HFD (p=0.026) groups compared to the NFD group. There is a significant difference between the Emb females and the HFD females (p=0.018). There was an effect of sex on Layer 2/3, with Emb females showing a higher cell density than males (p=0.002), but no differences in NFD (p=0.347) or HFD (p=0.446).

Layer 4 shows a significant increase in cell density between HFD males and the NFD group (p=0.005), but no significant differences in the Emb males (p=0.699). There is a significant difference between the Emb males and the HFD males (p=0.014). In females, there are no significant differences between the HFD (p=0.640) or Emb (p=0.757) and the NFD group. There is not a significant difference between the Emb females and the HFD females (p=0.469). There was not an effect of sex on Layer 4 in NFD (p=0.058), Emb (p=0.347) or HFD groups (p=0.405).

Layer 5 did not show significant differences between the number of cells in the HFD or Emb males compared to the NFD male group (p=0.072 and p=0.796, respectively). There is a significant difference between the Emb males and the HFD males (p=0.050). In females, the HFD group showed increased cell density (p=0.022), and the Emb group (p=0.001) as well when compared to the NFD group. There is not a significant difference between the Emb females and the HFD females (p=0.086). There was an effect of sex on Layer 5, in the NFD and HFD groups showing changes in cell density (p=0.024, and p=0.049, respectively), but no differences in Emb (p=0.053) group.

Layer 6 did not show a significant difference in the HFD females (p=0.937), but the Emb males had a higher number of cells compared to the NFD male group (p=0.025). There is not a significant difference between the Emb males and the HFD males (p=0.167). In females, there are no significant differences between the HFD (p=0.235) or Emb (p=0.798) and the NFD group. There is a significant difference between the Emb females and the HFD females (p=0.019). There was an effect of sex on Layer 6, with HFD females showing a higher cell density than males (p=0.020), but no differences in NFD (p=0.458) or Emb groups (p=0.114).
Figure 4.4. Cell density per layer of the cortex quantified from DAPI stained sections;

a. Cell density was higher in the HFD males in layers 1, 2/3, and 4, whereas the Emb group was showing a higher density in layer 1. HFD females showed higher cell density in layers 2/3, 5, and 6; and Emb in layers 2/3, and 5. In Layer 1, Emb males showed a higher cell density than females ($\alpha$ $p=0.008$), but in layer 2/3, Emb females showed a higher cell density than males ($\beta$ $p=0.002$). In layers 5, HFD males showed a higher cell density than females ($\gamma$ $p=0.049$), and in layer 6, HFD females showed a higher cell density than males and ($\delta$ $p=0.020$). NFD males had a higher cell density than females in layer 5 ($\epsilon$ $p=0.024$). All bars represent means ± SEM. n=6 mice from 6 litters in each group * $p<0.05$, ** $p<0.01$, *** $p<0.001$. 

NFD group (blue), Emb group (green), and HFD (red).
4.3.2 Maternal HFD does not causes alterations of neuronal populations in the adult offspring brain.

We evaluated the density of neurons in the cortex since these cells are a significant component of this region in the brain. The number of cells stained with NeuN (Figure 4.5) was counted and given the number of NeuN positive cells/mm² (Tables 4.3 and 4.4; Figure 4.6).

Figure 4.5. Coronal sections of the somatosensory cortex stained with NeuN and DAPI; Immunostained cortex tissue for the detection of mature neurons using the NeuN marker. (a, b and c) DAPI staining (blue, general cell marker); (d, e and f) neurons positive for NeuN (green, neuron-specific marker); (g, h and i) merged channels for NeuN+/DAPI+ cells. These images were further magnified in panel (j, k, and l), showing some examples of NeuN+/DAPI+ cells (yellow arrows). (a, d, g and j) NFD group. (b, e, h, and k) Emb group. (c, f, i, and l) HFD group. The scale bar represents 100µm.
Layer 1 did not show a significant difference between the mean of the three diet groups in males. The HFD males did not show changes in terms of neuronal cell density (p=0.302), similar to the Emb group (p=0.165) when compared to the NFD group. There is not a significant difference between the Emb males and the HFD males (p=0.658). In females, there are no significant differences between the HFD (p=0.673) or Emb (p=0.305) and the NFD group. There is not a significant difference between the Emb females and the HFD females (p=0.484). There was an effect of sex in layer 1 in the NFD group showing a higher cell density in females than males (p=0.029), but no differences between females vs males in the Emb (p=0.099) or HFD (p=0.563) groups.

We did not observe changes in the number of neurons in layer 2/3 between HFD males and the NFD group (p=0.463), neither between Emb males (p=0.510) and the NFD group. There is not a significant difference between the Emb males and the HFD males (p=0.180). In females, there were no modifications in the neuronal cell density in Emb (p=0.156) or HFD (p=0.145) groups compared to the NFD group. There is not a significant difference between the Emb females and the HFD females (p=0.853). There was not an effect of sex (females vs males) in layer 2/3 in the NFD (p=0.956), Emb (p=0.864) or HFD groups (p=0.173).

Layer 4 shows a significant decrease in NeuN cell density between HFD males and the NFD group (p=0.036), but in the Emb males, the neuronal cell density showed a decreasing trend but did not reach significance (p=0.056). There is not a significant difference between the Emb males and the HFD males (p=0.900). In females, there are no significant differences between the HFD (p=0.626) or Emb (p=0.794) and the NFD group. There is not a significant difference between the Emb females and the HFD females (p=0.489). There was not an effect of sex (females vs males) in layer 4 in the NFD (p=0.232), Emb (p=0.925) or HFD groups (p=0.431).

In layer 5, we observed a significant increase in the number of neurons between the HFD males (p=0.005) compared to the NFD male group; however, no changes were observed in the Emb males (p=0.127). There is not a significant difference between the Emb males and the HFD males (p=0.141). In females, the HFD group did not show changes in neuronal cell density (p=0.603), similar to the Emb group (p=0.115) when compared to the NFD group. There is a significant difference between the Emb females and the HFD females (p=0.046). There was an effect of sex in layer 5 in the NFD group showing a higher cell density in females than males (p=0.005), but no differences between females vs males in the Emb (p=0.832) or HFD (p=0.616) groups.

We did not observe changes in the number of neurons in layer 6 between HFD males and the NFD group (p=0.231), neither between Emb males (p=0.446) and the NFD group. There is not a significant difference between the Emb males and the HFD males (p=0.066). In females, there were
no modifications in the neuronal cell density in Emb (p=0.418) or HFD (p=0.955) groups compared to the NFD group. There is not a significant difference between the Emb females and the HFD females (p=0.432). There was not an effect of sex (females vs males) in layer 6 in the NFD (p=0.128), Emb (p=0.051) or HFD groups (p=0.087).

These data showed that the maternal HFD in our model did not affect the neuronal density in most layers, indicating that it is unlikely that neurons are behind the changes in cell density in the cortex.

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Table 4.3. NeuN+ cells in the somatosensory cortex in males.

Cells/mm² ± SEM estimated cell count of NeuN cell density of the somatosensory cortex (CTX) and each cortical layer for each group.
Table 4.4. NeuN+ cells in the somatosensory cortex in females.

Cells/mm² ± SEM estimated cell count of NeuN cell density of the somatosensory cortex (CTX) and each cortical layer for each group.
Figure 4.6. Neuronal cell density in the different cortex layers;

a. There were significant differences in HFD in layers 4 and 5 compared to the NFD group. b. No differences were observed in females neither in HFD or Emb groups. In Layer 1, NFD females showed a higher NeuN cell density than males ($\alpha p=0.029$). In layers 5, NFD females showed a higher cell density than females ($\beta p=0.005$). NFD group (blue), Emb group (green), and HFD (red). All bars represent means ± SEM. n=6 mice from 6 litters in each group. *$p<0.05$. 

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4.3.3 Effect of Maternal HFD on NeuN Expression in the Adult Cortex

Neuronal Nuclei (NeuN) is the DNA-binding, neuron-specific protein NeuN and is expressed in most neuronal cell types throughout the nervous system. However, some types of neurons appear not to be reactive to this marker, such as Cajal-Retzius cells in the neocortex\textsuperscript{539,540}.

Western Blot analysis carried out on whole cell lysates extracted from the cortex reveals NeuN as two bands in the 46-48kDa range (Figure 4.7). NeuN expression in the HFD group (males \( p=0.962 \), females \( p=0.770 \)) or Emb group mice (males \( p=0.816 \), females \( p=0.481 \)) was not significantly different from the levels in the NFD-fed mice (Figure 4.8). NeuN expression in the HFD group (males \( p=0.778 \), females \( p=0.331 \)) was not significantly different from the levels in the Emb group. There was an effect of sex on NeuN expression, with females expressing higher levels than males in the NFD, Emb and HFD groups (\( p=0.041, p=0.007, \) and \( p=0.049, \) respectively).

**Figure 4.7.** NeuN protein expression in the cortex in males and females; Western Blot used for analysis, in males (a) and females (b). NeuN (top bands) detected as a band at 46kDa - 48kDa and GAPDH (bottom band) at 37kDa. NFD: normal fat diet; Emb: embryonic high-fat diet; HFD: high-fat diet.
Figure 4.8. NeuN protein level analysis in the cortex;

Analysis of the NeuN western blot did not show significant differences in males or females. n=6 mice from 6 litters in each group. Females expressed higher levels than males in the NFD (α p=0.041), Emb (β p=0.007), and HFD (γ p=0.049) groups. NFD group (blue), Emb group (green) and HFD (red) at 26 weeks of age. All bars represent means ± SEM.

Because NeuN presents two isoforms 46 and 48 kDa, the analysis was based on the intensity of these two bands together. It has been described that NeuN is expressed in higher proportion in the cellular nuclei, and to a lesser extent, in the perinuclear cytoplasm\textsuperscript{39,40}. It has been suggested that these two isoforms (46-48kDa) are present in both the cell nuclei and the perinuclear cytoplasm; however, their concentration may vary. The two isoforms have a higher concentration in the nucleus, with the 46kDa isoform being the most predominant, but at the cytoplasmic level, the most predominant isoform is 48kDa\textsuperscript{41,42}. This difference in concentration between these two isoforms could explain the intensity variation between bands, with the 48 kDa band more intense in some samples than the 46 kDa band.
4.3.4 Maternal HFD causes alterations of astrocytic populations in the cortex of the adult offspring brain.

4.3.4.1 Maternal HFD alters astrocytic density in the adult brain cortex

We further investigated whether the altered number of cells in the cortex was due to modifications in the glial population. Here we decided to analyse astrocytic cells since we did not observe significant changes in the neural population. Astrocytes have a range of functions, including nutrient provision, energy metabolism, blood flow regulation, and neurotransmission\(^{543}\). To examine astrocytic cell density in the cortex, we stained tissue with GFAP (Figure 4.9). Results from immunostaining demonstrated that the number of GFAP positive cells was increased in the cortex of the animals in the HFD group as compared with the NFD group animals (Tables 4.5 and 4.6).
Figure 4.9. Representative images of coronal sections showing DAPI and GFAP staining in the cortex of mice from the different maternal diet groups.

Immunostained cortex tissue for the detection of Astrocytes using the GFAP marker. (a, b and c) DAPI staining (blue, general cell marker); (d, e and f) positive astrocytes for GFAP (green, Glial Fibrillary Acidic protein marker); (g, h and i) merged channels for GFAP+/DAPI+ cells. These images were further magnified in panel (j, k, and l), showing some examples of GFAP+/DAPI+ cells (yellow arrows). (a, d, g and j) NFD group. (b, e, h, and k) Emb group. (c, f, i, and l) HFD group. The scale bar represents 100μm, except in j, k, and l panels; the scale bar is 250μm.
Layer 1 did not show a significant difference between GFAP cell density in HFD or Emb male groups (p=0.105, and p=0.484, respectively). There is not a significant difference between the Emb males and the HFD males (p=0.359). In females, there was a significant difference between the HFD group (p=0.014) and the NFD group, but not between the Emb (p=0.274) and the NFD groups. There is a significant difference between the Emb females and the HFD females (p=0.003). There was an effect of sex on the number of GFAP cells, with NFD and HFD males showing a lower cell density than females (p=0.012, and p=0.020, respectively), but no differences in the Emb group (p=0.957).

Layer 2/3 showed an increase in GFAP cell density between HFD males and the NFD group (p=0.035); however, we did not observe a significant difference in the Emb males (p=0.135). There is not a significant difference between the Emb males and the HFD males (p=0.522). In females, we did not observe differences in GFAP cell density in the Emb (p=0.843), and the HFD showed an increasing trend but did not reach significance (p=0.067) compared to the NFD group. There is not a significant difference between the Emb females and the HFD females (p=0.072). There was an effect of sex on the number of GFAP cells, with Emb males showing a lower cell density than females (p=0.003), but no differences in the NFD or HFD groups (p=0.897, and p=0.697, respectively).

We did not observe changes in the number of GFAP positive cells in layer 4 between HFD males and the NFD group (p=0.101), neither between Emb males (p=0.226) and the NFD group. There is not a significant difference between the Emb males and the HFD males (p=0.683). We observed a significant increase in the number of GFAP positive cells between the HFD females (p=0.004) compared to the NFD female group; however, no changes were observed in the Emb females (p=0.532). There is a significant difference between the Emb females and the HFD females (p=0.004). There was an effect of sex on the number of GFAP cells, with HFD males showing a lower cell density than females (p=0.012), but no differences in the NFD and Emb groups (p=0.368, and p=0.112, respectively).

Layer 5 shows a significant increase in GFAP cell density between HFD males and the NFD group (p=0.008). However, in the Emb males, GFAP cell density did not show significant differences compared to the NFD male group (p=0.125). There is not a significant difference between the Emb males and the HFD males (p=0.201). In females, the HFD group showed increased cell density (p=0.0001), and the Emb group (p=0.00074) as well when compared to the NFD group. There is a significant difference between the Emb females and the HFD females (p=0.007). There was not an effect of sex on the number of GFAP cells in layer 5 in NFD (p=0.262), Emb (p=0.861) or HFD groups (p=0.633).
Layer 6 did not show a significant difference in the HFD males (p=0.104), or in the Emb (p=0.233) compared to the NFD male group. There is not a significant difference between the Emb males and the HFD males (p=0.680). In females, we observed a significant increase in the number of astrocytes between the HFD females (p=0.00001) compared to the NFD female group; however, no changes were observed in the Emb females (p=0.093). There is a significant difference between the Emb females and the HFD females (p=0.0003). There was not an effect of sex on the number of GFAP cells in layer 6 in NFD (p=0.108), Emb (p=0.297) or HFD groups (p=0.191).

The immunofluorescent results revealed that the number of GFAP positive cells was increased significantly in different layers of the cortex in mice that were exposed to a maternal-HFD during gestation and lactation. These results showed an increase in the number of astrocytes in both Emb and HFD groups, with the HFD female group showing the highest density of GFAP+ cells in most of the layers (Figure 4.10).

<table>
<thead>
<tr>
<th></th>
<th>NFD</th>
<th>Emb</th>
<th>HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cells/mm²</td>
<td>SEM</td>
<td>cells/mm²</td>
</tr>
<tr>
<td>L1</td>
<td>746.53</td>
<td>54.94</td>
<td>832.18</td>
</tr>
<tr>
<td>L2/3</td>
<td>255.56</td>
<td>30.05</td>
<td>326.85</td>
</tr>
<tr>
<td>L4</td>
<td>214.81</td>
<td>12.96</td>
<td>250.00</td>
</tr>
<tr>
<td>L5</td>
<td>195.06</td>
<td>29.61</td>
<td>303.09</td>
</tr>
<tr>
<td>L6</td>
<td>259.88</td>
<td>32.56</td>
<td>308.02</td>
</tr>
</tbody>
</table>

Table 4.5. GFAP cell density in the somatosensory cortex in males.
Cells/mm² ± SEM estimated cell count of GFAP cell in each layer of the somatosensory cortex (CTX) for each group.
<table>
<thead>
<tr>
<th></th>
<th>NFD</th>
<th>Emb</th>
<th>HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>L6</td>
<td>188.27 ± 12.74</td>
<td>246.91 ± 15.82</td>
<td>425.00 ± 24.43</td>
</tr>
<tr>
<td>L5</td>
<td>151.85 ± 13.79</td>
<td>283.95 ± 34.69</td>
<td>456.17 ± 30.17</td>
</tr>
<tr>
<td>L4</td>
<td>248.15 ± 24.12</td>
<td>285.19 ± 13.58</td>
<td>417.59 ± 30.10</td>
</tr>
<tr>
<td>L3/2</td>
<td>258.64 ± 18.41</td>
<td>248.77 ± 28.49</td>
<td>329.63 ± 23.70</td>
</tr>
<tr>
<td>L1</td>
<td>946.76 ± 92.71</td>
<td>884.26 ± 88.71</td>
<td>1294.85 ± 78.91</td>
</tr>
</tbody>
</table>

Table 4.6 GFAP cell density in the somatosensory cortex in females.

Cells/mm² ± SEM estimated cell count of GFAP cell in each layer of the somatosensory cortex (CTX) for each group.
Figure 4.10. Number of GFAP+ cells in the different cortex layers.

a. There were diet-induced differences in the number of astrocytes in layer 2/3 and 5 in the HFD group males compared to the NFD group. b. Differences were found in astrocyte quantity in most of the layers when the HFD group was compared to NFD (layers 1, 4, 5, and 6), and Emb had a significantly higher astrocyte cell in layer 5. Emb females were significantly different from HFD females in layers 1, 4, 5, and 6. In Layer 1, NFD and HFD females showed a higher GFAP cell density than males (\(\alpha p=0.012\), and \(\beta p=0.020\), respectively). In layer 2/3, Emb females showed a higher cell density than males (\(\gamma p=0.003\)). In layer 4, HFD females showed a higher cell density than males (\(\delta p=0.0012\)). NFD group (blue), Emb group (green), and HFD group (red). All bars represent means ± SEM. n=6 mice from 6 litters in each group * p<0.05, ** p<0.01, *** p<0.001, **** p≤0.0001.
4.3.4.2 Maternal HFD causes alterations of S100b astrocytic density in the cortex

Astrocytes are morphologically and functionally heterogeneous; therefore, their identification is complex and challenging. Glial fibrillary acidic protein (GFAP) is the main component of the intermediate filament in the cytoskeleton in astrocytes\textsuperscript{544}. GFAP expression is limited to processes and the cell body, which suggests that GFAP is involved in maintaining mechanical strength and shape. Antibodies against GFAP protein have helped to identify and to distinguish astrocytes from other types of glial cells\textsuperscript{545}. One of the main problems of GFAP as a marker is the variation of its expression levels in different brain regions, for example, GFAP is expressed only in approximately 15-20 percent of astrocytes in the cortex of mature animals because GFAP expression differs widely between different astrocyte populations\textsuperscript{239,546}. For this reason, additional analysis to clarify the relationship between GFAP cell density and the number of astrocytes in the brain cortex was needed.

In addition to GFAP as an astrocytic marker, S100 calcium-binding protein B (S100) has been a widely used marker\textsuperscript{547}. The protein S100b belongs to the S100 family of calcium-binding proteins, and astrocytes represent the brain cell type with the highest expression of S100B. Astrocytes produce and secrete S100b to control astrocytic proliferation, cell structure, energy metabolism, and calcium homeostasis\textsuperscript{548}. To verify the number of astrocytes in the cortex, immunohistochemistry was conducted to quantify the expression of S100b positive cells (Figure 4.11 shows GFAP\textsuperscript{+}, S100b\textsuperscript{+} and S100b\textsuperscript{+}/GFAP\textsuperscript{+} cells). Results from immunostaining demonstrated that the number of S100b positive cells was increased in the cortex of the animals in the HFD group as compared with the NFD group animals (Tables 4.7 and 4.8).
Figure 4.11. Cells expressing S100b and GFAP in the cortex.

Images of coronal cortex sections analysed for S100b (red first column from the left), and GFAP (green second column from the left), DAPI (blue, third column from the left), and double staining S100b+/GFAP+ (fourth column from the left). NFD group (Top row), Emb group (middle row) and HFD (bottom row). Scale bars, 100 μm.
Layer 1 did not show significant differences in the HFD and Emb groups in males (p=0.131, and p=0.615, respectively) when compared to the NFD group. There is not a significant difference between the Emb males and the HFD males (p=0.321). In females, The HFD group showed increased S100b cell density compared to the NFD group (p=0.00098), but no changes were observed in the Emb group (p=0.134). There is not a significant difference between the Emb females and the HFD females (p=0.059) (Tables 4.7 and 4.8). There was an effect of sex on the number of S100b cells, with Emb and HFD males showing a lower cell density than females (p=0.005, and p=0.005, respectively), but no differences in the NFD group (p=0.409).

The percentage of GFAP+ cells expressing S100b did not vary between HFD males and the NFD group (p=0.311), neither between Emb males (p=0.223) and the NFD group. There is not a significant difference between the Emb males and the HFD males (p=0.784). In females, there were no modifications in the percentage of GFAP+ cells expressing S100b in Emb (p=0.358), or in HFD (p=0.881) groups compared to the NFD group (Tables 4.7 and 4.8). There is not a significant difference between the Emb females and the HFD females (p=0.509). There was an effect of sex on the percentage of GFAP+ cells expressing S100b with NFD females showing a lower percentage than males (p=0.032), but no differences in the NFD or Emb groups (p=0.310, and p=0.626, respectively).

In layer 2/3, we did not observe differences in S100b cell density in Emb males (p=0.894), whereas the HFD showed an increasing trend, but did not reach significance (p=0.060) compared to the NFD group. There is not a significant difference between the Emb males and the HFD males (p=0.088). In females, we observed an increase in S100b cell density between the HFD and the NFD group (p=0.00087); however, we did not find a significant difference in the Emb group (p=0.627) (Tables 4.7 and 4.8). There is a significant difference between the Emb females and the HFD females (p=0.006). There was an effect of sex on the number of S100b cells, with HFD males showing a lower percentage than females (p=0.011), but no differences in the NFD or Emb groups (p=0.081, and p=0.852, respectively).

The percentage of GFAP+ cells expressing S100b in layer 2/3 did not vary between the Emb groups and the NFD groups (males p=0.354, females p=0.908). In the HFD, we observed an increase in the percentage of GFAP+ cells expressing S100b in males (p=0.034) and females (p=0.022) groups compared to the NFD group (Tables 4.7 and 4.8). There is not a significant difference between the Emb males and the HFD males (p=0.214); however, there is a significant difference between the Emb females and the HFD females (p=0.032). There was not an effect of sex on the percentage of GFAP+ cells expressing S100b cells in layer 2/3 in NFD (p=0.739), Emb (p=0.507), and HFD groups (p=0.385).
Layer 4 did not show significant differences between the number of S100b cells in the HFD or Emb males compared to the NFD male group (p=0.490 and p=0.392, respectively). There is not a significant difference between the Emb males and the HFD males (p=0.836). In females, the HFD group showed increased S100b cell density (p=0.000031), and the Emb group (p=0.030) as well when compared to the NFD group (Tables 4.7 and 4.8). There is a significant difference between the Emb females and the HFD females (p=0.011). There was an effect of sex on the number of S100b cells, with NFD females showing a lower cell density than males (p=0.015), but no differences in the Emb or HFD groups (p=0.520, and p=0.082, respectively). We did not observe changes in the percentage of GFAP+ cells expressing S100b in the HFD males and the NFD group (p=0.082), neither between Emb males (p=0.426) and the NFD group. There is a significant difference between the Emb males and the HFD males (p=0.020). In females, there were no modifications in the percentage of GFAP+ cells expressing S100b in Emb (p=0.170) or HFD (p=0.284) groups compared to the NFD group (Tables 4.9 and 4.10). There is a significant difference between the Emb females and the HFD females (p=0.028). There was an effect of sex on the percentage of GFAP+ cells expressing S100b cells in layer 4 in Emb females showing a lower percentage than males (p=0.011), but no differences in the NFD or Emb groups (p=0.647, and p=0.920, respectively).

Layer 5 showed an increase in S100b cell density between HFD males and the NFD group (p=0.033); however, we did not observe a significant difference in the Emb males (p=0.338). There is not a significant difference between the Emb males and the HFD males (p=0.217). In females, we observed an increase in S100b cell density in the Emb (p=0.019) and the HFD (p=0.00014) groups compared to the NFD group (Tables 4.7 and 4.8). There is not a significant difference between the Emb females and the HFD females (p=0.081). There was not an effect of sex on the number of S100b cells in layer 5 in NFD (p=0.738), Emb (p=0.661) or HFD groups (p=0.055).

The percentage of GFAP+ cells expressing S100b did not vary in layer 5 between the Emb groups and the NFD groups (males p=0.081, females p=0.875). In the HFD, we observed an increased percentage of GFAP+ cells expressing S100b in males (p=0.00077), and females (p=0.045) groups compared to the NFD group (Tables 4.9 and 4.10). There is a significant difference between the Emb males and the HFD males (p=0.004); however, there is not a significant difference between the Emb females and the HFD females (p=0.054). There is a significant difference between the Emb females and the HFD females (p=0.028). There was an effect of sex on the percentage of GFAP+ cells expressing S100b cells in layer 5 in Emb females showing a lower percentage than males (p=0.025), but no differences in the NFD or HFD groups (p=0.167, and p=0.108, respectively).
Layer 6 did not show a significant difference in the HFD males (p=0.227), or in the Emb males (p=0.360) compared to the NFD male group. There is no significant difference between the Emb males and the HFD males (p=0.798). In females, we observed a higher S100b cell density in the HFD group (p=0.00001), but no changes in cell density in the Emb group (p=0.188). There is a significant difference between the Emb females and the HFD females (p=0.0035). There was an effect of sex on the number of S100b cells, with Emb and HFD males showing a lower cell density than females (p=0.026, and p=0.002, respectively), but no differences in the NFD group (p=0.162). We observed a significant increase percentage of GFAP+ cells expressing S100b in the HFD males and the NFD group (p=0.002), but not in Emb males (p=0.319) (Tables 4.9). There is a significant difference between the Emb males and the HFD males (p=0.016). In females, there were no modifications in the percentage of GFAP+ cells expressing S100b in Emb (p=0.572) or HFD (p=0.979) groups compared to the NFD group. There is not a significant difference between the Emb females and the HFD females (p=0.576). There was an effect of sex on the percentage of GFAP+ cells expressing S100B cells in layer 6 in HFD males showing a lower percentage than females (p=0.002), but no differences in the NFD or Emb groups (p=0.411, and p=0.068, respectively).

Here we observe an increase in the number of S100b positive cells in the different layers of the cortex and that most of the cells that express GFAP are also S100b positive. (Figure 4.12 and 4.13). As in our previous results using GFAP as a marker, we observed a higher cell density in the group exposed to the maternal HFD. This data supports the idea that maternal exposure to an HFD during pregnancy and lactation is enough to modify the cellular density of astrocytes in the cerebral cortex, especially in females where even Emb exposure can be enough to induce this.
Table 4.7 S100b cell density in the somatosensory cortex in males.

Cells/mm$^2$ ± SEM estimated cell count of S100b cell in each layer of the somatosensory cortex (CTX) for each group.

<table>
<thead>
<tr>
<th>Layer</th>
<th>NFD</th>
<th>SEM</th>
<th>Emb</th>
<th>SEM</th>
<th>HFD</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>712.96</td>
<td>36.78</td>
<td>773.15</td>
<td>52.51</td>
<td>839.12</td>
<td>80.16</td>
</tr>
<tr>
<td>L2/3</td>
<td>597.22</td>
<td>14.89</td>
<td>601.85</td>
<td>20.07</td>
<td>651.85</td>
<td>21.36</td>
</tr>
<tr>
<td>L4</td>
<td>685.19</td>
<td>35.74</td>
<td>726.85</td>
<td>32.88</td>
<td>725.00</td>
<td>38.30</td>
</tr>
<tr>
<td>L5</td>
<td>633.95</td>
<td>37.47</td>
<td>718.52</td>
<td>33.90</td>
<td>747.53</td>
<td>37.57</td>
</tr>
<tr>
<td>L6</td>
<td>586.42</td>
<td>44.71</td>
<td>630.86</td>
<td>26.94</td>
<td>648.15</td>
<td>35.55</td>
</tr>
</tbody>
</table>

Table 4.8 S100b cell density in the somatosensory cortex in females.

Cells/mm$^2$ ± SEM estimated cell count of S100b cell in each layer of the somatosensory cortex (CTX) for each group.

<table>
<thead>
<tr>
<th>Layer</th>
<th>NFD</th>
<th>SEM</th>
<th>Emb</th>
<th>SEM</th>
<th>HFD</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>774.69</td>
<td>84.51</td>
<td>1075.23</td>
<td>63.12</td>
<td>1215.28</td>
<td>78.48</td>
</tr>
<tr>
<td>L2/3</td>
<td>521.60</td>
<td>25.74</td>
<td>568.52</td>
<td>62.00</td>
<td>809.88</td>
<td>37.92</td>
</tr>
<tr>
<td>L4</td>
<td>540.74</td>
<td>26.59</td>
<td>659.26</td>
<td>43.26</td>
<td>870.37</td>
<td>49.77</td>
</tr>
<tr>
<td>L5</td>
<td>611.73</td>
<td>30.89</td>
<td>757.41</td>
<td>30.61</td>
<td>898.77</td>
<td>51.79</td>
</tr>
<tr>
<td>L6</td>
<td>663.58</td>
<td>20.36</td>
<td>750.62</td>
<td>24.13</td>
<td>931.48</td>
<td>31.42</td>
</tr>
</tbody>
</table>
Figure 4.12. The cell density of S100b+ cells in the different cortex layers;

a. There were diet-induced differences in the number of astrocytes (S100b+) in layer 5 in the HFD group males compared to the NFD group. b. Differences were found in astrocytes (S100b+) quantity in all the layers when the HFD group was compared to NFD, and Emb had a significantly higher number of astrocytic cells in layers 4, and 5. Emb females were significantly different from HFD females in layers 2/3, 4, and 6. In Layer 1, Emb and HFD females showed a higher S100b cell density than males (α p=0.005, and β p=0.005, respectively). In layer 2/3, HFD females showed a higher cell density than males (γ p=0.011). In layer 4, NFD females showed a higher cell density than males (δ p=0.015). In Layer 6, Emb and HFD females showed a higher S100b cell density than males (ε p=0.026, and λ p=0.002, respectively). NFD group (blue), Emb group (green), and HFD (red). All bars represent means ± SEM. n=6 mice from 6 litters in each group * p<0.05, ** p<0.01, *** p<0.001.
### Table 4.9 Percentage of GFAP\(^{+}\) cells expressing S100b in the somatosensory cortex in males.

Percentage ± SEM in each layer of the somatosensory cortex (CTX) for each group.

<table>
<thead>
<tr>
<th>Layer</th>
<th>NFD (GFAP(^{+})/S100b(^{+})/GFAP(^{+}))</th>
<th>SEM</th>
<th>Emb (GFAP(^{+})/S100b(^{+})/GFAP(^{+}))</th>
<th>SEM</th>
<th>HFD (GFAP(^{+})/S100b(^{+})/GFAP(^{+}))</th>
<th>SEM</th>
</tr>
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<tbody>
<tr>
<td>L1</td>
<td>92.78%</td>
<td>2.70</td>
<td>88.84%</td>
<td>1.57</td>
<td>89.61%</td>
<td>1.03</td>
</tr>
<tr>
<td>L2/3</td>
<td>85.40%</td>
<td>1.37</td>
<td>87.65%</td>
<td>1.90</td>
<td>90.09%</td>
<td>0.23</td>
</tr>
<tr>
<td>L4</td>
<td>88.43%</td>
<td>1.44</td>
<td>86.17%</td>
<td>2.05</td>
<td>90.59%</td>
<td>0.91</td>
</tr>
<tr>
<td>L5</td>
<td>85.36%</td>
<td>1.38</td>
<td>89.67%</td>
<td>1.05</td>
<td>93.20%</td>
<td>1.32</td>
</tr>
<tr>
<td>L6</td>
<td>88.11%</td>
<td>1.54</td>
<td>89.87%</td>
<td>1.07</td>
<td>95.70%</td>
<td>1.19</td>
</tr>
</tbody>
</table>
### Table 4.10 Percentage of GFAP+ cells expressing S100b in the somatosensory cortex in females.

Percentage ± SEM in each layer of the somatosensory cortex (CTX) for each group.

<table>
<thead>
<tr>
<th>Layer</th>
<th>NFD (GFAP+/S100b+)/GFAP+</th>
<th>SEM</th>
<th>Emb (GFAP+/S100b+)/GFAP+</th>
<th>SEM</th>
<th>HFD (GFAP+/S100b+)/GFAP+</th>
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<td>L6</td>
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<td>87.29%</td>
<td>1.83</td>
<td>88.95%</td>
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</tbody>
</table>
Figure 4.13. Quantitative analysis of GFAP+ cells expressing S100b in the different cortex layers;
a. There were diet-induced differences in the percentage of GFAP+ cells expressing S100b in layers 2/3, 5, and 6 in the HFD group males compared to the NFD group. b. Differences were found in the percentage of GFAP+ cells expressing S100b in layers 2/3 and 5 when the HFD group was compared to NFD in females. Emb males were significantly different from HFD males in layers 4, 5, and 6. Emb females were significantly different from HFD females in layers 2/3 and 4. In layer 1, NFD males showed a higher percentage than males (α p=0.032). In layer 2/3, HFD males showed a higher percentage than females (β p=0.011). In layer 4, Emb males showed a higher percentage than females (γ p=0.011). In layer 5, Emb males showed a higher percentage than females (δ p=0.028). In layer 6, HFD males showed a higher percentage than females (ε p=0.002). NFD group (blue), Emb group (green), and HFD (red). All bars represent means ± SEM. n=6 mice from 6 litters in each group * p<0.05, ** p<0.01, *** p<0.001.
4.3.4.3 Astrocytic markers in the cortex

To infer on the modification of the astrocytic markers used in this work (GFAP and S100b), we measured the levels of mRNA of these astrocyte-specific markers in the mouse cortex. In the cortex, we observed that Gfap mRNA levels were not modified by maternal HFD exposure in males or females. When analysing the HFD and Emb males, we observed that the mRNA level of Gfap did not vary when compared to their NFD group (p=0.157, and p=0.355, respectively). There is not a significant difference between the Emb males and the HFD males (p=0.156). Female Emb and HFD did not display significantly different expression levels of Gfap (p=0.759, and p=0.598, respectively), compared to the NFD group. There is not a significant difference between the Emb females and the HFD females (p=0.604). There was not an effect of sex on the mRNA level of Gfap in the cortex in NFD (p=0.861), Emb (p=0.968) or HFD groups (p=0.086). On the other hand, in the cortex, S100b mRNA expression in Emb males was increased (p=0.022) compared to the NFD group, but HFD males were not significantly different from the NFD group (p=0.193). There is a significant difference between the Emb males and the HFD males (p=0.002). No significant differences were observed in females (Figure 4.14), neither in the HFD group (p=0.252) or the Emb group (p=0.095). There is no significant difference between the Emb females and the HFD females (p=0.438). There was a significant effect of sex on the mRNA level of S100b in the cortex in the HFD group (p=0.003), but no differences in the NFD (p=0.806), or Emb (p=0.069) groups. Here, we tried to see if variations in astrocytic protein expression were related to levels of Gfap or S100b mRNA. In general, mRNA levels of these two astrocyte-specific genes did not significantly change between the three diet groups in the cortex, indicating that maternal HFD did not affect Gfap or S100b gene expression in the offspring (Figure 4.14).
Figure 4.14. Effect of maternal HFD on the astrocytic marker in the cortex.

The mRNA levels of Gfap and S100b in males (a) and females (b) were assessed using RT-qPCR. S100b mRNA levels in Emb males were significantly different from HFD males. S100b mRNA levels in HFD females were higher than males (α p=0.003). The mRNA levels of the selected markers were normalized to Fbxw2, and Htatsf1 transcripts in the cortex. NFD group (blue), Emb group (green) and HFD (red) at 26 weeks of age. n=6 mice from 6 litters. All bars represent means ± SEM. * p<0.05, ** p<0.01.

In order to correlate the immunostaining result with the protein expression, Western blot analysis was carried out on whole cell lysates extracted from the cortex. Quantitation of GFAP and S100b relative to GAPDH was performed by western blot (Figure 4.15). GFAP protein expression in the cortex of HFD females was increased relative to NFD group (p=0.035), but not in the Emb group (p=0.847). There is no significant difference between the Emb females and the HFD females (p=0.085). In males, there were no significant differences for NFD vs Emb (p=0.970), or NFD vs HFD (p=0.213) in the cortex concerning protein expression of GFAP (Figure 4.16). There is no significant difference between the Emb males and the HFD males (p=0.242). There was a significant effect of sex on the protein level of GFAP in the cortex in the HFD group (p=0.037), but no differences in the NFD (p=0.734) or Emb (p=0.056) groups. S100b expression in males did not show significant differences between the HFD (p=0.600) or Emb (p=0.092) and the NFD group. There is no significant difference between the Emb males and the HFD males (p=0.186). In females, the HFD group showed increased levels in the cortex (p=0.023), but not in the Emb group (p=0.736) (Figure 4.16). There is no significant difference between the Emb females and the HFD females (p=0.059). There was not an effect of sex on the protein level of S100b in the cortex in NFD (p=0.069), Emb (p=0.868) or HFD groups (p=0.641).

Here we observe that the results of Western Blot have the same trend observed in the cell counting in the cortex, suggesting that exposure to a maternal HFD may be associated with an increase of glial specific proteins, namely GFAP and S100b in the cortex.
Figure 4.15. GFAP and S100b protein level in the cortex;

Representative Western Blot used for the analysis of GFAP (A) detected as a band at 48kDa and S100b (B) detected at 10kDa. Both proteins were normalized with GAPDH protein (37kDa). n=6 mice from 6 litters in each group. NFD: normal fat diet; Emb: embryonic high-fat diet; HFD: high-fat diet.
In the cortex, analysis of GFAP Western blot did not show significant differences in males (a), whereas, in females, the protein expression levels of GFAP were increased in the HFD group (b). Also, the S100b Western blot did not show significant differences in males (c), whereas, in females, the protein expression levels of GFAP were increased in the HFD group (d). The protein level of GFAP in the cortex in the HFD females was higher than males (α $p=0.037$). n=6 mice from 6 litters in each group. NFD group (blue), Emb group (green) and HFD (red) at 26 weeks of age. All bars represent means ± SEM. * $p<0.05$. 

Figure 4.16. GFAP & S100b expression analysis in the cortex;
4.3.5 Expression of cortical plasticity markers

BDNF is one of the neurotrophin families that modulates the synaptic plasticity and facilitates the differentiation, maturation, survival of neurons. BDNF is widely distributed in different areas in the brain, including the cortex, hippocampus, olfactory bulb, and hypothalamus. Synapsin I is a neuron-specific phosphoprotein located at the presynaptic level, bound to the synaptic vesicle, which controls the availability of the synaptic vesicles and neurotransmitter release by exocytosis. Finally, PSD-95, a postsynaptic protein, highly expressed in the cortex, hippocampus, and striatum, plays an essential role in synaptic plasticity learning and memory, stabilizing the neuronal circuitry. In this study, we assessed how maternal HFD affects the mRNA expression of plasticity markers Bdnf, synapsin, and Psd95 in the cortex and hippocampus.

When analysing the HFD males, no differences were observed in Bdnf (p=0.370), Synapsin (p=0.120), and Psd95 (p=0.114) genes. In Emb males, we observed that the levels of Synapsin (p=0.014) were increased compared to the NFD group, no differences in Bdnf (p=0.625), and Psd95 (p=0.191) mRNA levels. When comparing the HFD vs Emb males, no differences were observed in Bdnf (p=0.184), Synapsin (p=0.233) genes, but there is a significant difference in the Psd95 (p=0.010) gene.

Females Emb did not display significant differences in the expression levels of Bdnf (p=0.693), Synapsin (p=0.741), and Psd95 (p=0.919) genes than the NFD group. In HFD females, we observed differences in Synapsin (p=0.035) gene, but not in Bdnf (p=0.715) and Psd95 (p=0.914) genes. When comparing the HFD vs Emb females, a significant difference was observed in the Synapsin (p=0.031) gene, but not in the Bdnf (p=0.485) and Psd95 (p=0.993) genes. Finally, comparing males vs females we observed that there was not an effect of sex on the mRNA levels of Bdnf and Psd95 in the cortex in NFD (p=0.867, and p=0.875, respectively), Emb (p=0.479, and p=0.777, respectively) or HFD groups (p=0.161, and p=0.086 respectively). However, there was a significant effect of sex on the mRNA level of Synapsin in the cortex in the HFD group (p=0.011), but no differences in the NFD (p=0.681), or Emb (p=0.235) groups.
Figure 4.17. Quantification of plasticity markers expression.

The mRNA levels of *Bdnf*, *synapsin*, and *Psd95* in males (a) and females (b) were assessed using RT-qPCR. Emb males were significantly different from HFD males in the mRNA levels of the *Psd95* gene. mRNA levels of the *Synapsin* gene were significantly different between Emb and HFD females. *Synapsin* mRNA levels in HFD males were higher than females (α p=0.011). The mRNA levels of the selected markers were normalized to *Fbxw2* and *Htatsf1* transcripts in the cortex. NFD group (blue), Emb group (green) and HFD (red) at 26 weeks of age. n=6 mice from 6 litters. All bars represent means ± SEM. * p<0.05.

4.3.6 Maternal HFD alters microglia cells in the cortex in the PND01 and the adult offspring brain.

Microglia cells play an essential role in the brain, especially at the level of protection, exerting their function as macrophages in the brain. Different studies have identified microglial activation in neuroinflammation after exposure to an HFD\(^{92-94}\). Taking into account this data, we decided to explore whether our maternal HFD is enough to alter the microglia population in the cortex, in the same way as observed for astrocytes, in the offspring brain at PND 01 and in the adulthood at 26 weeks of age.

4.3.6.1 Maternal HFD affects cortical microglia at PND01.

At first, we hypothesized that most of the changes in cell density occurred during gestation, which is a critical phase during brain development that might lead to alteration in glial cells. In mice, it is possible to identify the first microglia progenitor cells in the brain on embryonic day \(^9263,553\). This
indicates that embryonic microglia appears in the brain before neurons or astrocytes, suggesting that inadequate microglia development during gestation might affect the development of these cells\textsuperscript{265}.

Iba1 is frequently used as a marker for microgliosis and is involved in remodelling the actin cytoskeleton that allows motility, proliferation, and phagocytosis in activated microglia\textsuperscript{192}. To evaluate microglia cells, we count the number of Iba1 positive cells in the cortex in PND 01 brains (Figure 4.18).

Figure 4.18. Expression of Iba1 during brain development at PND01.
DAPI positive cells in blue (left column), Iba1 positive cells in green at x10 (right column). CTX, cortex. Scale bars are 100 μm.
Quantification demonstrated that the number of Iba1+ cells in the NFD group was 173.45 cells/mm² in males and 140.27 cells/mm² in females. In the Emb group, the number of cells was similar to 196.83 cells/mm² in males and 158.37 cells/mm² in females. However, the HFD groups displayed an increase in Iba1 cell density in males (240.69 cells/mm²), and female (215.68 cells/mm²) compared to the NFD group. When analysing the HFD groups, we observed that the number of Iba1 positive cells were significantly increased compared to the NFD group (males $p=0.046$, females $p=0.013$). In contrast, no differences in Iba1 cell density were observed in the Emb males ($p=0.472$), or females ($p=0.353$) (Figure 4.19). There is no significant difference between the Emb females and the HFD females ($p=0.110$) neither between the Emb males and the HFD males ($p=0.116$). There was not an effect of sex on the number of Iba1 cells in the cortex in NFD ($p=0.155$), Emb ($p=0.654$) or HFD groups ($p=0.357$).

Figure 4.19. Microglia cell density in the cortex at PND1, quantified from Iba1 stained sections; a. Cell density was higher in the HFD males in the cortex. b. HFD females showed higher cell density in the cortex. NFD group (blue), Emb group (green), and HFD (red). All bars represent means ± SEM. n=4-6 mice from 4-6 litters in each group * $p<0.05$. 
4.3.6.2 Microglial density in the adult cortex is altered by maternal HFD

Different studies have identified microglial activation in neuroinflammation after exposure to an HFD\textsuperscript{92-94}. Microglia are the immune cells of the CNS, protecting the brain\textsuperscript{95,96}. In normal conditions, microglia cells maintain a constant cell environment by cleaning debris and modulating synapses. Also, microglial cells produce both pro- or anti-inflammatory cytokines and chemokines according to the stimulus\textsuperscript{95}. Iba1 is involved in remodelling the actin cytoskeleton that allows motility, proliferation, and phagocytosis in activated microglia\textsuperscript{192}.

Considering the data from the cortex at PND 01, we decided to explore whether a maternal HFD may alter the microglia population in the adult cortex, in the same way as observed in the newborn brain cortex. The number of cells stained with Iba1 antibody was counted and given as the number of Iba1 positive cells/mm\textsuperscript{2} (Figure 4.20). Table 4.11 and Table 4.12 shows the number of microglia in the different layers in the cortex for each diet group. Results showed a significant increase in microglia in the HFD and Emb groups in different layers (Figure 4.21).
Figure 4.20. Representative images of coronal sections showing DAPI and Iba1 staining in the adult cortex:

Immunoprocessed tissue for the detection of microglia using Iba1 as a marker. DAPI staining (blue, a, b, c); Positive microglia for Iba1 (green, d, e, f); merged channels at x10 for Iba1+/DAPI+ cells (g, h, i); and merged channels magnified at x40 for Iba1+/DAPI+ cells (j, k, l). NFD group (left column), Emb group (middle column,) HFD group (right column). The scale bar represents 250μm at x10, and 100μm at x40.
Layer 1 showed a significant difference between Iba1+ cell density in the HFD group in males (p=0.004) compared to the NFD male in the HFD female group, cell density showed an increasing trend. Still, it did not reach significance (p=0.065). The Emb groups were not significantly different from the NFD groups (male p=0.674 and female p=0.404). There is a significant difference between the Emb males and the HFD male groups (p=0.002), but no differences between Emb females and HFD females (p=0.393). There was a significant effect of sex on the density of Iba1 positive cells in the cortex in the HFD group (p=0.029), but no differences in the NFD (p=0.798), or Emb (p=0.898) groups.

Layer 2/3 showed an increase in Iba1 cell density between HFD males and the NFD group (p=0.002); however, we did not observe a significant difference in the Emb males (p=0.825). There is a significant difference between the Emb males and the HFD male groups (p=0.001). In females, we did not observe differences in Iba1 cell density in the Emb (p=0.289), and the HFD showed an increase in cell density (p=0.001) compared to the NFD group; however, there is a significant difference between the Emb females and the HFD females (p=0.021) There was not an effect of sex on the density of Iba1 positive cells in the cortex in NFD (p=0.780), Emb (p=0.193) or HFD groups (p=0.545).

In layer 4, we observe changes in the number of Iba1 positive cells between all the diet groups. The HFD groups show increased Iba1 cell density compared to the NFD groups (male p=0.003, female p=0.000264). We observed a significant increase in the number of Iba1 positive cells between the Emb groups (male p=0.045, female p=0.049) compared to the NFD group. There was not a significant difference between the Emb males and the HFD male groups (p=0.243), neither between Emb females and HFD females (p=0.055). There was not an effect of sex on the density of Iba1 positive cells in the cortex in NFD (p=0.220), Emb (p=0.152) or HFD groups (p=0.680).

Layer 5 shows a significant increase in Iba1 cell density between HFD males and the NFD group (p=0.004), and in the Emb males (p=0.034) when compared to the NFD group. There is not a significant difference between the Emb males and the HFD male groups (p=0.332). In females, the HFD group showed increased cell density (p=0.006), but not in the Emb group (p=0.151) compared to the NFD group. There is not a significant difference between the Emb females and the HFD female groups (p=0.217). There was not an effect of sex on the density of Iba1 positive cells in NFD (p=0.210), Emb (p=0.570) or HFD groups (p=0.682).

We observed in layer 6, a significant difference in the HFD males (p=0.002), but not in the Emb (p=0.396) compared to the NFD male group. There is a significant difference between the Emb
males and the HFD male groups (p=0.011). In females, we observed a significant increase in the number of microglia cells between the HFD females (p=0.029) compared to the NFD male group; however, no changes were observed in the Emb males (p=0.068). There is not a significant difference between the Emb females and the HFD female groups (p=0.911). There was not an effect of sex on the density of Iba1 positive cells in NFD (p=0.697), Emb (p=0.797) or HFD groups (p=0.370). Our data demonstrate how the HFD and Emb groups presented higher numbers of microglial cells compared to the NFD group, in different layers of the cortex, supporting the idea that early nutritional status has long-term effects on the cellular composition of the brain cortex.

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Table 4.11 Microglia cell density in the somatosensory cortex in males. Cells/mm² ± SEM estimated cell count of Iba1 cells in each layer of the somatosensory cortex (CTX) for each group.

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Table 4.12 Microglia cell density in the somatosensory cortex in females. Cells/mm² ± SEM estimated cell count of Iba1 cells in each layer of the somatosensory cortex (CTX) for each group.
Figure 4.21. Cell densities of Iba1+ cells in the different adult cortex layers;

a. There were diet-induced differences in the number of microglia in all the layers in the HFD group males, and layers 4 and 5 in the Emb group compared to the NFD group. b. Differences were found in microglial quantity in most of the layers when the HFD group was compared to NFD, and Emb had significantly higher microglia cells in layer 4. Emb males were significantly different from HFD males in layers 1, 2/3, and 6. Emb females were significantly different from HFD females in layer 2/3. In layer 1, HFD males showed a higher cell density than females (α p=0.029). NFD group (blue), Emb group (green), and HFD (red). All bars represent means ± SEM. n=6 mice from 6 litters in each group * p<0.05, ** p<0.01, *** p<0.001.
4.3.6.3 Microglial markers mRNA levels in the cortex

Stem cells in the yolk sac (mesenchymal origin) will migrate and colonize the central nervous system (CNS) during development, becoming these microglia cells. Microglia are macrophages resident in the brain and are traditionally considered a critical element in response to inflammation in the CNS caused by harmful influences. Microglia require the expression of different proteins to exert an adequate function. Monocyte and Macrophage Protein CD68 is a transmembrane glycoprotein protein involved in the interaction of macrophages with low-density lipoproteins, highly expressed by activated microglia, indicating phagocytic activity.

Cd11b protein is part of the type 3 complement receptor that aids in the recognition and phagocytosis of antigens. CD11b is expressed by both resting and activated microglia.

Cx3cr1 or CX3C receptor-1 is a fractalkine receptor that modulates neuronal-microglial interactions with its CX3C Ligand-1 (CX3CL1) in the neurons. CX3CR1 signalling plays a critical role in synaptic pruning, neuronal survival, and synaptic transmission and plasticity.

Csf1r or colony-stimulating factor 1 receptor is a cell surface protein activated by two ligands, CSF1 and IL-34. Csf1r regulates, development, proliferation, maintenance, and survival of microglia, and is involved in neuroinflammation.

Iba1 is a member of the calcium-binding group of proteins. Iba1 is widely distributed in the cytoplasm and processes of ramified microglia. Iba1 binds actin molecules in order to form microfilaments. This protein takes part in reorganizing the cytoskeleton and altering the configuration of the cell membrane during phagocytosis. Due to the critical role of these markers in microglia function and homeostasis, we analysed the mRNA expression of Cd68, Cd11b, Cx3xr1, Csf1r, and Iba1 in the adult mouse brain.

When analysing the HFD males, we observed that the levels of Cx3cr1 (p=0.000012), Csf1r (p=0.41) and Iba1 (p=0.038) were higher compared to the NFD male group, no differences in Cd68 (p=0.446), and Cd11b (p=0.411) genes. No differences in expression levels of mRNA were observed in the Emb males in Cd68 (p=0.616), Cd11b (p=0.399), Csf1r (p=0.725), and Iba1 (p=0.089), whereas Cx3cr1 expression was increased (p=0.002) compared to the NFD male group (Figure 4.22). When comparing the HFD vs Emb in males, a significant difference was observed in the Cx3cr1 (p=0.021) gene, but not in the Cd68 (p=0.215), Cd11b (p=0.949), Csf1r (p=0.092), and Iba1 (p=0.072) genes.
In females, no significant differences between groups (HF or Emb vs. NFD) were observed regarding the expression levels of *Cd68* (Emb *p*=0.470, HFD *p*=0.205), *Cd11b* (Emb *p*=0.300, HFD *p*=0.157), *Cx3xr1* (Emb *p*=0.458, HFD *p*=0.051), *Csf1r* (Emb *p*=0.227, HFD *p*=0.127). However, the expression of *Iba1* in HFD females was increased (**p**=0.028), but not in the Emb females (**p**=0.613) (Figure 4.22). When comparing the HFD vs Emb in females, there were not significant differences in the *Cd68* (**p**=0.838), *Cd11b* (**p**=0.949), *Cx3cr1* (**p**=0.294), *Csf1r* (**p**=0.928), and *Iba1* (**p**=0.105) genes.

Finally, comparing males vs females we observed that there was not an effect of sex on the mRNA levels of *Cd68*, *Csf1r*, and *Iba1* genes in the cortex in NFD (**p**=0.847, 0.987, and 0.949, respectively), Emb (**p**=0.369, 0.161, and 0.454, respectively) or HFD groups (**p**=0.832, 0.394, 0.988, respectively). However, there was a significant effect of sex on the mRNA level of *Cd11b* in the cortex in the HFD group (**p**=0.047), but no differences in the NFD (**p**=0.934), or Emb (**p**=0.200) groups. There was a significant effect of sex on the mRNA level of *Cx3cr1* in the cortex in the HFD group (**p**=0.002) and Emb group (**p**=0.005), but no differences in the NFD (**p**=0.851) group.

**Figure 4.22. Quantification of microglial marker expression.**

The mRNA levels of *Cd68*, *Cd11b*, *Cx3xr1*, *Csf1r* and *Iba1* in males (a) and females (b) were assessed using RT-qPCR. The mRNA levels of the selected markers were normalized to *Fbxw2* and *Htatsf1* transcripts in the cortex. Emb males were significantly different from HFD males in the mRNA levels of the *Cx3cr1* gene. *Cd11b* and *Cx3cr1* mRNA levels in HFD males were different from females (α **p**=0.047, and δ **p**=0.002). *Cx3cr1* mRNA levels in Emb males were higher than females (β **p**=0.005). NFD group (blue), Emb group (green) and HFD (red) at 26 weeks of age. n=6 mice from 6 litters. All bars represent means ± SEM. * **p**<0.05, ** **p**<0.01, *** **p**<0.001.
Chapter 4

4.3.7 Maternal HFD and non-inflammatory changes in the adult offspring brain cortex.

Inflammation in the brain activates glial cells mainly microglia, which secrete inflammatory cytokines, nitric oxide and prostaglandin to generate inflammation in the brain\textsuperscript{183,192}. Our results show an increase in cell density of microglia and astrocytes in the cortex in Emb and HFD groups. It has been described that the basal gene expression of pro-inflammatory markers such as IL-1β, IL-6, or TNFα, can be increased after maternal HFD\textsuperscript{195}. Therefore, in this study, we analysed the gene expression of genes involved in immune homeostasis, including pro-inflammatory cytokines (interleukins (\textit{Il}-1\textit{b}, \textit{Il}-6, and \textit{Il}-18), and \textit{Tnfα}), and anti-inflammatory cytokines (interleukins (\textit{Il}-4, and \textit{Il}-10), and \textit{Tgfβ}) in the cortex in the three different diet groups.

In males, the mRNA expression levels of \textit{Il}-1\textit{b} a pro-inflammatory gene were significantly increased in the Emb group (\textit{p}=0.024) compared to the NFD group, but not in the HFD group (\textit{p}=0.236). \textit{Il}-6 (HFD \textit{p}=0.450, Emb \textit{p}=0.308), \textit{Il}-18 (HFD \textit{p}=0.273, Emb \textit{p}=0.067), and \textit{Tnfα} (HFD \textit{p}=0.132, Emb \textit{p}=0.148) pro-inflammatory genes did not display significant differences compared to NFD (Figure 4.23). When comparing the mRNA levels of pro-inflammatory genes in HFD vs Emb in males, there were no significant differences in \textit{Il}-1\textit{b} (\textit{p}=0.190), \textit{Il}-6 (\textit{p}=0.086), \textit{Il}-18 (\textit{p}=0.374), and \textit{Tnfα} (\textit{p}=0.992) genes.

For anti-inflammatory cytokines in males, only \textit{Tgfβ} mRNA expression levels did not significantly change (HFD \textit{p}=0.393, Emb \textit{p}=0.277). \textit{Il}-4 mRNA expression levels were significantly increased in HFD (\textit{p}=0.00013) and Emb (\textit{p}=0.018) groups, while \textit{Il}-10 was increased in the Emb group (\textit{p}=0.018) compared to NFD group, but not in the HFD group (\textit{p}=0.083) (Figure 4.23). When comparing the mRNA levels of anti-inflammatory genes in HFD vs Emb in females, there were no significant differences in \textit{Il}-4 (\textit{p}=0.108), \textit{Il}-10 (\textit{p}=0.355), and \textit{Tgfβ} (\textit{p}=0.771) genes.

In the females, the pro-inflammatory mRNA expression levels of the genes were not significantly different in the HFD (\textit{Il}-1\textit{b} \textit{p}=0.491, \textit{Il}-6 \textit{p}=0.599, \textit{Il}-18 \textit{p}=0.727, and \textit{Tnfα} \textit{p}=0.212) or Emb (\textit{Il}-1\textit{b} \textit{p}=0.117, \textit{Il}-6 \textit{p}=0.931, \textit{Il}-18 \textit{p}=0.351, and \textit{Tnfα} \textit{p}=0.797) groups compared to NFD group (Figure 4.23). When comparing the mRNA levels of pro-inflammatory genes in HFD vs Emb in females, there were no significant differences in \textit{Il}-1\textit{b} (\textit{p}=0.290), \textit{Il}-6 (\textit{p}=0.725), \textit{Il}-18 (\textit{p}=0.213), and \textit{Tnfα} (\textit{p}=0.178) genes.

Concerning anti-inflammatory cytokines in females, none of the three analysed genes showed significantly different expression levels in HFD or Emb females compared with NFDs \textit{Il}-4 (HFD
p=0.051, Emb p=0.545), Il-10 (HFD p=0.894, Emb p=0.773), and Tgfβ (HFD p=0.991, Emb p=0.588) (Figure 4.28b). When comparing the mRNA levels of anti-inflammatory genes in HFD vs Emb in females, there were no significant differences in Il-4 (p=0.108), Il-10 (p=0.857), and Tgfβ (p=0.584) genes.

Finally, comparing males vs females we observed that there was not an effect of sex on the mRNA levels of Il-1b, Il-6, Il-10, Il-18, and Tgfβ genes in the cortex in NFD (p=0.848, p=0.901, p=0.981, p=0.731, and p=0.828, respectively), Emb (p=0.713, p=0.183, p=0.227, p=0.093, and p=0.523, respectively) or HFD groups (p=0.411, p=0.611, p=0.227, p=0.338, and p=0.499 respectively). However, there was a significant effect of sex on the mRNA level of Tnfa in the cortex in the Emb group (p=0.010), but no differences in the NFD (p=0.458), or HFD (p=0.808) groups. There was a significant effect of sex on the mRNA level of Il-4 in the cortex in the HFD group (p=0.038) and Emb group (p=0.036), but no differences in the NFD (p=0.901) group.

These findings suggest that the maternal HFD in the offspring at 26 weeks of age does not cause neuroinflammation in the offspring. Therefore, more studies are needed to understand the relationship between maternal diet and systemic and cerebral inflammation.
Figure 4.23. mRNA levels of pro-inflammatory and anti-inflammatory cytokines in the cortex

The mRNA levels of pro-inflammatory (Il-1b, Il-6, Il-18, and Tnfα) and anti-inflammatory (Il-4, Il-10, and Tgfβ) markers in male (a) and female (b) were assessed using RT-qPCR. Tnfα and Il-4 mRNA levels in Emb males were different from females (α p=0.010, and β p=0.036, respectively). Il-4 mRNA levels in HFD males were different from females (δ p=0.038). The mRNA levels of the selected markers were normalized to Fbxw2 and Htatsf1 genes. NFD group (blue), Emb group (green) and HFD (red) at 26 weeks of age. n=6 mice from 6 litters. All bars represent means ± SEM. * p<0.05, *** p<0.001.
4.3.8  GFAP astrocytic density in the hippocampus is altered by maternal HFD

Different studies have indicated that HFD harms the hippocampus in terms of structure and function affecting learning and memory\(^{533,534}\). Therefore, in our model, we want to identify whether the exposure to a maternal HFD, during pregnancy and lactation, is enough to alter the population of astrocytes in the adult hippocampus (dentate gyrus, hilus, CA1, and CA3) of the offspring. Despite the non-uniform distribution of GFAP positive astrocytes in the brain, we decided to used GFAP (Figure 4.24) since astrocytes in the hippocampus usually are highly GFAP positive\(^{239,562,563}\).

![Representative images of coronal sections showing DAPI and GFAP staining in the hippocampus of mice from the different maternal diet groups.](image)

Immunoprocessed tissue for the detection of Astrocytes using the GFAP marker. DAPI staining (blue, top row); Positive Astrocytes for GFAP (green, middle row) merged channels for GFAP+/DAPI+ cells (bottom row).
In the dentate gyrus of the offspring, the analysis was based on the morphology of each of the cells (Figure 4.25). We only count the star-like shape astrocytes in the dentate gyrus, not including the radial glial-like cells since these cells are stem cells, and they will be described in more detail in the next chapter.

**Figure 4.25. A representative image is showing star-like shape astrocytes GFAP positive cells.** Red arrowheads are showing Star-like GFAP positive cells in the dentate gyrus (GCL and SGZ), and hilus. Blue: DAPI positive cells, Green: GFAP positive cells, Magenta: Sox2 positive cells. GCL: granule cell layer. SGZ: subgranular zone. Scale bar 25µm.

In the dentate gyrus, the number of GFAP$^+$ cells in males and females did not change significantly in the different diet groups. The Emb (p=0.968) and HFD (p=0.737) males had a slightly reduced number of cells compare to the NFD, but this difference did not reach significance (Figure 4.26). There is not a significant difference between the Emb males and the HFD males (p=0.771). The number of GFAP$^+$ cells was no significant difference in Emb (p=0.104), and HFD females (p=0.848) compared to the NFD female group (Figure 4.26). There is not a significant difference between the Emb females and the HFD females (p=0.109). There was not an effect of sex (females vs males) in the dentate gyrus in the NFD (p=0.282), Emb (p=0.502) or HFD groups (p=0.472).

In the Hilus, the statistical analysis performed in males and females did not show changes in the number of GFAP$^+$ cells. In males, there are no significant differences between the HFD (p=0.937) or Emb (p=0.490) and the NFD group. There is not a significant difference between the Emb males and the HFD males (p=0.535). The number of GFAP$^+$ cells in females was no significant difference
between the HFD (p=0.626) and Emb (p=0.794) groups (Figure 4.26). There is not a significant difference between the Emb females and the HFD females (p=0.109). There was not an effect of sex (females vs males) in the hilus in the NFD (p=0.543), Emb (p=0.639) or HFD groups (p=0.471).

Then we assessed GFAP+ cells in the CA1 region. Microscopy analysis revealed that the number of GFAP+ cells was increased in HFD (p=0.00024) and Emb (p=0.00093) male animals compared to the NFD male group. There is not a significant difference between the Emb males and the HFD males (p=0.456). The number of GFAP+ cells was significantly different in Emb females (p=0.004) compared to the NFD group. Similarly, in HFD females, the number of GFAP+ cells were also increased (p=0.00035) (Figure 4.26). There is not a significant difference between the Emb females and the HFD females (p=0.490). There was not an effect of sex (females vs males) in the CA1 region in the NFD (p=0.255), Emb (p=0.796) or HFD groups (p=0.643).

Finally, the CA3 region revealed that the number of GFAP+ cells in Emb males showed an increasing trend but did not reach significance (p=0.063) compared to the NFD group, and HFD males had a significantly increased number of cells (p=0.021) (Figure 4.26). There is not a significant difference between the Emb males and the HFD males (p=0.572). The number of GFAP+ cells in females, however, did not change significantly in the different diet groups. The Emb females were not significantly different (p=0.102), and HFD females had a slightly increased number of GFAP+ cells (p=0.063) (Figure 4.26). There is not a significant difference between the Emb females and the HFD females (p=0.935). There was not an effect of sex (females vs males) in the CA3 region in the NFD (p=0.583), Emb (p=0.228) or HFD groups (p=0.799).

This data suggests the number of astrocytes that express GFAP is increased in the HFD group in the CA1 and CA3, and Emb group in CA1, but no changes in the dentate gyrus or hilus.
Figure 4.26. Cell density in the hippocampus by gender quantified from GFAP staining;

a. Significant differences were observed in males in the CA1 and CA3 regions. b. Differences were seen in the females CA1 region. NFD group (blue), Emb group (green), and HFD (red). All bars represent means ± SEM. n=5 in each group. * p<0.05, ** p<0.01, *** p<0.001, **** p≤0.0001.
4.3.9 Astrocytic markers in the hippocampus

Interestingly in the hippocampus, we observed that Gfap mRNA levels were significantly reduced in the Emb group (male \( p=0.023 \) and female \( p=0.021 \)) and the HFD group (male \( p=0.010 \), and female \( p=0.001 \)) concerning their corresponding NFD group. There is not a significant difference between the Emb males and the HFD males (\( p=0.784 \)), neither between the Emb males and the HFD males (\( p=0.257 \)). There was not an effect of sex on the mRNA level of Gfap in the cortex in NFD (\( p=0.191 \)), Emb (\( p=0.498 \)) or HFD groups (\( p=0.261 \)).

When analysing S100b mRNA expression, we observed an opposite trend to the data obtained in the Gfap mRNA analysis. Here, the levels of S100b were significantly upregulated in the Emb females \( p=0.040 \), but not in the Emb males \( p=0.069 \), and HFD (male \( p=0.001 \), and female \( p=0.001 \)) groups, when compared to the NFD group. There is not a significant difference between the Emb males and the HFD males (\( p=0.076 \)), neither between the Emb males and the HFD males (\( p=0.246 \)). There was not an effect of sex on the mRNA level of S100b in the hippocampus in NFD (\( p=0.476 \)); however, there was a significant difference between females and males in the Emb (\( p=0.018 \)) and HFD (\( p=0.047 \)) groups (Figure 4.27).

**Figure 4.27.** Effect of maternal HFD on the astrocytic marker in the hippocampus.

The mRNA levels of Gfap and S100b in males (a) and females (b) were assessed using RT-qPCR. S100b mRNA levels in Emb and HFD males were higher than females (\( \alpha p=0.018 \), and \( \beta p=0.047 \)). The mRNA levels of the selected markers were normalized to Fbxw2, Pak1lp1 and Ap3d1 genes in the hippocampus. NFD group (blue), Emb group (green) and HFD (red) at 26 weeks of age. n=6 mice from 6 litters. All bars represent means ± SEM. * \( p<0.05 \), ** \( p<0.01 \).
Western blot analysis was carried out on whole cell lysates extracted from the hippocampus to correlate the immunostaining result with the protein expression (Figure 4.28). In the hippocampus, GFAP protein expression was not significantly different in Emb males (p=0.884) or females (p=0.538). In contrast, in HFD males (p=0.025) or females (p=0.045), the protein level was increased significantly (Figure 4.29).

There is no significant difference between the Emb males and the HFD males (p=0.511), neither between the Emb females and the HFD females (p=0.454). Finally, there was not an effect of sex on the protein level of GFAP in the hippocampus in the group NFD (p=0.425), Emb (p=0.790) or HFD groups (p=0.701).

![Males and Females Western Blot](image)

**Figure 4.28.** GFAP protein level in the cortex;

Representative Western Blot used for the analysis of GFAP detected as a band at 48kDa. GFAP protein was normalized with GAPDH protein (37kDa). n=6 mice from 6 litters in each group. NFD: normal fat diet; Emb: embryonic high-fat diet; HFD: high-fat diet.
Figure 4.29. GFAP & S100b expression analysis in the hippocampus;
In the hippocampus, analysis of GFAP Western blot shows in males (a), and females (b), that the protein expression levels were increased in the HFD group. n=6 mice from 6 litters in each group. NFD group (blue), Emb group (green) and HFD (red) at 26 weeks of age. All bars represent means ± SEM. * p<0.05.

4.3.10 Expression of hippocampal plasticity markers

Synaptogenesis is a process involving formation, maintenance, and elimination of synapses to establish an adequate neural network and developing brain circuits\textsuperscript{564}. Synapses are specialized cell to cell connections between a presynaptic neuron and a postsynaptic target, transferring either electrical or chemical signals. They are essential for optimal cognitive function (learning and memory)\textsuperscript{565}. Here, we assessed how maternal HFD affects the mRNA expression of plasticity markers Bdnf, synapsin, and Psd95 in the hippocampus.

In HFD males, the relative mRNA expression of Bdnf (\(p=0.022\)), and Psd95 (\(p=0.00001\)), was reduced. However, the expression of Synapsin (\(p=0.127\)) was not significantly different. Males Emb showed lower expression levels of Psd95 (\(p=0.00045\)) gene, but no differences in expression levels of Bdnf (\(p=0.087\)), and Synapsin (\(p=0.734\)) than NFD group (Figure 4.30). When comparing the HFD vs Emb males, no differences were observed in Bdnf (\(p=0.546\)), Synapsin (\(p=0.077\)) genes, but there is a significant difference in the Psd95 (\(p=0.020\)) gene.
The females HFD showed lower expression levels of \textit{PSD95} \( (p=0.037) \), but no changes in the expression levels of \textit{Bdnf} \( (p=0.076) \), and \textit{Synapsin} \( (p=0.719) \) genes. In Emb females, the relative mRNA expression of \textit{Bdnf} \( (p=0.059) \), \textit{Synapsin} \( (p=0.110) \), and \textit{Psd95} \( (p=0.297) \) genes were not significantly different (Figure 4.30). When comparing the HFD vs Emb females, no differences were observed in \textit{Bdnf} \( (p=0.672) \), \textit{Synapsin} \( (p=0.173) \), and \textit{Psd95} \( (p=0.375) \) genes.

Finally, comparing males vs females we observed that there was not an effect of sex on the mRNA levels of \textit{Bdnf} in the hippocampus in NFD \( (p=0.972) \), Emb \( (p=0.661) \) and HFD \( (p=0.470) \) groups. However, there was a significant effect of sex on the mRNA level of \textit{Synapsin} in the Emb group \( (p=0.039) \), but no differences in the NFD \( (p=0.350) \), or HFD \( (p=0.055) \) groups. Males and females were showing different \textit{Psd95} mRNA levels in the hippocampus in the Emb \( (p=0.028) \), and HFD \( (p=0.016) \) groups, but no differences in the NFD group.

\begin{figure}[!h]
  \centering
  \includegraphics[width=\textwidth]{figure4.30.png}
  \caption{Quantification of plasticity markers expression.}
  \end{figure}

The mRNA levels of \textit{Bdnf}, \textit{synapsin}, and \textit{Psd95} in males (a) and females (b) were assessed using RT-qPCR. \textit{Synapsin} mRNA levels in Emb females were higher than males \( (\alpha p=0.039) \). \textit{Psd95} mRNA levels in Emb and HFD in females were higher than males \( (\beta p=0.028, \text{ and } \delta p=0.016 \text{ respectively}) \). The mRNA levels of the selected markers were normalized to \textit{Fbxw2}, \textit{Pak1p1} and \textit{Ap3d1} genes in the hippocampus. NFD group (blue), Emb group (green) and HFD (red) at 26 weeks of age. \( n=6 \) mice from 6 litters. All bars represent means ± SEM. * \( p<0.05 \).
4.3.11 Maternal HFD alters microglial cells in the hippocampus of the PND01 and adult offspring brain.

4.3.11.1 Maternal HFD affects hippocampal microglia at PND1.

Iba1 is frequently used as a marker for microgliosis. To evaluate microglia cells, we count the number of Iba1 positive cells in the hippocampus (dentate gyrus, CA1, and CA3 regions) at x40 magnification in PND 01 brains (Figure 4.31).

![Figure 4.31. Expression of Iba1 during brain development at PND01.](image)

DAPI positive cells in blue (left column), Iba1 positive cells in green at x10 (middle column), and Iba1 positive cells in the dentate gyrus (DG) at x40 (right column). Scale bars are 100 μm.
In the dentate gyrus, the number of Iba1+ cells in Emb males was no significant different compared to the NFD males (p=0.436), while HFD males had a significantly increased number of Iba1+ cells (p=0.024). The number of microglia cells (Iba1+) in females, was significantly different in the HFD group (p=0.031) compared to the NFD females; however, in the Emb females, there were no differences (p=0.118). There is no significant difference between the Emb females and the HFD female groups (p=0.583) neither between the Emb males and the HFD male groups (p=0.090). There was not an effect of sex on the number of Iba1 cells in the dentate gyrus in NFD (p=0.066), Emb (p=0.220) or HFD groups (p=0.095).

In the CA1 region, there were no significant differences between the HFD female group (p=0.497), or Emb female group (p=0.181) and the NFD female group (Figure 4.32). There is not a significant difference between the Emb females and the HFD female groups (p=0.573). Emb males (p=0.035), and HFD males (p=0.001) had a higher Iba1+ cell density compared to the NFD male group. There is a significant difference between the Emb males and the HFD male groups (p=0.038). There was not an effect of sex on the number of Iba1 cells in the CA1 region in NFD (p=0.977), Emb (p=0.513) or HFD groups (p=0.275).

Finally, in the CA3 region, the analysis showed that the HFD groups had a significant increase in cell density compared to the NFD group (male p=0.008, female p=0.030). In contrast, no differences in Iba1 cell density were observed in the Emb males (p=0.872), or females (p=0.282) compared to the NFD group (Figure 4.32). There is no significant difference between the Emb females and the HFD female groups (p=0.286), but there is a significant difference between the Emb males and the HFD males (p=0.007). There was an effect of sex on the number of Iba1 cells in the CA3 region in NFD (p=0.048), and HFD (p=0.044) groups, but not in the Emb (p=0.220) group.

These data suggest that microglial cell density in the hippocampus may be affected by maternal HFD during gestation, suggesting that the increasing number of microglia cells could modify brain development processes such as neurogenesis or gliogenesis.
Figure 4.32. Microglia cell density in the hippocampus at PND 01, quantified from Iba1 stained sections;

a. Cell density was higher in the HFD males in the DG, CA1 and CA3. b. HFD females showed higher cell density in the DG and CA3. Emb males were significantly different from HFD males in CA1 and CA3. NFD and HFD males showed a higher cell density than females ($\alpha p=0.048$, $\beta p=0.044$, respectively). NFD group (blue), Emb group (green), and HFD (red). All bars represent means ± SEM. n=4-6 mice from 4-6 litters in each group * p<0.05, ** p<0.01.
Chapter 4

4.3.11.2 Maternal HFD affects microglial density in the adult hippocampus.

In the adult brain, microglia cells continually examine the local environment through their processes, interacting with other cell types, such as astrocytes, neurons, stem cells, and endothelial cells. Diet influences the function of microglia, for instance, HFD increases the number of microglia and apoptotic cells in the dentate gyrus, indicating a detrimental effect on the survival of the new cells. The purpose of analysing Iba1 cells was to investigate whether the maternal HFD has the same effect on the hippocampus, similar to what was observed in the new-born brain.

We counted the number of positive Iba1 stained cells present in the dentate gyrus, hilus, CA1, and CA3 (Figure 4.33). The number of cells stained with Iba1 antibody was counted and given as the number of Iba1 positive cells/mm2 (Figure 4.34) in the different regions of the hippocampus for each diet group.
Figure 4.33. Representative images of coronal sections showing DAPI and Iba1 staining in the adult hippocampus;
Immunoprocessed tissue for the detection of microglia using Iba1 as a marker. DAPI staining (blue, A, B, C); positive microglia for Iba1 (green, D, E, F); merged channels at x10 for Iba1+/DAPI+ cells (G, H, I); and merged channels magnified at x40 for Iba1+/DAPI+ cells (J, K, L). NFD group (left column), Emb group (middle column), HFD group (right column). The scale bar represents 100μm.
In the dentate gyrus of the offspring, the NFD male animals had a similar number of Iba1+ cells than the Emb males (p=0.0304), whereas the HFD males had an increased number of cells (p=0.002) compared to the NFD males. There is not a significant difference between the Emb males and the HFD male groups (p=0.447). The number of Iba1+ cells in the Emb females significantly increased (p=0.000146), same as in the HFD females (p=0.000025) compared to the NFD females. There is not a significant difference between the Emb females and the HFD female groups (p=0.512). There was a significant effect of sex (females vs males) in the dentate gyrus in the NFD group (p=0.007), but no differences in the Emb (p=0.613), or HFD (p=0.280) groups.

In the Hilus, the statistical analysis performed in males and females did not show changes in the number of Iba1+ cells. The number of Iba1+ cells in Emb males (p=0.220), and HFD males (p=0.077) were not significantly different from the NFD males. There is not a significant difference between the Emb males and the HFD male groups (p=0.386). The number of Iba1+ cells in Emb females (p=0.624), and HFD females (p=0.068) were not significantly different from the NFD females. There is not a significant difference between the Emb females and the HFD female groups (p=0.705). There was not an effect of sex on the number of Iba1 cells in the hilus in NFD (p=0.144), Emb (p=0.583) or HFD groups (p=0.084).

Then we assessed Iba1+ cells in the CA1 region. Microscopy analysis revealed that the number of Iba1+ cells was increased in the HFD males (p=0.00024) and Emb males (p=0.00026) compared to the NFD male group. There is not a significant difference between the Emb males and the HFD male groups (p=0.809). The number of Iba1+ cells was significantly different in Emb females (p=0.039), and in HFD females (p=0.001) compare to the NFD female group. There is not a significant difference between the Emb females and the HFD female groups (p=0.141). There was not an effect of sex on the number of Iba1 cells in the CA1 region in NFD (p=0.216), Emb (p=0.320) or HFD groups (p=0.575).

Finally, in the CA3 region, Emb males showed a significantly increased number of cells (p=0.012) compared to the NFD male group. HFD males had a significantly increased number of cells (p=0.002) when compared to the NFD group. There is not a significant difference between the Emb males and the HFD male groups (p=0.449). The number of Iba1+ cells in females was not significantly different (p=0.091), and HFD females had a significantly increased number of Iba1+ cells (p=0.026) compare to the NFD female group. There is not a significant difference between the Emb females and the HFD female groups (p=0.755). There was not an effect of sex on the number of Iba1 cells in the CA3 region in NFD (p=0.818), Emb (p=0.270) or HFD groups (p=0.912).
Figure 4.34. Cell density in the adult hippocampus quantified from Iba1 staining:
Significant differences were observed in males (a.) and females (b.) in dentate gyrus (DG), CA1 and CA3 regions. NFD males showed a higher cell density than females (α p=0.007). NFD group (blue), Emb group (green) and HFD (red). All bars represent means ± SEM. n=5 in each group. * p<0.05, ** p<0.01, *** p<0.001, **** p≤0.0001.
4.3.11.3 Microglial cell density at PND 01 and adulthood in the hippocampus

Here we compared the microglial cell density in the hippocampus at two different time points: at PND 01 and 26 weeks of age (Figure 4.35). The microscopy analysis revealed that the number of Iba1+ cells in the CA1 region was not different between NFD groups at PND 01 and 26 weeks (males p=0.978 and females p=0.897) of age. There is not a significant difference between the Emb males and Emb female groups at PND 01 and 26 weeks (males p=0.398 and females p=0.567) of age. Finally, there is not a significant difference between the Emb males and Emb female groups at PND 01 and 26 weeks (males p=0.624 and females p=0.997) of age.

In the CA3 region, NFD groups at PND 01 and 26 weeks of age did not show significant changes in the number of cells (males p=0.971 and females p=0.803). There is not a significant difference between the Emb groups at PND 01 and 26 weeks (males p=0.5542 and females p=0.949) of age. HFD groups at PND 01 and 26 weeks of age did not show significant changes in the number of cells (males p=0.243 and females p=0.715).

In the dentate gyrus of the offspring, NFD groups at PND 01 and 26 weeks of age did not show significant changes in the number of cells (males p=0.069 and females p=0.107). There is a significant difference between the Emb groups at PND 01 and 26 weeks (males p=0.004 and females p=0.032) of age. HFD groups at PND 01 and 26 weeks of age showed significant changes in the number of cells (males p=0.006 and females p=0.013).
Figure 4.35. Cell density in the PND 01 and adult hippocampus quantified from Iba1 staining; Microglia cells in males (a.) and females (b.) in CA1 region. Microglia cells in males (c.) and females (d.) in CA2 region. Microglia cells in males (e.) and females (f.) in the DG. NFD group (blue), Emb group (green) and HFD (red). All bars represent means ± SEM. n=5 in each group. * p<0.05, ** p<0.01.
4.3.11.4  Microglial markers mRNA levels in the hippocampus

Due to the critical role of these markers in microglia function and homeostasis, we analysed the mRNA expression of *Cd68*, *Cd11b*, *Cx3cr1*, *Csf1r* and *Iba1* in the adult mouse brain. In HFD males the relative mRNA expression of *Cd11b* (*p*=0.041) was reduced, but not in *Cd68* (*p*=0.086), *Cx3cr1* (*p*=0.200), *Csf1r* (*p*=0.500), and *Iba1* (*p*=0.398) mRNA expression. In Emb males no differences in expression levels of mRNA were observed in *Cd68* (*p*=0.413), *Cd11b* (*p*=0.313), *Cx3cr1* (*p*=0.624), *Csf1r* (*p*=0.328), and *Iba1* (*p*=0.885) compared to the NFD group (Figure 4.36). When comparing the HFD vs Emb in males, a significant difference was observed in the *Cd68* (*p*=0.020) gene, but not in the *Cd11b* (*p*=0.304), *Cx3cr1* (*p*=0.443), *Csf1r* (*p*=0.724), and *Iba1* (*p*=0.339) genes.

HFD females showed lower expression levels than NFD group of *Cd11b* (*p*=0.001), *Cx3cr1* (*p*=0.031), and *Csf1r* (*p*=0.002) genes, but no differences in expression levels of *Cd68* mRNA (*p*=0.957), and *Iba1* (*p*=0.628) genes. The relative mRNA expression of *Cd68* (*p*=0.829), *Cd11b* (*p*=0.510), *Cx3cr1* (*p*=0.083), *Csf1r* (*p*=0.194), and *Iba1* (*p*=0.463) genes in Emb females was not significant different (Figure 4.36). When comparing the HFD vs Emb in females, a significant difference was observed in the *C11b* (*p*=0.006) gene, but not in the *Cd68* (*p*=0.861), *Cx3cr1* (*p*=0.852), *Csf1r* (*p*=0.080), and *Iba1* (*p*=0.246) genes.

Finally, comparing males vs females we observed that there was not an effect of sex on the mRNA levels of *Cd68*, *Cd11b*, *Cx3cr1*, and *Iba1* genes in the cortex in NFD (*p*=0.942, *p*=0.882, *p*=0.792, and *p*=0.795 respectively), Emb (*p*=0.094, *p*=0.241, *p*=0.258, and *p*=0.300 respectively) or HFD groups (*p*=0.095, *p*=0.171, *p*=0.499, and *p*=0.449 respectively). However, there was a significant effect of sex on the mRNA level of *Csf1r* in the cortex in the HFD group (*p*=0.004), but no differences in the NFD (*p*=0.476), or Emb (*p*=0.803) groups.

It is well known that the population of microglia in the brain is heterogeneous, affected by the surrounding microenvironment\textsuperscript{568,569}, the maternal HFD may alter the environment of the hippocampus generating the observed changes.
The mRNA levels of *Cd68*, *Cd11b*, *Cx3xr1*, *Csf1r* and *Iba1* in males (a) and females (b) were assessed using RT-qPCR. Emb males were significantly different from HFD males in the mRNA levels of the *Cd68* gene, and Emb females were significantly different from HFD females in the mRNA levels of the *Cd11b* gene. *Csf1r* mRNA levels in HFD males were different from females (α p=0.004). The mRNA levels of the selected markers were normalized to *Fbxw2, Pak1lp1* and *Ap3d1* genes in the hippocampus. NFD group (blue), Emb group (green) and HFD (red) at 26 weeks of age. n=6 mice from 6 litters. All bars represent means ± SEM. * p<0.05, ** p<0.01, *** p<0.001, **** p≤0.0001.
4.3.12 Maternal HFD and non-inflammatory changes in the hippocampus in the adult offspring brain.

In this study, we analysed the gene expression of genes involved in immune homeostasis, including pro-inflammatory cytokines (interleukins (Il-1b, Il-6, and Il-18), Tnfα), and anti-inflammatory cytokines (interleukins (Il-4, and Il-10, Tgfβ)) in the hippocampus in the three different diet groups.

In males no altered expression levels for pro-inflammatory genes were observed in HFD (Il-1b p=0.941, Il-6 p=0.374, Il-18 p=0.938, Tnfα p=0.394) or Emb males (Il-1b p=0.628, Il-6 p=0.632, Il-18 p=0.864, Tnfα p=0.814) compared to the NFD group (Figure 4.36). When comparing the mRNA levels of pro-inflammatory genes in HFD vs Emb in males, there were no significant differences in Il-1b (p=0.575), Il-6 (p=0.702), Il-18 (p=0.804), and Tnfα (p=0.294) genes. For anti-inflammatory markers, Il-4 mRNA expression levels were significantly decreased in HFD males (p=0.027), but not in Emb males (p=0.742). However, no differences in expression levels of Il-10 (HFD p=0.435, Emb p=0.890), or Tgfβ (HFD p=0.355, Emb p=0.170), were observed when compared to the NFD group (Figure 4.36). When comparing the mRNA levels of anti-inflammatory genes in HFD vs Emb in males, there were no significant differences in Il-4 (p=0.059), Il-10 (p=0.537), and Tgfβ (p=0.595) genes.

We found in females that the expression levels of pro-inflammatory genes were not significantly different in the HFD (Il-1b p=0.149, Il-6 p=0.105, Il-18 p=0.055, and Tnfα p=0.703) and Emb (Il-1b p=0.981, Il-18 p=0.124, and Tnfα p=0.498) groups compared to NFD group, but Il-6 (p=0.017) Emb females were significantly different compared to the NFD female group (Figure 4.36).

When comparing the mRNA levels of pro-inflammatory genes in HFD vs Emb in females, there were no significant differences in Il-1b (p=0.193), Il-6 (p=0.218), Il-18 (p=0.875), and Tnfα (p=0.714) genes. When the mRNA expression levels of anti-inflammatory cytokines were analysed, we observed that all three genes were significantly decreased in the HFD group (Il-4 p=0.004, Il-10 p=0.027, and Tgfβ p=0.010), but not in Emb females (Il-4 p=0.523, Il-10 p=0.102, and Tgfβ p=0.056) compared with the NFD group (Figure 4.36).

When comparing the mRNA levels of anti-inflammatory genes in HFD vs Emb in females, there was a significant difference in Il-4 (p=0.030), but no differences in Il-10 (p=0.709), and Tgfβ (p=0.637) genes.
Finally, comparing males vs females we observed that there was not an effect of sex on the mRNA levels of Il-4, Il-10, Il-18, Tgfβ, and Tnfa genes in the hippocampus in NFD (p=0.571, p=1.0, p=0.819, p=0.838, and p=0.616 respectively), Emb (p=0.868, p=0.416, p=0.689, p=0.423, and p=0.799 respectively) or HFD groups (p=0.699, p=0.479, p=0.193, p=0.202, p=0.526 respectively). However, there was a significant effect of sex on the mRNA level of Il-1b in the hippocampus in the HFD group (p=0.002), but no differences in the NFD (p=0.981), or Emb (p=0.502) groups. There was a significant effect of sex on the mRNA level of Il-6 in the hippocampus in the Emb group (p=0.047), but no differences in the NFD (p=0.695) and HFD (p=0.063) group.

Taken together, our findings show a decrease in the expression of cytokines and anti-inflammatory mediators in the hippocampus in the HFD group, but not a significant increase in pro-inflammatory mediators. Therefore, the results reported here suggest that the maternal HFD in the offspring at 26 weeks of age does not cause neuroinflammation in the offspring by the age of 26 weeks.
Figure 4.37. mRNA levels of pro-inflammatory and anti-inflammatory cytokines in the hippocampus.
The mRNA levels of pro-inflammatory (II-1b, II-6, II-18, and Tnfα), anti-inflammatory (II-4, II-10, and Tgfβ) markers in male (a) and female (b) were assessed using RT-qPCR. Emb females were significantly different from HFD females in the mRNA levels of the II-4 gene. II-1b mRNA levels in HFD males were different from females (α p=0.002). II-6 mRNA levels in Emb males were different from females (β p=0.047). The mRNA levels of the selected markers were normalized to Fbxw2, Pak1lp1 and Ap3d1. NFD group (blue), Emb group (green) and HFD (red) at 26 weeks of age. n=6 mice from 6 litters. All bars represent means ± SEM. * p<0.05, ** p<0.01.
4.4 Discussion

In our maternal HFD model in the absence of obesity, we found that the offspring of mothers fed a HFD showed a significant increase in the number of DAPI positive cells in the adult cortex (Emb and HFD groups), being layers 1, 2/3, 4 and 5 in males, and layers 2/3, 5 and 6 in females where we observed significant differences from the NFD. These modifications in cell density were most likely due to the increased in astrocytes (GFAP and S100b positive cells) and microglial (Iba1 positive cells) populations in the cerebral cortex and the hippocampus. In the present study, we observed increased of astrocytes in the Emb female group (layer 5), in the HFD female group (layer 1, layer 4, layer 5 and layer 6), and in the HFD male group (layer 2/3 and layer 5). Interestingly, we observed that Emb females were significantly different from HFD females in layers 1, 4, 5, and 6. This result is in line with previous results were obesity caused by HFD produced an increasing number of astrocytes.  

On the other hand, although it is widely known that astrocytes are the most abundant cells of the CNS, it has been described that antibodies like GFAP only identify about 15-20% of astrocytes in the brain cortex. However, GFAP is not an absolute marker in non-reactive astrocytes, indicating that these astrocytes in a healthy CNS would not be easily identified with a GFAP antibody. For that reason, we used S100β as a marker which is abundantly expressed in astrocytes. Here, we observed increased cell density of S100β astrocytes in the Emb female group (layers 4 and 5), in the HFD female group (layers 1, 2/3, 4, 5 and 6), and in the HFD male group (layer 5). Interestingly, we observed that Emb females were significantly different from HFD females in layers 2/3, 4, and 6. There was a higher cell density in females compere to males in layer 1, 2/3, and 6 in the HFD and layers 1, and 6 in the Emb group. In the hippocampus, we observed an increasing number of astrocytes labelled as GFAP. The HFD males had a higher number of astrocytes in the CA1 and CA3 regions and HFD female only in the CA1 compared to the NFD group. The Emb males and females had a higher number of astrocytes in the CA1. These results in general (GFAP and S100b markers) showed an increasing number of astrocytes in the different layers. Also, indicating that the maternal HFD during pregnancy and gestation has a higher impact on the offspring at 26 weeks of age compared to the Emb group, especially in females.

Increased levels of GFAP and S100B are seen in astrogliosis, and several studies have indicated that increased levels of these markers are associated with degenerative and inflammatory brain disorders. These data led to the hypothesis that the increasing levels of GFAP and S100β
caused by maternal HFD might be involved in the pathophysiology of neurodegenerative processes in humans. Therefore, understanding how different brain cells during this developmental window are affected by the maternal HFD may be essential to determine disease aetiology. Therefore, future analyses are required to assess cognitive behaviour and functions in adult offspring in the Emb and HFD groups.

Astrocytes are essential in synapse development interacting with both presynaptic and postsynaptic neurons, together forming the tripartite synapse. In the hippocampus it has been described that one single astrocyte can cover and influence up to 140,000 synapses, regulating synapse development and synaptic plasticity. Neural plasticity defines the ability of the brain to adapt and respond to different challenges, through cellular and molecular mechanisms of synapse formation, neurite growth and behavioural adaptation. Considering that the maternal HFD modified the cellular density of astrocytes in adult offspring and that astrocytes play an important role in neural plasticity, we evaluated three markers involved in synaptogenesis PSD-95, Synapsin and BDNF.

In this study we did not observe changes in the mRNA levels of BDNF in the cortex, however in the hippocampus BDNF mRNA levels were reduced in HFD males compared to the NFD group, in HFD female there was a reduced trend but did not reach significance. BDNF is the most abundant neurotrophin in the brain and is mainly synthesized by neurons. Low levels of BDNF in the hippocampus decrease spatial learning, synaptic function and cognitive function. This changes in mRNA levels of BDNF are not reflecting changes at the protein level, and further analysis is needed to understand the impact of maternal HFD on the expression of BDNF protein. On the other hand, results showed a reduction in PSD95 mRNA levels in the hippocampus in HFD and Emb males and HFD females, but not in the cortex. PSD95 plays a critical role in organizing the postsynaptic, spatial learning and visual cortical plasticity. This changes in BDNF and PSD95 could suggest an alteration in synaptic function in the hippocampus and possibly spatial learning, synaptic function and cognitive function. Further analysis is required to understand the real impact at the protein level of pre-synaptic and post-synaptic markers.

On the other hand, microglia cells play an essential role in the protection of the CNS. Our data demonstrate that maternal HFD increases the number of microglia cells compared to the NFD group. In the cortex HFD males (layers 1, 2/3, 4, 5 and 6) and the hippocampus (DG, CA1, and CA3) had a higher cell density compared to the NFD males. The Emb males in the cortex in layers 4 and 5 and the hippocampus in the CA1 and CA3 regions had a higher cell density compared to the NFD males. In HFD females in layers 2/3, 4, 5 and 6 and the hippocampus in DG, CA1 and CA3 showed
an increasing number of microglial cells compared to the NFD females. We observed in the Emb female group more microglia cells in layer 4 in the cortex and DG and CA1 region in the hippocampus compared to the NFD females. Under physiological conditions, these cells are continually cleaning the waste, modulating synapses, as well as producing cytokines\textsuperscript{95}. Microglia can be reactive in response to an HFD or obesity, generating morphological changes in the microglia cells\textsuperscript{92,94,103}. It has been described that one of the components of HFD that can activate microglia are saturated fatty acids\textsuperscript{93}. We hypothesize that HFD might be enough to increase the number of microglia and astrocytes in the cortex and hippocampus in the offspring, during early embryo development.

Microglia are primarily known for mediating brain inflammation, and to adopt different phenotypes in response to an insult, a pro-inflammatory M1 phenotype, and an immunosuppressive M2 phenotype\textsuperscript{269,589,590}. However, the analysis of specific inflammatory markers did not show significant induction of pro-inflammatory cytokines in the offspring of HFD fed dams at 26 weeks of age (Emb or HFD males or females), suggesting that maternal HFD is not causing long-term neuroinflammation in the adulthood. However, we observed an increased number of microglia at PND 01 in the cortex (HFD males and females) and the hippocampus in the DG (HFD males and females), CA1 (HFD and Emb males), and CA3 (HFD males and females). In line with these results, in a maternal obese model caused by an HFD, the number of microglia was higher in offspring brain born from obese rat dams at PND 01\textsuperscript{126}. However, in this study, most of the microglia in the offspring brain from obese mothers had a higher proportion of M1 type\textsuperscript{126}. Here, we were unable to identify what type of microglia was observed in PND 01 brains. Additional experiments are needed to explore whether the microglia in PND 01 are M1 or M2. In the adult rat offspring, hippocampal M1 microglia increased in response to a neuroimmune challenge with LPS\textsuperscript{126}, indicating that maternal HFD could potentially exacerbate M1 microglial activation. Therefore, it is necessary to evaluate neuroinflammation markers and signalling pathways that regulate microglia in the early stages of development in the offspring.

To assess whether M1 microglia in the adult offspring of HFD fed dams was increased, we analysed CD11b (immune activation), and CD68 (phagocytic activity) as activated microglia markers. Our data obtained show that the microglia analysed is not of the M1 phenotype since the expression of these markers was not altered with respect to the NFD group in the cortex. Additionally, the expression of CD11b was reduced in the hippocampus. This suggests that the microglia cells in the cortex and hippocampus in adulthood are in their M2 phenotype. CX3CR1, which is uniquely expressed by microglia in the brain, is a crucial mediator of neuron–microglia interactions\textsuperscript{556,557}. In the present
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study, we demonstrate that cortical (male) and hippocampal (female) mRNA levels of \textit{Cx3cr1} were changed in the HFD group. In the hippocampus, the mRNA levels of \textit{Cx3cr1} were downregulated in the adult female HFD group compared to the NFD group. In humans, it has been suggested that mutations in this gene could contribute to neurodevelopmental disorders such as schizophrenia and ASD\textsuperscript{591}. A \textit{Cx3cr1}/- mice support this data showing deficiency in synaptic pruning, activation of hippocampal microglia cells, decreased hippocampal neurogenesis, and impaired cognitive function and social behaviour, resembling an ASD phenotype\textsuperscript{194,592,593}. Interestingly in our mouse model, we observed an increase in hippocampal microglia and impaired neurogenesis similar to that found in mice with \textit{Cx3cr1} deficiency\textsuperscript{592}. It is necessary to perform behaviour tests in the future to identify whether HFD maternal diet during the preimplantation period or gestation and lactation affects cognitive function.

\textit{Csf1r} regulates, development, proliferation, maintenance, and survival of microglia, and is involved in neuroinflammation\textsuperscript{559}. Our data reveal that in the cortex, the expression of the \textit{Csf1r} gene was increased in the HFD males, while the hippocampus was decreased in the HFD females. No changes were observed in the Emb groups. Recently, it has been described that there are different subpopulations of microglia in the brain, each with different expression profiles\textsuperscript{594,595}. Our data support the idea that cortex and hippocampal microglia may be distinct populations, with \textit{CSF1} mRNA expression being different, or having different functions. Expression profile analysis of purified cortex and hippocampal microglia should be done to observe whether maternal HFD leads to molecular changes in the different microglia subtypes. \textit{Csf1r} is essential for the proper functioning of the microglia since \textit{Csf1r} deficiency mice do not survive until adulthood, the cortex has no microglia, and there are problems in the architecture of the brain\textsuperscript{596,597}. Future research might be directed towards elucidating the role of \textit{CSF1R} in brain biology and establishing how the maternal HFD affects the normal function of the microglial markers in early stages of development.

4.5 Conclusion

Our data demonstrate that the HFD and Emb groups presented greater numbers of astrocytes and microglia cells compared to the NFD group, in different layers of the cortex and in the hippocampus (Figure 4.38). Different studies have shown that the nutritional environment in the early stages of development influences the maturation of microglial cells, which generates lasting effects on the response to neuroimmune challenge, and maternal obesity induced by HFD was enough to increase microglia density in adulthood\textsuperscript{126}. These data suggest that maternal HFD consumption during pregnancy and lactation is critical for the detrimental effects of HFD in offspring. However, the
preimplantation period showed an intermediate phenotype between HFD and NFD groups. Our data support the idea that early nutritional status has long-term effects on the cellular composition of the cortex and hippocampus.
Figure 4.38. Schematic diagram of overarching results in terms of cell density from Chapter 4. Overall effects of maternal HFD on the offspring in terms of cell density in the cortex (Ctx) and hippocampus (hippo) at PND 01 and 26 weeks of age. Only significant changes are shown.
Chapter 5. Maternal HFD during gestation and lactation alters neurogenesis in adult male and female offspring brains.

5.1 Introduction

Formation of new neurons in the brain is defined as neurogenesis. Neurogenesis is a complex process that occurs during embryonic and perinatal stages and continues in the adult brain, but declines with age. Neurogenesis comprises a complex multistep process (proliferation, differentiation and migration), which allows new cells to integrate the existing circuitry and to acquire physiological properties. In the adulthood, neurogenesis occurs in two brain regions, the subventricular zone (SVZ) of the lateral ventricles, and the subgranular zone (SGZ of the dentate gyrus) in the hippocampus. In the hippocampus, the neuronal precursors differentiate near their place of origin, giving rise to new granule cells. At the same time, in the SVZ the neuroblasts migrate through the rostral migratory stream to the olfactory bulb (OB). In the OB, neuroblasts will generate interneurons (periglomerular and granule cells) that participate in plasticity and learning processes.

Neurogenesis in SVZ occurs in the lateral ventricles of the adult brain, these ventricles are filled with cerebrospinal fluid (CSF), and the neural stem cells (NSCs) are located along the lateral ventricle walls. In the SVZ we could find different cell types: migrating neuroblasts (type A cells), primary progenitors neural stem cells (type B1 cells), transit-amplifying precursors (type C cells), and ependymal cells (type E cells). Type B1 cells share many characteristics of brain astrocytes, expressing astrocytic markers such as glial-fibrillary acidic protein (GFAP), glutamate aspartate transporter (GLAST), and brain lipid-binding protein (BLBP). Type B1 cells exist in two different states quiescent or activated, and activated type B1 cells give rise to type C cells, and in turn, these are responsible for generating neuroblasts (type A cells), which migrate to the OB. Type A cells move through the rostral migratory stream in elongated cellular aggregates forming a neuroblast chain. These neuroblasts will become new neurons to replace the dying neurons in the periglomerular region and the granule cell layer in the olfactory bulb.

The SGZ of the dentate gyrus in the hippocampus is the other neurogenic region in the adult brain. NSCs in this region, have multipotent and self-renewal capacities. There are two different types...
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of NSCs, type 1 and type 2. Type 1 NSCs have a radial process that elongate to the granule layer, expressing different markers such as GFAP, Sox2 and Nestin\textsuperscript{321,606}. Type 2 cells are originated from type 1, and it has been suggested that type 1 cells are NSCs in a quiescent state, and once activated they generate type 2 cells, which are considered as intermediate progenitors\textsuperscript{323,324}. Type 2 cells are positive for Sox2 and Nestin, but not for GFAP. These intermediate progenitors give rise to neuroblasts (DCX+) that integrate into the granule cell layer of the dentate gyrus\textsuperscript{340,348,607}. A different number of new-born cells naturally undergo apoptosis (up to 60%) during their first days of life, while they transit form type 2 cells, to neuroblasts, and are rapidly phagocytosed by microglia\textsuperscript{608}.

It is estimated that approximately 8,000 to 10,000 new neurons are generated in the DG per day, and only 50% of these cells will be functionally integrated\textsuperscript{335}. These immature cells integrate into the network and mature by establishing connections with pyramidal neurons of the hippocampal layer CA3\textsuperscript{326,342}. The addition of these new neurons plays a significant role in learning and memory function\textsuperscript{351,609}. Adult neurogenesis can be affected by extrinsic factors that increase the production of new neurons, such as physical exercise or environmental enrichment\textsuperscript{357,358}, or decrease their production such as ageing, HFD or inflammation\textsuperscript{611}. Neurogenesis is an essential, highly regulated process, since small imbalances may alter brain development and impair the cognitive function of the offspring. Maternal obesity caused by an HFD can cause abnormal activation of the Notch signalling pathway in the offspring. The Notch signalling pathway has been described as a crucial element regulating neurogenesis, especially during differentiation. Notch modulates cell cycle to avoid a declining in the number of NSCs in adulthood\textsuperscript{610}. Exposure to maternal obesity caused by an HFD in mice showed that this insult was sufficient to activate the Notch signalling pathway, increasing the number of NSCs in the neonatal mice\textsuperscript{611}.

Different studies have shown how maternal obesity caused by an HFD might affect neurogenesis\textsuperscript{162,612,613}. Decreased proliferation of progenitor cells in the dentate gyrus was observed during postnatal development until adulthood in the offspring of HFD fed dams, but not during embryonic development\textsuperscript{613}. On the other hand, maternal obesity-induced by an HFD was associated with decreased proliferation of neural progenitors in the dentate gyrus at embryonic day 17 in the offspring\textsuperscript{612}. Tozuka et al. found that maternal HFD-induced-obesity produced obesity in the adult offspring and impaired hippocampal BDNF production, which decreased the number of new hippocampal neurons\textsuperscript{613}. Conversely, a recent study in rats, where maternal consumption of HFD occurred only during gestation and lactation (which did not lead to overweight in dams), did not show any changes in terms of hippocampal neurogenesis\textsuperscript{162}. 

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The impact of maternal HFD on neurogenesis in the hippocampal DG has been reported in different studies. However, the data obtained do not allow clarifying exactly whether there is a significant impact on neurogenesis. Therefore, we want to identify whether the exposure to a maternal HFD, during gestation and lactation in a mouse model, is sufficient to alter neurogenesis in the SVZ and SGZ in the offspring.

5.2 Material and Methods

5.2.1 Immunofluorescence

PND1 animal bodies were fixed with 4% formaldehyde solution and stored at room temperature in a 70% ethanol solution for two years. Brains were flushed with 1XPBS, removed from the skull, and then slowly rehydrated in 50%, and 30%, 1X PBS, 24 hours each step. Then brains were cryoprotected by transferring them to 15% sucrose and then 30% sucrose solution in PBS until they sank, at 4°C. After overnight cryoprotection, tissues were equilibrated in 50:50 30% sucrose in PBS: OCT (Tissue Tek) for 1 hour and subsequently oriented and embedded in OCT. Tissue blocks were stored in -80°C. Tissue was sectioned at 14 µm thickness onto Superfrost Plus slides (Fisher Scientific) on a Leica cryostat and stored at -20°C. Coronal brain sections were made between bregma 3.39mm and bregma 3.63 mm for hippocampal samples, according to the stereotaxic coordinates of the mouse brain atlas.436

Adult brains were fixed in 4% PFA solution and then cryoprotected in 30% sucrose solution. Then, they were embedded in OCT (Tissue Tek) and stored at -80°C until sectioning. Coronal brain sections (14 µm thick) were made between bregma -1.22mm and bregma -2.70 mm for hippocampal samples, and from bregma 1.10mm to bregma 0.14 mm for lateral ventricle according to the stereotaxic coordinates of the mouse brain atlas.235 Twenty-one regions of interest in the DG from the dorsal hippocampus were selected in each animal to count GFAP, SOX2, DCX, and NeuN positive cells. The primary antibodies used include rabbit anti-GFAP (Dako, Z0334, 1:500), goat anti-Sox2 (Santa cruz, Sc-17320, 1:100), goat anti-DCX (Santa Cruz, Sc-8066, 1:100), and mouse anti-NeuN (Merck Millipore, MAB377, 1:200).

The secondary antibodies were Alexa Fluor 488, or 568-conjugated donkey anti-mouse, anti-rabbit IgG (1:200), and anti-goat. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) which was used as well to demarcate the subgranular zone (SGZ) from the granule cell layer (GCL).
Then Mowiol®4-88 Reagent (Calbiochem, Darmstadt, Germany) was used as mounting media. The detailed protocol is described in materials and methods chapter (Chapter 2). Primary antibodies were not used in the negative controls. Stained sections were examined under a Leica fluorescence microscope at x40, and x63 magnification (Leica, DM 5000B; Leica CTR 5000; Germany).

5.2.2 Imaging analysis

Postnatal day 1 (PND 01) brain sections were analysed by the fraction of a given region of interest (ROI) with the signal, and the mean intensity of that signal was measured using ImageJ. The regions of interest were determined based on the granular cell layer (GCL), and the hilus in the dentate gyrus. We used the auto-threshold algorithm to find an appropriate threshold value for each ROI of each picture. For each sample, 3 to 4 images were taken, and the average of the auto-threshold values was used to choose the value from the averages of each brain’s Area Fraction and Mean Intensity. This assay was done in collaboration with medical student Callum Robins (Faculty of Medicine, University of Southampton). The quality of the tissue was not ideal to perform the analysis of the SVZ in the PND 01 brains.

The quantification for NeuN and DCX markers was performed by counting positive cells with ImageJ in the different layer of the dentate gyrus. Three slices per animal and 21 non-overlapping fields were analysed at x40 magnification. For quantitative analysis of GFAP+/Sox2+ and GFAP-/Sox2+ located in the SGZ were counted at x63.

5.2.3 Western blot analysis

For Western blot, we followed the procedures according to the protocol described in materials and methods chapter (Chapter 2). The proteins were separated by SDS-PAGE, transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5 % fat-free milk in PBST for one h at 4°C, and then, incubated overnight with anti-NeuN (mouse, 1:5000; Millipore), and anti-GAPDH (rabbit, 1:10000; Sigma-Aldrich) at 4°C overnight. After washing, the membranes were incubated 1 hour with secondary antibodies IRDye®680RD goat anti-mouse red (1:10000), and IRDye®800CW Goat anti-rabbit green (1:10000). Detection and quantification were performed using the Odyssey imaging system (LI-COR®). Densitometric quantification of protein bands was performed with Image Studio Lite, version 5.2 (LI-COR, Lincoln, NE, USA).
5.2.4 Quantitative real-time PCR

Total RNA was isolated from frozen liver and frozen brain cortex and hippocampus using a RNeasy Lipid Tissue mini kit (Qiagen, UK) according to the manufacturer’s instructions. The isolated RNA was quantitated using the Nanodrop ND-1000 spectrophotometer (LabTech UK), and only samples with an adequate RNA concentration (A260/A280 $\geq$ 1.8) and purity (A230/A260 $\geq$ 2.0) were selected for reverse transcription. 1µg of RNA from each sample was reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad, UK) following the manufacturer’s protocol.

Gene expression of different markers were quantified using highly specific primers as listen in chapter 2, section 2.10. qPCR was then performed on a CFX96 Real-Time System (Bio-Rad, Hercules, CA). Melting analysis was carried out to verify the amplification specificity. Stability of 2–3 housekeeping genes was verified using GeNorm (gene stability value: 0.5; coefficients variation: 0.2) and used for normalization (qBase+3.0; Biogazelle, Ghent, Belgium). Data analysis was performed using the comparative CT method ($2^{-\Delta\Delta CT}$).

5.2.5 Statistical analysis

For all experiments data from the different diet groups was first normalized using Z-score transformation. The Z-score was calculated by the ratio of the difference each variable and its mean over the standard deviation of that variable. We used the z-transformed data to compare the different diet groups and also by gender using a multilevel random effects regression model accounting for different parameters (litter size, sex, and body weight) from individual animals (SPSS version 24). Normality of data was tested using the Shapiro–Wilk test. A probability of $p$ less than 0.05 was considered to be significant. All quantitative data were expressed as mean ± standard error of the mean (SEM). $P$ value is shown as * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p\leq0.0001$. 
5.3 Results

5.3.1 Maternal HFD does not affect hippocampal neurogenesis at PND1.

At first, we hypothesized that a maternal HFD in the absence of obesity during gestation (which is a critical phase during neurodevelopment) might lead to alterations in neurogenesis. In particular, we focused on GFAP positive cells in the dentate gyrus in PND1 mouse hippocampus. GFAP is exclusively expressed in radial glia and astrocytes during development throughout the CNS\(^ {236,614}\).

To evaluate GFAP positive cells, we analysed them based on intensity (mean greyscale value) and area fraction in the dentate gyrus (Figure 5.1), since it was impossible to perform an accurate cell count due to their high cell density.

![Figure 5.1. Expression of GFAP during dentate gyrus development at PND1.](image)

DAPI positive cells in blue (A1, B1, C1), and GFAP positive cells in red (A2, B2 and C2). The dotted line indicates the dentate gyrus area. Scale bars are 50µm.
We quantified a total of 102 dentate gyrus sections from the three different diet groups. In Emb males, maternal HFD had no significant effect on offspring in terms of GFAP intensity (pixels) in the dentate gyrus ($p=0.827$) or hilus ($p=0.179$). In the male mice from the HFD group, GFAP immunoreactivity was increased at trend level in the dentate gyrus ($p=0.095$) but did not reach significance when compared with the NFD group. No significant differences were observed in the hilus ($p=0.489$) (Figure 5.2a). In the female cohort, HFD ($p=0.368$) and Emb ($p=0.206$) offspring were not found to be significantly different compared with the NFD females in the dentate gyrus. No differences in GFAP immunoreactivity, in the hilus were observed in HFD females, but in Emb females GFAP immunoreactivity GFAP was increased at trend level in the dentate gyrus ($p=0.075$) but did not reach significance (Figure 5.2b). There is not a significant difference between the Emb and the HFD groups in males or females in the DG ($p=0.052$, and $p=0.561$ respectively), and hilus ($p=0.053$, and $p=0.307$ respectively). There is not a significant sex effect (females vs. males) in the DG (NFD $p=0.840$; Emb $p=0.114$; HFD $p=0.117$), or in the hilus (NFD $p=0.079$; Emb $p=0.081$; HFD $p=0.183$).
We then analysed area fraction in the dentate gyrus area and the hilus. In males (Figure 5.3a), neither the dentate gyrus area, nor the hilus showed significant differences in HFD (p=0.395, and p=0.195 respectively) or the Emb group (p=0.174, and p=0.180 respectively). There is not a significant difference between the Emb males and the HFD male groups in the DG (p=0.595) or the hilus (p=0.997).

No significant differences were observed in females (Figure 5.3b), neither in the dentate gyrus area, nor in the hilus in HFD (p=0.393, and p=0.347 respectively) or the Emb group (p=0.732, and p=0.752 respectively). There is not a significant difference between the Emb females and the HFD female groups in the DG (p=0.727) or in the hilus (p=0.291). There was not an effect of sex on the DG in NFD (p=0.276), Emb (p=0.324) or HFD groups (p=0.308) or the hilus NFD (p=0.131), Emb (p=0.511) or HFD groups (p=0.337). In general, GFAP positive cells, likely to be type 1 cells, did not significantly change between the three diet groups, indicating that maternal HFD did not affect GFAP immunoreactivity at PND 01.
Figure 5.3 Area fraction in the dentate gyrus area and hilus at PND 01.

a. GFAP area fraction in males. b. GFAP area fraction in females. NFD group (blue), Emb group (green) and HF (red). All bars represent means ± SEM. n=6-8 mice from 6-8 litters in each group.

5.3.2 Effect of maternal HFD on cell proliferation in the adult Ventricular-Subventricular zone

We decided to examine the presence of neuronal progenitor cells in the ventricular-subventricular zone (V-SVZ). The SVZ is one of few areas in the adult brain that are known to show neurogenesis. It is essential to mention that SVZ is a large area, and it has been observed that it is not a homogeneous zone; therefore, we developed our analysis of the SVZ dorsal area. NSCs in the V-SVZ or Type B cells were stained using antibodies against GFAP and SOX2, to visualise the location and quantify the number of NSCs in the region of interest within the brain (Figure 5.4). We examined SOX2+/GFAP- cells in the V-SVZ to evaluate the impact of maternal HFD on neural precursor cells (Table 3.1 and 3.2). Still, we did not observe significant changes in HFD and Emb males compared

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to the NFD group (p=0.743 and p=0.759, respectively). There is not a significant difference between the Emb males and the HFD male groups (p=0.333). In females, there were no significant differences between the HFD (p=0.319) or Emb (p=0.133) and the NFD group (Figure 5.5 A-B). There is not a significant difference between the Emb females and the HFD female groups (p=0.187). There was not an effect of sex (females vs males) in SOX2+/GFAP+ cells in the NFD (p=0.345), Emb (p=0.121) or HFD groups (p=0.420).

We then analysed SOX2+/GFAP+ cells to detect NSCs. Quantification demonstrated that the number of SOX2+/GFAP+ NSCs were not different between HFD males and the NFD group (p=0.222), neither between Emb males (p=0.527) and the NFD group. There is not a significant difference between the Emb males and the HFD male groups (p=0.079). In females, there were no modifications in SOX2+/GFAP+ NSCs density in Emb (p=0.673), or HFD (p=0.926) groups compared to the NFD group (Figure 5.5 C-D). There is not a significant difference between the Emb females and the HFD female groups (p=0.328). There was not an effect of sex (females vs males) in SOX2+/GFAP+ cells in the NFD (p=0.513), Emb (p=0.369) or HFD groups (p=0.121). To evaluate whether the number of astrocytes was altered, we analysed cells that were positive for GFAP, but negative for SOX2. There were no significant differences between the different diet groups when V-SVZ astrocytes were compared. The number of SOX2-/GFAP+ cells in HFD and Emb male animals was not significantly different compared to the NFD group (p=0.522 and p=0.605 respectively). There is not a significant difference between the Emb males and the HFD male groups (p=0.632). The number of SOX2-/GFAP+ cells in HFD (p=0.849) and Emb (p=0.985) in females was not found to be significantly different compared with the NFD females in the V-SVZ (Figure 5.5 E-F). There is not a significant difference between the Emb females and the HFD female groups (p=0.088). There was not an effect of sex (females vs males) in SOX2+/GFAP+ cells in the NFD (p=0.083), Emb (p=0.560) or HFD groups (p=0.081).
Figure 5.4. V-SVZ Neural Precursor Cells expressing GFAP and SOX2.

Images of coronal V-SVZ sections analysed by GFAP (green top row), SOX2 (red second row to bottom), and DAPI (blue third row to bottom), and merge (bottom row). NFD group (left column), Emb group (middle column) and HFD (right column). Scale bars, 100 μm.
Table 5.1 SOX2 and GFAP quantification in the V-SVZ in each diet group in males.
The mean ± SEM estimated cell count of SOX2 and GFAP positive cells in the V-SVZ for each group. n=6 in each group.

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Table 5.2 SOX2 and GFAP quantification in the V-SVZ in each diet group in females.
The mean ± SEM estimated cell count of SOX2 and GFAP positive cells in the V-SVZ for each group. n=6 in each group.

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<td>cells/mm²</td>
<td>SEM</td>
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<tr>
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<td>213.75</td>
<td>1611.11</td>
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Figure 5.5 SOX2 and GFAP intensity in the V-SVZ in each diet group. 
A-B. SOX2+/GFAP- cells, C-D SOX2+/GFAP+ cells. E-F SOX2-/GFAP+ cells. NFD group (blue), Emb group (green) and HF (red). All bars represent means ± SEM. n=6 mice from 6 litters in each diet group.
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5.3.3  The effect of maternal HFD on neurogenesis in the adult mouse brain

5.3.3.1  Total cell density in the Dentate Gyrus

Previous studies have observed that maternal HFD could lead to structural and cellular changes in the dentate gyrus\textsuperscript{149,162,612,613}. Therefore, we analysed in the dentate gyrus, the number of positive DAPI stained cells to evaluate total cell density. The total number of cells (DAPI positive cells), were analysed across the three maternal diet groups HFD, within the three regions of the dentate gyrus: Granule cell layer (GCL), Subgranular zone (SGZ), and Hilus. Cell density in the dentate gyrus at 26 weeks of age, showed differences between males or females and their respective NFD group in the different regions of the dentate gyrus (Figure 5.6). The statistical analysis performed in the HFD males showed the increase in the number of cells in the GCL ($p=0.015$), decrease in the number of cells in the SGZ ($p=0.031$), but no changes in the Hilus ($p=0.235$) regarding the NFD group. In Emb males, there were no significant differences in the number of DAPI cells in the different regions of the dentate gyrus (GCL $p=0.785$, SGZ $p=0.720$, Hilus $p=0.242$) compared to NFD males. There is a significant difference between the Emb males and the HFD male groups in the GCL ($p=0.030$), and hilus ($p=0.029$). However, there is not a significant difference between the Emb males and the HFD male groups in the SGZ ($p=0.072$).

HFD and Emb females did not show significant differences in the SGZ region ($p=0.156$; $p=0.222$, respectively). However, HFD females exhibited less DAPI-positive cells in the GCL ($p=0.019$), and more DAPI-positive cells in the Hilus ($p=0.028$). Similarly, there was also a decrease in the number of DAPI-positive cells in the GCL ($p=0.009$) in the Emb females, but no changes in cellular density in the Hilus ($p=0.262$). There is not a significant difference between the Emb females and the HFD female groups in the GCL ($p=0.426$), and the SGZ ($p=0.999$). However, there is a significant difference between the Emb males and the HFD male groups in the hilus ($p=0.005$).

There was not an effect of sex (females vs males) in the GCL in the NFD group ($p=0.086$), or Emb group ($p=0.369$), but it was a significant difference in the HFD group ($p=0.001$). In the SGZ, there was not an effect of sex in the NFD ($p=0.395$), Emb ($p=0.474$) or HFD groups ($p=0.057$). In the hilus, there was not an effect of sex in the NFD group ($p=0.562$), or Emb group ($p=0.077$), but it was a significant difference in the HFD group ($p=0.015$) In general, these results revealed that adult offspring of HFD fed dams during gestation and lactation exhibited changes in the density of DAPI-positive cells in the different regions of the dentate gyrus, compared to the NFD group.
Figure 5.6 Total cell density in the Dentate Gyrus by gender quantified by DAPI staining;

Each chart represents the different region in the dentate gyrus by sex. NFD group (blue), Emb group (green) and HFD (red). All bars represent means ± SEM. n=6 mice from 6 litters in each group. There was a sex-induced difference in total cell density in the SGZ (p=0.031) in males. No differences were observed in females in the SGZ. Differences were seen when GCL cell density was compared in females and males. In the hilus, only differences were found in females. Emb males were significantly different from HFD males in the GCL and the Hilus. Emb females were significantly different from HFD females in the Hilus. In the GCL, HFD males showed a higher DAPI cell density than males (α p=0.001). In the hilus, HFD females showed a higher DAPI cell density than males (β p=0.015). * p<0.05, ** p<0.01.
5.3.3.2 Maternal HFD changes cell density of progenitor cells in the Dentate Gyrus

GFAP is a characteristic marker of adult reactive mature astrocytes that is also expressed in quiescent neural precursors or type-1 cells. These type-1 cells are radial cells that have their cell body residing in the SGZ, and a broad apical process that extends towards the molecular layer of the dentate gyrus (Figure 5.7). Type 1 progenitor cells are characterized by being quiescent; that is, they stay non-dividing harbouring progenitor potential.

SOX2 protein, also known as SRY-box 2 (Sex Determining Region Y-box 2), is a transcription factor essential for maintaining the self-renewal of undifferentiated embryonic stem cells. SOX2 is a highly used marker to identify type-1 (radial glial-like cells) and type-2 cells (early progenitor cells) in the dentate gyrus. Therefore, in this document, we are going to describe the analysis of type-1 and type-2 cells to understand the impact of maternal HFD on NSCs in the adult offspring (more detailed information Chapter 1, section 1.2.3).

Type-1 and type 2 cells analysis and cell counting were based on their localization and cell morphology. Radial glial (RGL) stem cells are GFAP+/Nestin+/SOX2+ and have a triangular...
soma with a primary radial process extending through the GCL ending in the molecular layer of the dentate gyrus (Figure 5.7). Therefore, double immunofluorescent staining for GFAP/SOX2 and subsequent microscope analysis using the x63 objective, revealed labelled GFAP+/Sox2+ (type-1 cells) that are in the SGZ. Additionally, we analysed GFAP-/SOX2+ labelled cells, to identify amplifying progenitor cells or type-2 cells that also are in the SGZ but lack an apical process (Figure 5.8).

Figure 5.8. Type -1 and type2 cell in the dentate gyrus of the hippocampus; Example of immunoreactivity against GFAP (green), SOX2 (pink), counterstaining with DAPI (blue). Red arrowheads indicate type-1 (GFAP+/SOX2+) radial glia-like cells. Yellow arrowheads indicate type-2 (GFAP-/SOX2+) cells. GCL, granule cell layer; SGZ, subgranular zone. The broken lines show the border between the GCL and the SGZ. Scale bar 25µm.

After identifying the different cell types (Figure 5.9), we quantified the total number and the proportion of each of the cell types in the different diet groups (Table 5.3). We observed that adult males from the HFD group had more GFAP+/SOX2+ cells ($p=0.038$). On the contrary, in Emb males, maternal HFD did not affect the number of GFAP+/SOX2+ cells ($p=0.389$) in comparison to the NFD males. There is a significant difference between the Emb males and the HFD male groups ($p=0.013$). HFD Females showed very similar results to HFD males, as the number of GFAP+/SOX2+ cells was increased ($p=0.038$), although in Emb females the effect of maternal diet on type-1 cells was not
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significant (p=0.478) (Figure 5.10). There is a significant difference between the Emb females and the HFD female groups (p=0.025). There was not an effect of sex on GFAP+/SOX2+ cells in NFD (p=0.513), Emb (p=0.519) and HFD (p=0.096) groups.

The number of type-2 cells was calculated as the number of GFAP-/SOX2+ cells in the SGZ (Table 5.3). The data obtained show that in males and females from the HFD group, there were no significant changes compared to the NFD group (males p=0.121, females p=0.773). Emb mice did not show significant differences (males p=0.244, females p=0.175) in comparison to the NFD mice.

There is a significant difference between the Emb males and the HFD male groups (p=0.019), but no significant differences between the Emb females and the HFD female groups (p=0.218). There was not an effect of sex on GFAP+/SOX2- cells in NFD (p=0.345), Emb (p=0.261) and HFD (p=0.498) groups. These results suggest that maternal HFD might affect the maintenance of the NSCs reservoir in the offspring in the subgranular zone of the adult dentate gyrus (Figure 5.10).
Figure 5.9. SGZ Neural Precursor Cells expressing GFAP and SOX2.

Images of coronal SGZ sections analysed by DAPI (blue top row), GFAP (green second row to bottom), and SOX2 (red third row to bottom), and merge (bottom row). NFD group (left column), Emb group (middle column) and HFD (right column). A red arrowhead indicates an example of type-1 (GFAP+/SOX2+) radial glia-like cells. A yellow arrowhead indicates an example of type-2 (GFAP-/SOX2+) cells. GCL: granule cell layer; SGZ: subgranular zone Scale bars, 50 μm.
Table 5.3 SOX2 and GFAP quantification in the SGZ in each diet group in male and female.

The mean ± SEM estimated cell count of SOX2 and GFAP positive cells (Type-1 and Type-2 cells) in the dentate gyrus for each group. n=4-5 in each group.

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<tr>
<td><strong>Males</strong></td>
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<td></td>
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<tr>
<td>GFAP+/SOX2+</td>
<td>209.20 ± 14.98</td>
<td>211.90 ± 21.26</td>
<td>265.30 ± 12.84</td>
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<tr>
<td>GFAP+/SOX2-</td>
<td>200.20 ± 7.69</td>
<td>215.00 ± 10.67</td>
<td>180.50 ± 6.21</td>
</tr>
<tr>
<td><strong>Females</strong></td>
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<td></td>
<td></td>
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<tr>
<td>SOX2+/GFAP-</td>
<td>190.40 ± 13.92</td>
<td>177.10 ± 11.51</td>
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</tr>
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<td>SOX2+/GFAP-</td>
<td>182.80 ± 12.05</td>
<td>193.60 ± 6.04</td>
<td>183.70 ± 13.48</td>
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</table>

Figure 5.10. Cell density in the SGZ by gender quantified from GFAP and SOX2 staining;

Significant differences were observed in males and females after counting type-1 (GFAP+/SOX2+) cells. Differences were not seen when Type-2 cell (GFAP-/SOX2+) density was compared in females and males. Emb males and females were significantly different from HFD males and females respectively, in type-1 cells. Emb males were significantly different from HFD males in type-2 cells. NFD group (blue), Emb group (green) and HF (red). All bars represent means ± SEM. n=4-5 mice from 4-5 litters in each group. * p<0.05.
5.3.3.3 Maternal HFD alters neuronal maturation in the dentate gyrus

Doublecortin (DCX) is a protein associated with cytoskeletal microtubules that promote their polymerization\textsuperscript{620-622}. In the adult brain, DCX is still expressed by immature neurons, and its expression correlates with the extent of adult neurogenesis\textsuperscript{324}. DCX plays a crucial role in neuronal migration, axogenesis, synaptogenesis, and is required for proper acquisition of spatial learning\textsuperscript{324,623,624}. DCX expression begins with an overlap, when Nestin ends, following neuronal specification, the DCX expression ends with some overlap with the onset of NeuN marker expression\textsuperscript{324,620,621} (more detailed information Chapter 1, section 1.2.3).

NeuN is a soluble nuclear protein in postmitotic neurons\textsuperscript{540}. NeuN is expressed in the nuclei, and perinuclear cytoplasm of most granule cells in the CNS but is not detected in immature nerve cells\textsuperscript{541}. NeuN has never been detected in glial cells, suggesting that it is a specific neuronal marker. In adult hippocampal, NeuN is expressed by all post-mitotic cells and is used to distinguish the different neuronal populations by double labelling them with other markers such as DCX, Calretinin and Calbindin\textsuperscript{348}. These markers were utilized to confirm the presence of differentiating neuronal cells stained with DCX (neuroblasts), as well as mature neurons with NeuN staining.

Therefore, to investigate the extent of neuronal differentiation, neurons were double-stained with DCX and NeuN in the dentate gyrus to estimate the number of 3 different cell types: a) labelled DCX+/NeuN- new-born neurons, b) labelled DCX+/NeuN+ immature neurons and c) labelled DCX-/NeuN+ mature neurons (Figure 5.11).
Figure 5.11. Cells expressing DCX and NeuN in the dentate gyrus.
Images of coronal dentate gyrus sections analysed for DCX (green top row), and NeuN (red second row to bottom), DAPI (blue third row to bottom), and merge (bottom row. NFD group (left column), Emb group (middle column) and HFD (right column). GCL: granule cell layer. Scale bars, 50 μm.
The number of new-born neurons and mature neurons was significantly different for HFD males, but not HFD females at 26 weeks of age (Figure 5.12). Emb male animals were not significantly different to NFD males (p=0.854), while HFD males were significantly different from NFD males (p=0.033) having nearly 1.5 times more DCX+/NeuN- cells. There is not a significant difference between the Emb males and the HFD male groups in the number of DCX+/NeuN- cells (p=0.055). The number of DCX+/NeuN- cells in females, did not change significantly in the Emb (p=0.410), and HFD (p=0.888) compared to the NFD females. There was not an effect of sex on DCX+/NeuN- cells in NFD (p=0.159), Emb (p=0.963) and HFD (p=0.889) groups.

Dual-labelling DCX+/NeuN+ cells were analysed to assess the number of immature neurons (Figure 5.12). Unlike new-born neurons, there were no significant differences between the different diet groups when immature neurons were compared. In the Emb and HFD males, the average of DCX+/NeuN+ cells was not significantly different (p=0.450, p=0.751, respectively) compared to the NFD male group. There is not a significant difference between the Emb male and the HFD male groups in the number of DCX+/NeuN+ cells (p=0.291). The number of DCX+/NeuN+ cells in Emb females (p=0.654), and HFD females (p=0.735) were not different compared to the NFD female group. There is not a significant difference between the Emb females and the HFD female groups in the number of DCX+/NeuN+ cells (p=0.868). There was not an effect of sex on DCX+/NeuN+ cells in NFD (p=0.069), Emb (p=0.097) and HFD (p=0.460) groups.

Finally, we assessed mature neurons (DCX-/NeuN+) in the dentate gyrus (Figure 5.12). Microscopy analysis revealed that the number of DCX-/NeuN+ cells was decreased in HFD male animals (p=0.019) compared to their NFD group. The number of DCX-/NeuN+ cells was not significantly different in Emb males (p=0.960) compared to the NFD male group. There is a significant difference between the Emb males and the HFD male groups in the number of DCX-/NeuN+ cells (p=0.020). In females, there were no significant differences for Emb group (p=0.823) and HFD group (p=0.191) when compared to the NFD female group in the hippocampal dentate gyrus concerning to the number of DCX-/NeuN+ cells. There was not an effect of sex on DCX+/NeuN+ cells in NFD (p=0.266), and Emb (p=0.765), but it was a significant difference in between females and males in the HFD (p=0.024) group.

Although the number of type 1 and new-born neurons cells is higher in males of the HFD group, the number of mature neurons has been reduced. This indicates that exposure to maternal HFD during gestation and lactation might alter the neuronal maturation process, which leads to a reduction of mature neurons, either, by a delay in this process, or by an increase in apoptosis of mature neurons.
Figure 5.12. Neuron density in the dentate gyrus by gender quantified from DCX and NeuN staining;
Significant differences were observed in males in new-born neurons (DCX+/NeuN-) and mature neurons (DCX-/NeuN+). Differences were not seen in females. Emb males were significantly different from HFD males in the DCX-/NeuN+ cells. HFD females showed a higher DCX-/NeuN+ cell density than males ($\alpha \ p=0.024$). NFD group (blue), Emb group (green) and HF (red). All bars represent means ± SEM. n=6 mice from 6 litters in each group. * p<0.05.
5.3.4 Early neuronal differentiation and stem cell markers in adult neurogenesis

NSC markers were used to confirm our immunostaining results in the hippocampus. For this, we used *Nestin, Notch1, Sox2* and *Pax6* as markers. The mRNA expression levels of the neuronal genes *Nestin, Notch1, Sox2,* and *Pax6* in the HFD and Emb males was not significantly different from those observed in the NFD group (Figure 5.13). There are no differences between Emb and HFD males in terms of mRNA expression levels of the neuronal genes *Nestin* (p=0.112, and p=0.061, respectively), *Notch1* (p=0.100, and p=0.087, respectively), *Sox2* (p=0.543, and p=0.118, respectively), and *Pax6* (p=0.160, and p=0.075, respectively). In females, no significant differences between groups (HF or Emb vs NFD) were found concerning the expression levels of the Neural stem cell markers genes mentioned here (Figure 5.13). There are no differences between Emb and HFD females in terms of mRNA expression levels of the neuronal genes *Nestin* (p=0.351), *Notch1* (p=0.160), *Sox2* (p=0.563), and *Pax6* (p=0.937). Here we did not observe significant changes in the expression of different markers of NSCs. These data suggest NSCs are not in an active division process, and instead are in a quiescent state. These data are in line with the data observed when analysing type-2 cells, where we do not observe changes in their cell density (See figure 5.10).

Finally, comparing males vs females we observed that there was not an effect of sex on the mRNA levels of *Nestin, Notch1, Sox2, and Pax6* in the hippocampus in NFD (p=0.932, p=0.607, p=0.784, and p=0.791 respectively), Emb (p=0.535, p=0.054, p=0.057, and p=0.340 respectively) or HFD groups (p=0.054, p=0.947, p=0.056, and p=0.057 respectively).
Figure 5.13. Effect of maternal HFD on early neuronal differentiation stem cell markers.

Hippocampal mRNA levels of Nestin, Notch1, Sox2, and Pax6 in males (a.) and females (b.) were assessed using quantitative RT-PCR. The mRNA levels of the selected markers were normalized to Fbxw2, Pak1lp1, and Ap3d1 transcripts. NFD group (blue), Emb group (green) and HF (red) at 26 weeks of age. All bars represent means ± SEM. n=6 mice from 6 litters in each group.
5.3.5 Decreased expression of mature neuron gene (β-tubulin III), but not at protein level (NeuN)

Numbers of mature neurons in HFD males were considerably less compared to their controls in the dentate gyrus. Therefore, we evaluated B-tubulin III (B3-Tub) gene expression and NeuN protein expression in the hippocampus, since it was not possible to dissect only the dentate gyrus. B3-Tub is localised explicitly in neurons, and it is expressed in the earliest phases of neuronal differentiation, overlapping somewhat DCX and NeuN expression. When analysing the HFD males, we observed that the levels of B3-Tub (p=0.031), were significantly downregulated concerning the NFD group (Figure 5.14). In Emb males, we do not find changes in the expression levels of B3-Tub mRNA (p=0.119). There is not a significant difference between the Emb males and the HFD male groups in the number of B3-Tub mRNA (p=0.520). Female Emb and HFD did not display significantly different expression levels of B3-Tub (p=0.765, and p=0.183, respectively) compared to the NFD group (Figure 5.14). There is not a significant difference between the Emb females and the HFD female groups in the number of B3-Tub mRNA (p=0.143). We demonstrate that B3-Tub mRNA levels are decreased, consistent with our counting data in which the number of DCX-/NeuN+ cells was reduced in HFD males when compared to their NFD group (See Figure 5.12). There was not an effect of sex on B3-Tub mRNA in NFD (p=0.848), Emb (p=0.122) and HFD (p=0.319) groups.

![Figure 5.14. Effect of maternal HFD on B-tubulin III in the hippocampus.](image-url)

The mRNA levels of B3-Tub in males (a) and females (b) were assessed using quantitative RT-PCR. The mRNA levels of the selected markers were normalized to Fbxw2, Pak1p1, and Ap3d1 transcripts. NFD group (blue), Emb group (green) and HF (red) at 26 weeks of age. All bars represent means ± SEM. n=6 mice from 6 litters in each group. * p<0.05.
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Western blot analysis was carried out on whole cell lysates extracted from the hippocampus to validate results at the protein level. Quantitation of NeuN relative to GAPDH was performed by western blot (Figure 5.15). NeuN expression in the hippocampus of HFD group (males p=0.576, females p=0.487) or Emb group mice (males p=0.646, females p=0.223) was not significantly different to the levels in the NFD-fed mice (Figure 5.16). NeuN expression in the hippocampus of HFD group vs Emb group mice was not significantly different (males p=0.940, and females p=0.502). There was not an effect of sex on NeuN expression in the hippocampus in the NFD (p=0.994), Emb (p=0.152) and HFD (p=0.506) groups.

Analysis of neuron maturity using Western blot with NeuN did not reproduce previous findings employing RT-PCR of B3-Tub or immunofluorescence of DCX/NeuN and cell counting. One possible explanation is the number of neurons in the hippocampus since there are neurons that express NeuN in different regions of the hippocampus such as CA1, CA2, and CA3, which could dilute the results observed in the dentate gyrus.

Figure 5.15. NeuN protein expression in the hippocampus;
Western Blot used for analysis, NeuN (top bands) detected as a band at 46kDa -48kDa and GAPDH (bottom band) at 37kDa. n=6 mice from 6 litters in each group. NFD: normal fat diet; Emb: embryonic high-fat diet; HFD: high-fat diet.
Figure 5.16. NeuN expression analysis in the hippocampus; Analysis of NeuN western blot did not show significant differences in males or females. \( n=6 \) mice from 6 litters in each group. NFD group (blue), Emb group (green) and HF (red) at 26 weeks of age. All bars represent means ± SEM.

5.3.6 Regulation of neurogenesis by phagocytic microglia

Microglia are crucial elements in the adult hippocampal neurogenic niche since microglia regulate neurogenesis through phagocytosis. Most of the new cells in the adult dentate gyrus do not survive beyond the first week, and this is mostly due to apoptotic cell death, whose debris is cleared by the microglia. Considering the significant findings observed in the number of DCX+/NeuN- cells and DCX-/NeuN+ cells, in HFD males, we decided to explore whether there is an association between neurogenesis and microglia (more detailed information about microglia analysis see Chapter 4, section 4.3.6.3). There was no significant correlation between the number of microglial cells in the dentate gyrus and new-born cells in males \( (r=0.4133, p=0.0882) \) nor in females \( (r=0.3168, p=0.2003) \). The analysis of microglia cells and immature neurons did not show a correlation in males \( (r=0.0787, p=0.7560) \), nor in females \( (r=0.1205, p=0.6337) \). However, in males, there was a significant negative correlation between microglial cells in the DG (Iba1+ cells) and mature neurons (DCX-/NeuN+ cells) \( (r=-0.4702, p=0.0489) \), but we do not observe this in females \( (r=0.157, p=0.5339) \) (Figure 5.17).
Figure 5.17. Correlation between Iba1 positive cells and new-born, immature, and mature neurons.

A and B, New-born neurons vs Iba1 cells in males and females. C and D, there is no correlation between the number of immature neurons and Iba1 positive cells in males and females. E, in males, we observed a negative correlation between mature neurons (DCX-/NeuN+) and microglia cells ($r=0.4702$, $p=0.0489$), nevertheless, we did not observe this correlation in females. NFD group (blue), Emb group (green) and HFD (red). n=6 mice from 6 litters per diet group.
5.3.7 Expression of caspase-3, Bax and Bcl-2 in the hippocampus

To verify the contribution of caspase-dependent apoptosis on the decrease of mature neurons, we performed qPCR to evaluate changes at the mRNA level in the hippocampus. The results show that caspase-3 may be activated since caspase-3 mRNA levels were significantly increased in HFD males (p=0.000462), but not in the Emb males (p=0.212) compared with the male NFD group (Figure 5.18a). There is a significant difference between the Emb males and the HFD male groups in the number of caspase-3 mRNA (p=0.008). In females, no significant differences between groups (HFD p=0.575, or Emb p=0.624 vs NFD) were observed concerning caspase-3 mRNA levels (Figure 5.18b). There is not a significant difference between the Emb females and the HFD female groups in the number of caspase-3 mRNA (p=0.997). There was not an effect of sex on caspase-3 mRNA in the hippocampus in the NFD (p=0.960), and Emb (p=0.068) groups, but there was a significant effect of sex in HFD (p=0.002) group.

Level of Bax mRNA was significantly increased in HFD males (p=0.000016), and in the Emb males (p=0.000001) compared with the male NFD group (Figure 5.18a). There is a significant difference between the Emb males and the HFD male groups in the number of Bax mRNA (p=0.000029). The expression level of Bax mRNA was significantly higher in HFD (p=0.000175) and Emb (p=0.00012) females than in female NFD group (Figure 5.18b). There is a significant difference between the Emb females and the HFD female groups in the number of Bax mRNA (p=0.027). There was not an effect of sex on Bax mRNA in the hippocampus in the NFD (p=0.425), Emb (p=0.052), and HFD (0.563) groups.

The expression level of Bcl-2 mRNA was significantly increased in HFD males (p=0.00073), and in the Emb males (p=0.000010) compared with the male NFD group (Figure 5.18a). There is a significant difference between the Emb males and the HFD male groups in the number of Bcl-2 mRNA (p=0.012). The expression level of Bcl-2 mRNA was significantly higher in HFD (p=0.000458) and Emb (p=0.000067) females than in female NFD group (Figure 5.18b). There is not a significant difference between the Emb females and the HFD female groups in the number of Bcl-2 mRNA (p=0.087). There was not an effect of sex on Bcl-2 mRNA in the hippocampus in the NFD (p=0.163), Emb (p=0.947), and HFD (0.550) groups.
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a. Males

b. Females

Figure 5.18. Effect of maternal HFD on the apoptotic markers in the hippocampus.

The mRNA levels of Caspase-3, Bax and Bcl2 in males (A.) and females (B.) were assessed using quantitative RT-PCR. Emb males were significantly different from HFD males in the mRNA levels of Caspase 3, Bax and Bcl2. Emb females were significantly different from HFD females in the mRNA levels of Bax and Bcl2. HFD males showed a higher mRNA level of Caspase 3 than females (α p=0.002). The mRNA levels of the selected markers were normalized to Fbxw2, Pak1lp1, and Ap3d1 transcripts. NFD group (blue), Emb group (green) and HF (red) at 26 weeks of age. N=6 mice from 6 litters. All bars represent means ± SEM. * p<0.05, *** p<0.001, **** p≤0.0001
5.4 Discussion

Neurogenesis or the formation of new neurons has been described in different species of mammals, including humans, and has been observed to continue through the life span of most mammals\textsuperscript{291,627}. Neurogenesis comprises different steps (proliferation, differentiation and migration), concluding when the new mature neurons are integrated into existing neural circuits.

In hippocampal neurogenesis, NCSs are located in the subgranular zone in the dentate gyrus\textsuperscript{348}. These NSCs are essential to maintain the reservoir of stem cells throughout the life of the individual, due to their quiescence characteristics\textsuperscript{628}. After differentiation, cells migrate a short distance to the granular cell layer, where they mature and form dendrites and axons. The axons extend to connect with the pyramidal cells of the CA3 layer. Once the connections have been established and the new cells can generate neuron action potentials, the maturity process will end, and formally these new neurons will become part of the hippocampal circuit\textsuperscript{325}.

Our intention in the present work was to study neurogenesis in the postnatal and adult stage. At PND 01, we observed GFAP\textsuperscript{+} cells with short processes located in the DG area, and spread around the hilus, below the hippocampal fissure, without a clear radial orientation. However, exposure to maternal HFD during the preimplantation period (3.5 days) or gestation did not affect GFAP intensity in the DG of the offspring. The maturation of the DG, and therefore the development of the SGZ occurs between PND 07 and PND 15 in the mouse brain\textsuperscript{629}. It is possible that due to the immature state of the dentate gyrus, changes derived from the maternal HFD (in morphology or structure) were not visible. As the study of this region at this age (PND 01) would only allow us to analyse young and immature cells, we decided to focus the research towards a mature phenotype (at 26 weeks of age).

Adult neurogenesis is restricted to two specific neurogenic brain niches: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) in the dentate gyrus of the hippocampus. We have conducted a study of the NSC of SVZ in mice at 26 weeks of age to understand how maternal HFD affects neurogenesis in this region. We have found that there was no significant effect of maternal HFD on the number of SOX2\textsuperscript{+}/GFAP\textsuperscript{−}, SOX2\textsuperscript{+}/GFAP\textsuperscript{+}, and SOX2\textsuperscript{−}/GFAP\textsuperscript{+}. This indicates that exposure to the maternal HFD during the pre-natal period has no long-term effects on stem cell production in the V-SVZ. The SGZ of the dentate gyrus in the hippocampus is the other neurogenic region in the adult brain. NSCs in this region have multipotent and self-renewal capacities\textsuperscript{299}. Here, maternal HFD (HFD group) in the absence of obesity altered adult hippocampal neurogenesis, producing a higher density of type-1 cells (GFAP\textsuperscript{+}/SOX2\textsuperscript{+}) in the SGZ in
males and females. In the offspring from the Emb groups (males and females), the neurogenesis was not found to be altered by maternal HFD during the preimplantation period. However, there was a significant difference between Emb groups and HFD groups, where the HFD groups have a higher number of type-1 cells. These results suggest that maternal exposure to HFD during pregnancy and lactation leads to an increase in type-1 cells, but not a 3.5-day exposure.

Although the molecular mechanisms underlying the increased number of type-1 remain to be elucidated. Different mechanisms control and regulate adult neurogenesis, which could be related to our data. Notch signalling is highly expressed in the adult hippocampus, mainly because it regulates the reservoir, maintenance and proliferation of undifferentiated NSCs\textsuperscript{630}. Ehm et al. demonstrated that the inactivation of RBPj, a Notch pathway component increased the neurogenesis rate and differentiation of Sox2 positive cells in the hippocampus, generating a depletion of the NSC reservoir pool\textsuperscript{631}. In a maternal obese mouse model caused by an HFD (35% fat), different molecules in the Notch signalling pathway were overexpressed (Notch1, MSI1, and Hes1) in the neonatal NSCs in the offspring\textsuperscript{611}, suggesting that exposure to maternal HFD is sufficient to trigger an early activation of neonatal NSCs. Contrary to these findings, when we analysed Notch1 mRNA hippocampal expression in our animals, in the different diet groups (Figure 5.3.3.4), we did not observe significant differences in males nor females.

It has been described that adiponectin plays an important role not only in energy homeostasis but also in neurogenesis\textsuperscript{632}. Although we did not measure the plasma levels of adiponectin in our animals, here we demonstrate that maternal HFD induced downregulation of AdipoR1 and AdipoR2 genes in males, and AdipoR2 gene in females (more detailed information about AdipoR1 and AdipoR2 analysis see Chapter 3 Figure 3.21). In the hippocampus, \textit{in vitro} studies have shown that adiponectin signalling regulates neurogenesis, increasing proliferation of hippocampal NSCs through activating the p38MAPK/GSK-3β/β-catenin signalling cascade\textsuperscript{633}. In an Adiponectin deficiency mouse model (Adipo\textsuperscript{−/−}, and Adipo\textsuperscript{+/−}), it was observed that the number of proliferating cells was reduced in the dentate gyrus and was restored after intracerebroventricular infusion of adiponectin\textsuperscript{634}. Specific data on the effect of maternal HFD in the absence of obesity on adiponectin and its impact on adult neurogenesis are lacking. We speculate that adiponectin receptors are critical for the activation and proliferation of NSCs, especially AdipoR2 receptor since its mRNA expression was reduced in male and female offspring of HFD fed dams.

A significant finding of the present study is that early exposure to HFD during gestation and lactation decreased adult neurogenesis in the dentate gyrus, but no 3.5-day exposure. Indeed, maternal HFD exposure in males but not in females led to a decrease in the number of the new-mature neuron (DCX-/NeuN+), and downregulation of mature neuron markers (B3-Tub). Our findings indicate that
males in the HFD group were more susceptible to maternal diet compared to females in the HFD group. Males in the HFD group showed an increase in the number of new-born neurons and a decrease in the number of mature neurons. No differences were observed between males and females in the Emb group. Different studies have described that female rats have a higher cell proliferation rate of new neurons than males. This difference in cell proliferation during neurogenesis is dependent on the oestrous cycle phase; for example, the rate is higher during proestrus compared to non-proestrus. One limitation of our work was not knowing the phase of the oestrous cycle in which the females were. Although females generally have a higher proliferation rate, here our data showed that maternal diet during pregnancy and lactation could lead to a greater number of new-born neurons in males and not in females.

Production of new neurons in the dentate gyrus depends on intra- or extracellular factors that promote NSC proliferation. Age-related changes in these factors have been associated with a decline in the production of new-mature neurons like a decrease in the expression of neurotrophic factors such as the brain-derived neurotrophic factor (BDNF), and the insulin-like growth factor-1 (IGF-1). Interestingly, our maternal HFD model led to downregulated Bdnf, Psd95 and Igf1R mRNA levels in male offspring (See result chapter 3 and 4), which is common in the ageing brain. We cannot state whether maternal HFD causes overall ageing in the mouse brain (we did not evaluate ageing in this thesis). Still, it would be interesting to examine the effect of maternal HFD during the juvenile/adolescent period in the offspring, and its impact on hippocampal neurogenesis.

One of the effects of our maternal HFD was the increased mRNA level of apoptotic markers (Caspase-3 and Bax) in the hippocampus. In HFD-fed male mice study, the researchers found a significant increase in the number of TUNEL-positive cells (considered apoptotic cells) in the hippocampus of this animals. These data from Song et al. might indicate that in our model, a portion of immature neurons exits the neuronal proliferation phase via cell death, decreasing the number of mature neurons in the HFD male group, but we do not know yet which cells may be dying.

In this study, microglial cell density was higher in the dentate gyrus of HFD males (See chapter 4), suggesting that HFD during gestation increased microglia in the adult offspring, confirming the results obtained by different studies. Microglia regulate neurogenesis through phagocytosis of the excess of new-neurons in the dentate gyrus. Most of the new cells in the adult dentate gyrus do not survive beyond the first week, and this is mostly due to apoptotic cell death, whose debris is cleared by the microglia. Microglia cells can regulate the neurogenic niche, mediating the maturation of new-mature neurons by pruning synaptic contacts between them and old neurons.
Interestingly, we found in males a significant negative correlation between microglia and mature neurons in the dentate gyrus ($r=-0.4702, p=0.0489$), supporting the idea that the decrease in neurogenesis may be the result of a disruption in microglial surveillance capacity mediated by the maternal HFD. However, the analysis of phagocytic microglia is necessary to understand better if the decrease in the maturation of new neurons may be the result of excessive cell death.

### 5.5 Conclusion

Here, we provide data supporting the hypothesis that neurogenesis is altered by the exposure to maternal HFD (without causing obesity) during gestation and lactation and not during the preimplantation period only. The findings presented here showed that maternal HFD during pregnancy and lactation caused changes in the stem cell reservoir and decreasing the number of mature neurons. We suggest that microglia might be essential in the modulation of neurogenesis, and maternal HFD may modify their role. Overall, our findings highlight the importance of maternal nutrition on early-life stages and hippocampal neurogenesis.
Figure 5.19. Schematic diagram of overarching results in terms of neurogenesis from Chapter 4.
Overall effects of maternal HFD on the offspring in terms of neurogenesis in the dentate gyrus and mRNA levels of B3-Tub and apoptotic markers in the hippocampus (hippo) at 26 weeks of age.
Chapter 6. **General Discussion**

### 6.1 Introduction

Obesity is a global problem affecting developed and developing countries, and it is estimated that by 2025 more than 21% of women in the world will be obese\(^6\). Maternal nutrition is essential for the proper development of the fetus. Maternal obesity before and during pregnancy has been described as a factor involved in various pregnancy complications, including diabetes, pre-eclampsia, and large-for-gestational-age infants\(^5\). However, the adverse effects of obesity during pregnancy are not only limited to the mother but also extend to the offspring, increasing the risk of developing noncommunicable diseases later in life in the offspring\(^1\).

Maternal obesity seems to affect the neurological development of the new-born\(^4\). The first 1,000 days of life, which correspond to the time between conception and the second year of life, is a critical period for optimal neurocognitive development\(^5\). Therefore, improving maternal nutrition in the prenatal period and during breastfeeding will help reduce the rates of psychiatric and psychological disorders\(^6\).

Early nutritional intervention in the mother promises a long-term contribution to adequate neurological development in the offspring, however, if nutritional needs are unmet in this period, this could irreversibly affect neurological development. This situation has led governments to pay more attention to the impact that obesity in women of reproductive age has on the health of the mother and child, and the importance of developing strategies to address the problem\(^6\). Thus, deciphering effect of diet independently of obesity, which might bring more knowledge of the mechanisms of the diet effects rather than having diet and obesity effects mixed in an obesity model. Therefore, the study of the impact of the maternal diet during pregnancy and lactation is of great relevance, since it would help to understand the different aspects of brain development in the offspring.
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6.2 Overview of findings

The broad aim of this thesis was to evaluate the effects of maternal HFD without obesity, on metabolic-related parameters, brain physiology and neurogenesis in the offspring. The first major new finding of this thesis is that the maternal HFD in the absence of obesity, generates metabolic changes without modifying the body weight in the offspring. We observed an increase in the daytime activity and increase in the respiratory quotient in the females of the HFD group. In HFD males, although the activity was not altered, we observed a decrease in oxygen consumption, carbon dioxide production and energy expenditure. The mRNA expression analysis showed alterations in the receptors of essential molecules associated with energy metabolism in the brain and the liver. In the liver of HFD males we observed an increased in the expression of the leptin receptor, and in the cortex and the hippocampus a decreased expression of glucose transporters, and leptin and insulin receptors. In the HFD females we observed that in the cortex and hippocampus the mRNA levels of the glucose transporters, and leptin receptor were reduced, but not of the insulin receptor.

The second new finding is that the maternal diet modifies the cellular density of glial cells (astrocytes and microglia) in the cortex and hippocampus, especially in the offspring of mothers fed with HFD. These findings were showing the same trend in males and females. One of the most interesting results was the increase in microglia cell density on post-natal day 1 and in adulthood, however, we did not find significant changes in the expression of inflammatory markers in adults.

Finally, in this project we demonstrate how sensitive the hippocampus can be and the processes that occur there (neurogenesis) to the maternal diet. Here we describe in the adult males of the HFD group, the increase in the number of radial glial cells, and decrease in neuronal maturation. This highlights the importance of proper nutrition during pregnancy and breastfeeding for an adequate brain physiology.
6.3 Maternal HFD during pregnancy and lactation impairs metabolism

Our study shows that the offspring developed metabolic disturbances in adulthood after exposure to maternal HFD in the absence of obesity even during a short period (3 days after conception) and during pregnancy and lactation. Here we describe that 45% of calories from fat during gestation and lactation is not enough to generate obesity neither in the mother nor the offspring. This data is in line with a rat model using a similar maternal HFD (45% of fat) during gestation and lactation, without causing obesity in the dams.

Additionally, we found that maternal HFD in the Emb and HFD groups did not alter the offspring's body weight at week 26 of age. These data are very similar to other models where HFD replaced the maternal diet before or after mating. At week 26 of age, no changes in locomotor activity were observed in HFD or Emb males. In HFD females, an increase in activity was observed during the day. Although the biological mechanism behind this increase in daytime activity in females is unclear, various studies have linked maternal obesity caused by HFD and increased locomotor activity. These results could suggest that females of mothers exposed to HFD during the day are more hyperactive, which could be extrapolated to a hyperactivity phenotype in children of obese mothers in humans.

Maternal HFD affected the volume of oxygen consumed and the carbon dioxide produced during the day and night in males of mothers who were fed HFD during pregnancy and lactation. It is interesting to note that the offspring (males and females) of mothers fed with HFD during the preimplantation period did not show changes in the \( \text{vO}_2 \) and \( \text{vCO}_2 \). This data indicates that maternal exposure to an HFD during pregnancy and lactation can modify respiration in the adult male offspring, but not the exposure during the preimplantation period. Lower oxygen consumption has been observed in humans and animal models either by the failure of the heart to maintain cardiac output and \( \text{O}_2 \) or by mitochondrial mutations.

Recently, a maternal HFD obese model (45% fat, before and during gestation and lactation), showed reduction in mitochondrial electron transport chain enzyme complex activity in the liver in the offspring at 15 weeks of age. Another recent study supports the link between decreased \( \text{vO}_2 \), mitochondrial function, and HFD. In this study, mice were fed an HFD containing 56% fat, and they had mitochondrial dysfunction in the liver, decrease in beta-oxidation and decrease in oxygen consumption. These two studies suggest that HFD can directly modify mitochondrial function, and therefore alter respiration. Although in our study, we did not evaluate mitochondrial function in any tissue, it is possible that in utero environment leads to epigenetic modifications during
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Embryonic development (such as mitochondrial DNA methylation, mitochondrial DNA copy number) causing persistent changes to mitochondrial activity.$^{654}$

Interestingly, the results obtained indicated that, when analysing the RQ, the males and females of the HFD group showed higher values than the NFD group ($\geq 1.0$). The males and females of the Emb group showed increased values in some parts of the day, but not at night. The RQ indicates which macronutrients are being metabolised, with a value of 0.7 indicating lipids are being metabolised, 0.8 for proteins, and 1.0 for carbohydrates.$^{472}$ The data suggest that maternal HFD might drive the offspring in the HFD group to utilise carbohydrates other than lipids as a source of energy. This could confirm our observations of the vO$_2$ and vCO$_2$ analysis by suggesting that the changes observed could be a consequence of impaired mitochondrial function. Therefore, it is necessary to determine the efficiency of the metabolism in these animals, for example by calculating the amount of ATP produced relative to the oxygen consumed in different regions in the brain, since glucose metabolism relates to oxygen consumption.$^{655}$

The analysis of energy expenditure showed that females belonging to the Emb group had higher values of energy expenditure during the first part of the day and those of the HFD group in the second part of the night. Multiple studies have proved that increasing energy expenditure is an effective way to prevent obesity.$^{656,657}$ Although in our model we did not observe obesity in the dams or offspring, it is important to highlight that the energy expenditure was high in the Emb and HFD females, which could suggest a compensatory effect that led to the non-generation of obesity in them. Energy expenditure in males in the HFD group had lower values compared to the NFD group, but not in the Emb males. HFD models have described that this diet reduces energy expenditure, due to a positive energy balance resulting from a decrease in energy expenditure relative to energy intake.$^{658,659}$. Choi et al. described that the reduction in energy expenditure induced by HFD might be caused by the down-regulation of the expression of genes involved in catabolism and oxidation of fatty acid, and genes controlling energy transduction pathways in the mitochondria.$^{659}$ These different studies support the idea that maternal dietary composition could affect mitochondrial function and alter the different metabolic parameters studied (activity, energy expenditure, vO$_2$ and vCO$_2$). Additional studies that evaluate the mitochondrial function are necessary to elucidate the link between maternal HFD and alterations in respiration in the offspring.

Another factor associated with low energy expenditure is impaired insulin and leptin sensitivity.$^{30,473}$. Leptin and insulin main action is to control energy homeostasis and energy expenditure, mediated by leptin receptor (ObRb) and insulin receptor (InsR)$^{496}$. Hormones like leptin and insulin control energy homeostasis, mainly by increasing energy expenditure mediated by leptin receptor (ObRb) and insulin receptor (InsR)$^{496}$, therefore we focus our attention on these
two receptors in the liver and brain. Firstly, the analysis of leptin receptor (ObRa and ObRb) show that HFD males had higher levels of ObRa mRNA but not ObRb in the liver. There were no changes in Emb males or females compared to the NFD groups. In hepatic cells, leptin binds ObRb to activate the Jak2/Stat3 and PI3K/Akt pathways\(^{501}\), whereas ObRa is unable to activate the Jak2/Stat3 pathway, nevertheless in the liver ObRa mediates leptin-regulated gluconeogenesis\(^{506,507}\). Our data raised the possibility of compensatory upregulation of ObRa expression in hepatic gluconeogenesis, associated with an altered leptin or insulin signal. Further studies are needed to evaluate the protein expression of the ObRa receptor and try to identify the real impact of the upregulation of the mRNA levels of ObRa in the liver.

In the brain, we evaluated to regions the cortex and the hippocampus. The cortex is responsible for processing sensations and sensory information\(^{660}\). The hippocampus regulates different functions such as memory, spatial orientation and neurogenesis\(^{661}\). We observed in the cortex that the mRNA levels of ObRb in HFD males and levels of InsR in Emb and HFD males were downregulated, whereas in Emb females the mRNA levels of ObRa and ObRb in the Emb group were upregulated. In the hippocampus, we observed in HFD males that mRNA levels of ObRa and ObRb were upregulated, but InsR were downregulated. Emb males had higher mRNA levels of ObRb, but mRNA levels of InsR were downregulated. In females, mRNA levels of ObRa and ObRb were downregulated in HFD and Emb females.

Different models of obesity caused by HFD have described that in rodents, hippocampal-dependent learning and memory behaviour is altered, as in models where leptin signalling is affected\(^{662-665}\). Some of these studies have illustrated that in these behavioural deficits, InsR signalling was reduced\(^{663}\). Our data demonstrate a more significant effect on the hippocampus (males and females of the Emb and HFD groups) compared to the cortex. The hippocampus is a region with a great diversity of neural populations that makes the hippocampus an area of high metabolic demand\(^{666,667}\). Here, we observed that the mRNA expression of the ObRa and ObRb was overexpressed and the expression of InsR was decreased, which has led us to suggest that the decreases in InsR mRNA levels could be compensated by the increase in the expression of leptin receptors. However, it is necessary to evaluate protein levels of all these receptors to understand the impact of the HFD maternal diet on leptin and insulin signalling in the hippocampus.

The liver and brain are critical organs for proper energy balance and body metabolism. One of the functions of the liver is to keep plasma glucose levels balanced\(^{475}\). In the liver, glucose is transported into the cells by a set of membrane receptors called glucose transporters (Gluts). To analyse the effect of maternal HFD-induced glucose uptake in hepatocytes, we investigated the liver mRNA
expression of different glucose transporters such as Glut1, Glut3, Glut4 and Glut5. However, we did not observe changes in mRNA levels in the liver in the HFD and Emb offspring.

Different models of HFD have shown opposite results, as HFD-induced obesity leads to higher insulin levels that lead to insulin resistance, triggering the induction of GLUT expression in the liver. Different models of HFD have shown opposite results, as HFD-induced obesity leads to higher insulin levels that lead to insulin resistance, triggering the induction of GLUT expression in the liver. In these studies, HFD intake has a direct effect on mice; besides, a recent article has shown that HFD-induced maternal obesity reduces GLUT expression in the liver in the offspring. These discrepancies could be because maternal HFD in our study was limited to the preimplantation period and gestation and lactation. Possibly alterations in the liver of offspring are caused only if the mother is obese during pregnancy.

On the other hand, the brain uses glucose as the main source of energy, and Gluts facilitate glucose transport across the BBB and the uptake of glucose into neurons and glial cells. In the mouse brain cortex, Glut1 and Glut8 mRNA expression was decreased in HFD males. In the hippocampus, we observed decrease expression of Glut1, Glut3, Glut4, and Glut8 in HFD males and Glut1, Glut3, Glut4, and Glut5 in HFD females. Emb males and females had lower values of Glut3, Glut4, and Glut5. Glucose transporters in the hippocampus seem to be more sensitive to maternal HFD than in the cortex. One possible explanation is that neurons in the hippocampus continually undergo dendritic pruning and reorganisation, which is lower compared to the cortex. Reduction in the levels of mRNA GLUTs in the hippocampus may reflect an increased neuronal pruning, possibly affecting neuronal-glial interactions for nutrient transport.

Glut1 is the most abundant glucose transporter in the brain, is present in endothelial cells and astrocytes, and is essential for healthy brain development. It has been demonstrated that mutations in the GLUT1 gene in humans can cause seizures, intellectual disability, ataxia and dystonia that begin in childhood due to low levels of glucose in the brain. The recent findings of Lee and colleagues revealed that ageing affected the expression of Glut1 when the expression of Glut1 was compared in mice at 18 and 24 months of age, they observed that the expression of Glut1 decreased as the mouse aged. In humans, different studies in Alzheimer's disease brains have shown decreased GLUT1 expression in the cortex and hippocampus. This observation suggests that ageing is a factor that must be taken into account, basically because as we get older, there is a metabolic decline, and a reduction in brain glucose utilisation mainly in men. Unfortunately, in this study, we were not able to compare Glut mRNA levels between the PND 01 and 26 weeks mice. The main reason was that all PND 01 brains were fixed in formaldehyde so we could not extract good quality RNA from this tissue. Although the mice in our study were 26 weeks of age (adults, not aged adults), exposure to maternal HFD may contribute to deficiencies in Glut1 function, and we could speculate to brain ageing in males.
Glut3 expressed in neurons, is one of the most abundant glucose transporters in the brain, and is the main transporter of glucose in the cortex and hippocampus\textsuperscript{514}. Heterozygous Glut3 mutant mice show normal body development and brain size but display cognitive abnormalities\textsuperscript{465,515}. Glut4 and Glut8 are expressed in neurons and are insulin-sensitive glucose transporters, located in selective areas of the brain, such as hippocampus, hypothalamus, and cortex, and Glut5 is expressed explicitly in microglia\textsuperscript{465}. However, the role of Glut8 in the cortex is less known. Schmidt et al.\textsuperscript{513} showed Glut8 was strongly expressed in hippocampus and cortex, compared to other brain regions, highlighting the importance of Glut8 in these areas. In Glut8\textsuperscript{-/-} mice, Glut8 has been associated with neurogenesis (increasing cell proliferation in the dentate gyrus) and hyperactivity\textsuperscript{511-513}.

Here, for the first time, we are showing how maternal HFD decreases Glut8 expression in the HFD male offspring in the hippocampus. Our data showed an increasing number of NSCs and newborn neurons in the dentate gyrus of the HFD males. This data suggests that Glut8 could be associated with the changes we observed in the adult neurogenesis in the hippocampus. To date, the role of Glut8 in the cortex is not clear; future analyses should focus on understanding the role of Glut8 in the cerebral cortex. Hippocampal expression of Glut1, Glut3, and Glut4 was decreased in both sexes. In general, the reduction of these transporters in the hippocampus can modify behaviour, and explicitly learning\textsuperscript{518,519}. Experimental models of maternal obesity caused by a HFD suggest that maternal diet leads to an increased risk of cognitive impairment and behavioural dysfunctions in the offspring\textsuperscript{128}. However, to date, no model correlates reduced expression of glucose transporters (caused by a maternal HFD), and cognitive deficits, or memory impairment in adulthood.

Adiponectin is made exclusively by adipose tissue, and its primary function is to increase insulin sensitivity. There is an inverse correlation between adiponectin levels and obesity\textsuperscript{520,521}. Adiponectin binds to two membrane receptors, AdipoR1 and AdipoR2 that stimulate Glut4 transcription, increasing glucose uptake\textsuperscript{522}. Animals fed an HFD (35\%) for eight weeks showed a reduction of AdipoR1 and AdipoR2 expression in the cortex, and altered neurogenesis in the hippocampus, suggesting that AdipoR1 signalling might be neuroprotective against metabolic insults\textsuperscript{523}. In the present study, the expression of AdipoR2, and AdipoR1, AdipoR2 and Glut4 mRNA decreased in the cortex and hippocampus respectively in the adult HFD offspring. These results may indicate a possible adiponectin signalling alteration that may correlate with insulin resistance; therefore, a future analysis that allows quantifying serum insulin levels is necessary. Finally, for the first time we are showing how a maternal HFD, in the absence of obesity can alter some parameters of the metabolic cage, and the mRNA levels of some glucose transporters and some hormone receptors in the offspring at 26 weeks of age, significant changes are summarized in the following table (Table 6.1).
### Table 6.1 Summary of significant data of the metabolic analysis in the adult offspring.

Only the significant changes observed in both parts of the day or night are shown as day or night. Significantly changes in mRNA levels of glucose transporters and metabolic genes in the cortex (Ctx) and hippocampus (hippo).
6.4 Maternal HFD potentially alters cell composition in the offspring brain in the cortex and hippocampus.

Previous works have reported that maternal obesity caused by HFD might affect hormonal and metabolic pathways, which also elicits changes in the development of the offspring brain\textsuperscript{252,535,536}. Therefore, here we have investigated the influence of maternal HFD on the offspring brain cortex and hippocampus morphology. To explore eventual changes in different cell populations, we have analysed the cell density of neurons, astrocytes, and microglia in the cortex and hippocampus.

In the cortex, the quantification of NeuN positive neurons showed that other cell populations, and not neurons, were altered in the offspring’s cortex. An increase in the number of glial cells (astrocytes and microglia) was seen in both Emb (in some layers) and HFD males and females in the cortex and hippocampus. This result shows that maternal HFD leads to a higher density of astrocytes and microglia in the adult offspring. Importantly, astrocytes are the only cells in the CNS capable of oxidizing β fatty acids\textsuperscript{676,677}. Therefore, a higher concentration of fatty acids in the maternal diet during early life-development could change the function of astrocytes (astrogliosis) affecting their role in energy balance or modifying their cell density\textsuperscript{676,677}. However, the molecular mechanisms that led to an increase in astrocytic cell density have not yet been elucidated.

The data in this project have shown an increase for both astrocytic markers GFAP and S100β within the cortex in the Emb female group (layers 4 and 5), in the HFD female group (layers 1, 2/3, 4, 5 and 6), and the HFD male group (layer 5). In different degenerative and inflammatory brain disorders, increased levels of GFAP and S100β have been observed\textsuperscript{574,576,577}, which led us to postulate the hypothesis that increased levels of GFAP and S100β caused by exposure to a maternal HFD could be involved in the pathophysiology of neurodevelopmental disorders in humans. Epidemiological studies have associated maternal obesity and HFD with an increased risk of mental health and neurodevelopmental disorders, such as attention-deficit hyperactivity disorder (ADHD), and autism spectrum disorders (ASD)\textsuperscript{140,678-681}. For instance, in ASD, it is thought that its pathogenesis initiate during early to mid-fetal brain development\textsuperscript{683}, which in mice corresponds to embryonic day 14.5\textsuperscript{683}. Therefore, new studies are needed to understand better how maternal HFD during the preimplantation period and gestation and lactation alters brain development and function and design experiments to specifically assess mental health (or behaviour) in our model.

Astrocytes are involved in many different processes crucial for brain and cognitive functioning, for example, in synapse development, interacting with both presynaptic and postsynaptic neurons\textsuperscript{578}. Considering that astrocytes play an important role in neural plasticity\textsuperscript{582,583}, we evaluated three markers involved in synaptogenesis PSD95, Synapsin and BDNF. We observed reduced mRNA levels
of BDNF, and PSD95 in the hippocampus in HFD and Emb males and HFD females. In line with these different studies have shown that HFD reduces BDNF protein expression in different brain regions such as the cortex and the hippocampus. BDNF is the most abundant neurotrophin in the brain and is mainly, and low levels of BDNF in the hippocampus decrease spatial learning, synaptic function and cognitive function. PSD95 plays a critical role in organizing the postsynaptic, spatial learning and visual cortical plasticity, and changes in PSD95 expression could suggest an alteration in synaptic function in the hippocampus and possibly spatial learning, synaptic function and cognitive function. It has been reported that HFD reduced the expression of PSD-95 protein in both hippocampus. This idea is supported with data from humans, where changes in protein PSD95 expression has been observed in Alzheimer’s disease, Huntington’s disease, schizophrenia. Future studies in this model are required to explore whether maternal HFD leads to reduction of synaptic protein expression.

Our data demonstrate that the HFD and Emb groups presented higher densities of microglia cells compared to the NFD group, in the cortex and hippocampus. Microglia cells play an essential role in the protection of the CNS. Under physiological conditions, these cells are continually cleaning the waste, modulating synapses, as well as producing cytokines. Different studies have shown that the nutritional environment in the early stages of development influences the maturation of microglial cells, which generates lasting effects on the response to neuroimmune challenges. In line with our results, it has been demonstrated in rodent models, that maternal HFD during gestation was enough to activate microglial cells in the hippocampus of the offspring at birth, as well as increase microglia density in adulthood.

Also microglia regulate neurogenesis through phagocytosis of the excess of new-neurons in the dentate gyrus. In this study, microglia cell density was higher in the dentate gyrus of HFD males, also we found in males a significant negative correlation between microglia and mature neurons in the dentate gyrus (r=-0.4702, p=0.0489), supporting the idea that the decrease in neurogenesis may be the result of a disruption in microglial surveillance capacity mediated by the maternal HFD. However, the analysis of the phagocytic activity of microglia is necessary to better understand if the decrease in the maturation of new neurons might be the result of excessive cell death.

On the other hand, in PND 01 HFD mice (males and females) we found increased microglia in different regions of the hippocampus and cortex, which could indicate a possible activation of the microglia in response to the maternal HFD. Additional experiments are needed to explore whether the microglia at PND 01 HFD or Emb brains are causing neuroinflammation. However, the analysis of specific inflammatory markers did not show significant induction of pro-inflammatory cytokines in the adult offspring, suggesting that maternal HFD is not causing long-term neuroinflammation in
adulthood. Therefore, it is necessary to evaluate neuroinflammation markers and signalling pathways that regulate microglia in the early stages of development in the offspring.

Additionally, we assess in the adult offspring microglial markers for immune activation (CD11b) and phagocytic activity (CD68). Our data show that the analysed microglia did not show changes in the mRNA expression of these markers in the adult brain. However, we were unable to identify CD11b and CD68 markers in the PND 01 brains. This possibly suggests that the increase in the number of microglia cells in the cortex and hippocampus is programmed from pregnancy, and simply remains until adulthood. Another microglia marker is CX3CR1 which is a critical mediator of neuron–microglia interactions. We observed that mRNA levels of Cx3cr1 were changed in the HFD group, in the cortex (up-regulated) and hippocampus (down-regulated). Here, our results in the hippocampus showed an increase in microglia density, similar to the results obtained by Rogers in a Cx3cr1 deficiency mice model. Also, Xu and colleagues observed that hippocampal Cx3cr1 gene expression was up-regulated in a fructose-fed mice model, and the number of microglia cells was increased too. Although in the study by Xu et al., they do not describe whether microglia or the expression of the Cx3cr1 gene were affected in the cortex of the brain, we could suggest that an imbalance in the expression of the Cx3cr1 gene (up or down) leads to altered microglia cell density. However, analysis at the protein level is required to confirm the impact of Cx3cr1 expression in the brain after the maternal HFD exposure in the offspring. Interestingly in our mouse model, we observed an increase in hippocampal microglia and impaired neurogenesis similar to that found in mice with Cx3cr1 deficiency. Finally, when we analyse the mRNA expression of the Csf1r gene was increased in the HFD males, while the hippocampus was decreased in the HFD females, similar to the Cx3cr1 mRNA levels.

Csf1r regulates development, proliferation, maintenance, and survival of microglia, and is involved in neuroinflammation. Csf1r is essential for the proper functioning of the microglia since Csf1r deficiency mice do not survive until adulthood, the cortex has no microglia, and there are problems in the architecture of the brain. Taking into account the crucial role that Csf1r plays in the homeostasis of microglia, and in the brain, the variations observed in the Csf1r mRNA level may be due to an alteration of the transcription factors that regulate Csf1r expression such as PU.1, C/EBP, and PAX5. Future research might be directed towards elucidating the role of CSF1R in brain biology and establishing how the maternal HFD affects the normal function of the microglial markers in early stages of development. Significant changes in the cortex and hippocampus are summarized in the following table (Table 6.2).
Table 6.2 Summary of significant data of the cortex and hippocampal analysis in the offspring.
6.5 Maternal HFD during gestation alters adult neurogenesis

Neurogenesis is the process by which new neurons are formed and has been observed to continue through the life span of most mammals\(^{291,627}\). Neurogenesis comprises different steps (proliferation, differentiation and migration), concluding when the new mature neurons are integrated into existing neural circuits.

In this thesis, we evaluate neurogenesis at two different time points, at birth (PND 01) and adulthood (week 26). In newborns, we analyze hippocampal neurogenesis and in adults hippocampal and lateral ventricle neurogenesis. This difference in the regions was due to the quality of the tissue in the new-borns (PND 01) since the later ventricle was not in good condition, so it was not possible to study these two regions. We evaluated hippocampal neurogenesis in PND 01 mice using GFAP as a marker, but we did not observe significant changes in any of the animals of the Emb or HFD group. Previous studies have described that maternal obesity caused by HFD leads to alterations in neurogenesis in the fetal brain\(^{156,611,612}\). However, opposed to these studies, Tozuka et al. observed no differences in the neurogenesis in the DG at embryonic day E18\(^{613}\). The main difference in the studies mentioned above was the time of exposure to maternal HFD, suggesting that a more prolonged maternal exposure to HFD leads to a more significant difference in offspring neurogenesis. Here, maternal exposure to HFD during pregnancy or the preimplantation period does not lead to alterations in the neurogenesis at PND 01, when GFAP was used as a marker. However, the use of different markers\(^{629}\) of cell proliferation (Nestin, Ki67, phosphorylated histone H3), apoptosis (Caspase-3) and early neuronal differentiation (Calretinin) are required to validate these observations.

Adult neurogenesis is restricted to two specific neurogenic brain niches: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) in the dentate gyrus of the hippocampus. We have conducted a study of the NSC of SVZ in mice at 26 weeks of age to understand how maternal HFD affects neurogenesis in this region. In the V-SVZ in the adult lateral ventricle, we found that maternal HFD does not affect the total number type A (SOX2+/GFAP-), type B (SOX2-/GFAP+), and type C (SOX2+/GFAP+) cells. This data indicates that exposure to maternal HFD during the preimplantation period and gestation and lactation has no long-term effects on the stem cell production in the V-SVZ. A recent study has shown in an HFD-induced obesity mouse model that adult neurogenesis in the SVZ is affected by diet\(^{695}\). Two factors might support our findings; firstly, the offspring were not exposed directly to the HFD and secondly this neurogenic niche is an area with a high rate of cellular renewal, much higher than that described in the hippocampus\(^{293}\). These hypotheses could be the reasons why we did not observe alterations in the adult brain in the Emb and HFD groups. However, further analysis should be performed to provide
an accurate description of whether any of the cellular events involved in the neurogenesis process are altered, such as the formation of new-born neurons or their maturation.

In the adult dentate gyrus, we saw a higher number of type-1 cells or radial glial cells (GFAP+/SOX2+) in the SGZ in HFD males and females, but not in the number of type-2 cells (GFAP-/SOX2+). No differences were observed in the animals exposed to the maternal HFD during the preimplantation period. Although the molecular mechanisms underlying the increased number of type-1 and no changes in the number of type-2 cells remain to be elucidated. We might suggest that there are molecular mechanisms influenced by maternal HFD exposure, which are preventing the activation of radial glial cells, evading maturation and formation of new cells. In line with this idea, it has been described that Notch signalling regulates the reservoir, maintenance and proliferation of NSCs in the hippocampus. Yu and colleagues, using a maternal obese mouse model caused by an HFD (35% fat), observed that different molecules in the Notch signalling pathway were overexpressed (Notch1, MSI1, and Hes1) in the NSCs of the offspring, suggesting that exposure to HFD is sufficient to trigger an early activation of NSCs in the offspring. However, our data indicated that the expression of Notch1 in the hippocampus did not change when compared to the control group, meaning that in our model Notch pathway is not affected by the maternal HFD in the adult offspring since there is no increase in type-2 cells.

On the other hand, in vitro studies have shown that adiponectin signalling increases proliferation of hippocampal NSCs. In contrast, the adiponectin deficiency mice (Adipo−/−, and Adipo+/−) decrease the number of proliferating cells in the dentate gyrus. These studies suggested that adiponectin not only plays an essential role in energy homeostasis but also in neurogenesis. Although we did not measure the plasma levels of adiponectin in our animals, we saw that maternal HFD induced downregulation of AdipoR1 and AdipoR2 genes in males, and AdipoR2 gene in females. Here we suggested that the reduced expression levels of AdipoR1 and AdipoR2 receptors could generate a similar phenotype in our animals (reduced NeuN cells, and changes in DCX) as observed in Adipo−/−, Adipo−/− mice. It is known that adiponectin in the brain is produced by adipose tissue; however, it has been described that the brain can produce adiponectin endogenously. Adiponectin regulates lipid and carbohydrate metabolism, but it has also been reported to influence neurogenesis and synaptic plasticity in the hippocampus. Therefore, the decrease in adiponectin could alter the functioning of the hippocampus, similar to that observed in animal models where BDNF levels were reduced. Further investigations are required to investigate the specific roles of AdipoR1 and AdipoR2 in regulating NSCs.

A significant finding of the present study is that early exposure to HFD during gestation and lactation decreased adult neurogenesis in the male dentate gyrus. Indeed, maternal HFD exposure in males
led to a decrease in the number of new-mature neurons (DCX-/NeuN+), and downregulation of mature neuron markers (B3-Tub), but not to a proliferative status of type-2 cells (GFAP-/Sox2+) in the SGZ. Also, our maternal HFD model led to downregulated Bdnf, Psd95 and Igf1R mRNA levels in male offspring. Decreased neurogenesis has been described during ageing, especially as early as middle age. Ageing studies have illustrated that from the different regulatory processes during neurogenesis (production of new cells from NSCs, neuronal differentiation, and neuronal maturation), the decreased production of new cells or mature neurons is the major problem. This data suggests that declining neurogenesis with age is not the result of stem cell depletion, but instead because intra- or extracellular factors may influence neuronal proliferation and maturation. Downregulation of factors such as BDNF, and the insulin-like growth factor-1 (IGF-1) have been associated with a decline in the production of mature neurons in the ageing brain. We are not saying that maternal HFD causes accelerated ageing in the mouse brain. However, our findings resemble the neurogenesis phenotype during ageing. Therefore, it would be interesting to examine the effect of maternal HFD during the juvenile/adolescent period in the offspring, and its impact on hippocampal neurogenesis.

Different factors such as BDNF and Glut8 have been described as neurogenic regulators in the hippocampus, influencing neuronal proliferation and maturation. BDNF is involved in neuronal differentiation in neurogenesis in the hippocampus. In a BDNF conditional mutant mice, BDNF has been shown to act as an anorectic factor in regulating body weight. BDNF regulates obesity and appears to play a critical role in central energy balance. In our maternal HFD model, we did not observe changes in body weight, but we did see alterations in terms of energy expenditure, hippocampal neurogenesis and reduction of hippocampal BDNF mRNA expression. These data could suggest that in addition to neurogenesis, BDNF could regulate balance energy in the HFD mouse. Another factor is Glut8, which is expressed by hippocampal neurons. Glut8-/- mice have been associated with neurogenesis and hyperactivity since cell proliferation was increased. Here, we are showing an increased number of new-born neurons that could be related to decreased Glut8 expression in the male offspring. However, it is not clear how the absence of Glut8 increased cell proliferation in the dentate gyrus.
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td></td>
<td>Emb</td>
<td>HFD</td>
</tr>
<tr>
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<tr>
<td>New-born neurons</td>
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<td>↑</td>
</tr>
<tr>
<td>Mature neurons</td>
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Table 6.3 Summary of significant data of the adult neurogenesis in the adult offspring after maternal HFD exposure.
DG: Dentate gyrus, GCL: Granular cell layer, SGZ: Subgranular zone, ↑: Increased, ↓: Decreased.

6.6 Limitations

There are some limitations to this work, first, many studies have suggested that maternal HFD and associated obesity could lead to changes in cognitive behaviour, but due to financial constraints, we could not analyse it. To show if our maternal HFD model leads to an impairment of offspring behaviour, an elevated plus maze\textsuperscript{705} and open field\textsuperscript{706} test should be done to evaluate anxiety response to open environment. In order to evaluate depressive-like behaviour Porssolt swim test\textsuperscript{707} should be done, and finally a novel place/object recognition test to evaluate recognition memory\textsuperscript{708}.

Our next limitation was the way our PND 01 mice were fixed and stored. Since many of the brains from these mice showed no positive signal for any of the antibodies used, and even DAPI staining showed some irregularities, when compared to a freshly fixed same-age brain. For this reason, the number of PND 01 samples was lower compared to the number of adult brains, and only a small number of antibodies worked in these tissues. Therefore, being able to perform a new analysis with new mice could explain in greater detail the different changes observed in the cortex and in the hippocampus in adulthood. In addition, to analyse whether at PND 01 the mice presented neuroinflammation and systemic inflammation, using plasma markers or a mesoscale immunoassay panel, would be beneficial.
In the adult mice brains, we also faced many difficulties performing Western blots, since the material we had was limited (amount of cortex and hippocampus tissue available). Therefore, the concentration of total proteins obtained in each brain was low, which represents a challenge when analysing less abundant proteins such as cleaved Caspase3 (abcam, ab49822), doublecortin (abcam, ab18723), Iba1 (wako, 016-20001), and Cx3Cr1 (abcam, ab8021). This could have been improved by having a larger sample size, but we did not have time within this PhD. Another significant limitation in our study was the absence of blood or plasma samples to analyse the level of different hormones such as leptin, or insulin. For all these reasons, we could only perform the mRNA analysis for most of the markers and not a detailed protein expression analysis. Due to the limitation of the brain regions, we could not study the hypothalamus which is a region closely related to activity and metabolism, in order to elaborate a better conclusion about the effect of maternal HFD on the offspring phenotype.

Finally, one of the significant limitations in this thesis is that the stage of the estrous cycle was not examined in the female offspring at the time of the metabolic cage test, since this could affect activity and energy expenditure\(^{709-712}\). However, female mice were housed in the same cages/same conditions until the age of 26 weeks, so we believe it is possible that they have synchronized their estrous cycle\(^{713}\). However, it is necessary for subsequent studies that the analysis of the estrous cycle stage deserves more attention.

### 6.7 Conclusion

Our data showed that the HFD and Emb-HFD groups developed metabolic disturbances in adulthood and had a higher density of astrocytes and microglia cells, in the cortex and hippocampus. Similarly, we observed that the offspring of mothers fed an HFD had a higher density of new-born neurons and a reduced density of mature neurons in the dentate gyrus, all these finding indicate that exposure to a maternal HFD (during gestation or lactation or the preimplantation period) can generate changes in adult neurogenesis (Figure 6.1).

Different studies based on maternal obesity have shown that the nutritional environment influences the maturation of astrocytes and microglial cells. Here, we observe how maternal HFD, in the absence of obesity in both mother and offspring, is capable of inducing changes in cell densities in a similar degree as the maternal obesity, suggesting that an unhealthy diet without causing maternal obesity is causing these changes in the brain of the offspring.

In many cases, we observed a graded response (NFD<Emb<HFD) suggesting a very early induction of diet-induced responses, well before any neural differentiation, with exacerbated effects upon continued HFD challenge. This indicates that not only diet during pregnancy is crucial for proper
glial cell density and neurogenesis, but also the time of exposure is critical to determine the size of the effects in the offspring brain, caused by maternal HFD.

Figure 6.1. Summary of the main findings in this thesis.
6.8 Future work and implications for offspring health

Throughout this thesis, we have described the impact that the HFD has during pregnancy and lactation on the neurological development of the brain. Although here we have only globally described the consequences of maternal HFD on the cortex and the hippocampus of the offspring, it is clear that much more work is needed to understand the molecular mechanisms that led to such modifications. Such future work would help to propose new interventions in the mother and/or offspring that rescue the phenotype in the offspring.

Although this project has shown clear changes in the adult brain, it would be interesting to evaluate different time points in the embryonic and postnatal development of the mouse. In this way, we could generate a more precise conclusion about the consequences that the exposure to a maternal HFD has in the development of the mouse brain, so we would need to collect brains from mice at three-time points, at embryonic day 17.5 (once the formation of the BBB is completed\(^ {14} \)), at postnatal day 1 (after delivery), and after weaning (longest time of exposure to maternal HFD). These brains might be analyzed with different markers:

- **Cortex and hippocampus:** NeuN, Map2, NeuroD or doublecortin (immature neuronal markers).
- **Cortex:** GFAP and Vimentin staining.
- **Lateral ventricle region:** GFAP and SOX2 staining.

Here we do not observe changes in the number of neurons in the cortex. We do observe changes in astrocytes and microglia. Because the interaction of astrocytes and microglia can change the dendritic morphology and spine number\(^ {15} \), it would be interesting to see if the maternal diet is contributing to these changes. We observed that the mRNA expression of some markers related to neuronal plasticity was altered, electrophysiology experiments would give us some information about any alteration in neurotransmitters. Furthermore, an analysis aimed at identifying and quantifying changes in specific cell subtypes in the cortex would help to understand if another type of cell is involved in increasing cell density. It is required to analyse the number of interneurons using somatostatin, parvalbumin, and neuropeptide Y. It would also be useful to quantify at protein level GAD65 (the 65 kDa isoform of the enzyme glutamate decarboxylase, which catalyses the formation of GABA from glutamate), GAD67 (the 67 kDa isoform of the enzyme glutamate decarboxylase), and vGLUT (vesicular glutamate transporter) for glutamatergic neurons.

In order to understand the impact of maternal HFD on the generation of the various cell populations in the offspring brain, neural stem cells (NSCs) are excellent candidates to investigate how HFD could influence the production of neurons and glial cells. The culture of neurospheres derived from neural progenitor cells in two different time points (E17.5 and P1) from fetus and pups from the
different maternal diet groups will allow us to explore in vitro how NSCs respond to an HFD or NFD. To develop this objective, the following procedures could be performed:

- Obtainment of mouse offspring (females and males) at the age of E17.5 and P1, in order to correlate with the staining at the same age.
- Neocortices collection for future cell dissociation.
- Cell culture in media in order to obtain neurospheres.
- Neurosphere dissociation to get isolated cells that can be characterized by FACS to differentiate the heterogeneous population of cells.

Considering that our data do not show neuroinflammation in the cortex and hippocampus after exposure to a maternal HFD in the adult offspring. We wonder whether a second dietary (HFD 45% fat) challenge in adulthood could lead to a poorer metabolic profile and trigger an aggressive immune response in the offspring’s brain.
Appendix A  Normal Fat Diet

This diet was designed by Special Diet Services and is property of the company.

### Calculated Analysis

#### NUTRIENTS

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</tr>
<tr>
<td>Linolenic (g)</td>
<td>0.47</td>
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#### Amino Acids

<table>
<thead>
<tr>
<th>Component</th>
<th>Total</th>
<th>Supp (%)</th>
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<tbody>
<tr>
<td>Arginine (g)</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>Methionine (g)</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>Cystine (g)</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine (g)</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>Threonine (g)</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>Isoleucine (g)</td>
<td>0.54</td>
<td></td>
</tr>
<tr>
<td>Valine (g)</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>Tyrosine (g)</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>Lysine (g)</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>Asparagine (g)</td>
<td>0.87</td>
<td></td>
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</tbody>
</table>

### Rat and Mouse No.1 Maintenance

- **β-Carotene (g)**: 0.06 mg/kg
- **Rat (g)**: 2 mg/kg
- **Vitamin A (g)**: 800 μg/kg
- **Cholecystokinin (g)**: 150 μg/kg
- **Vitamin D (g)**: 300 μg/kg
- **Vitamin E (g)**: 80 μg/kg
- **Vitamin B6 (g)**: 600 μg/kg
- **Vitamin B12 (g)**: 100 μg/kg
- **Vitamin B1 (g)**: 600 μg/kg
- **Vitamin C (g)**: 2000 mg/kg
- **Folic Acid (g)**: 250 mg/kg
- **Nicotinic Acid (g)**: 250 mg/kg
- **Pantothenic Acid (g)**: 250 mg/kg
- **Choline (g)**: 2000 mg/kg
- **Inositol (g)**: 2000 mg/kg
- **Biotin (g)**: 100 mg/kg

### Notes

1. All values are calculated using a mixture base of 10%.
2. Specific nutrient levels will range between 95-105%.
3. The standard for Vitamin A does not detect β-carotene.
4. The standard for β-carotene is equivalent to 1.6 x Vitamin A activity.
5. The standard for Vitamin A does not detect β-carotene.
6. These nutrients coming from natural raw materials such as corn may have low availability due to the interactions with other components.
7. Based on in-vivo digestibility analysis.
8. AFE Energy = Atwater Fuel Energy (kcal) x %ME x 0.9225.
9. Supplemented nutrients from manufacturer and other sources.
10. Calculated.
## Appendix B  High Fat Diet

This diet was designed by Special Diet Services and is property of the company.

### CALCULATED ANALYSIS:

<table>
<thead>
<tr>
<th>PROXIMATES</th>
<th>FRESH</th>
<th>10% H2O</th>
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<tbody>
<tr>
<td>CRUDE OIL</td>
<td>22.56</td>
<td>21.26</td>
</tr>
<tr>
<td>CRUDE PROTEIN</td>
<td>23.04</td>
<td>21.72</td>
</tr>
<tr>
<td>CRUDE FIBRE</td>
<td>4.60</td>
<td>4.34</td>
</tr>
<tr>
<td>ASH</td>
<td>4.17</td>
<td>3.93</td>
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<tr>
<td>NFE</td>
<td>39.82</td>
<td>37.53</td>
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<table>
<thead>
<tr>
<th>ENERGY</th>
<th>Kcal/kg</th>
<th>MJ/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF ENERGY</td>
<td>4.54</td>
<td>4.26</td>
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<td>AF ENERGY</td>
<td>19.00</td>
<td>17.90</td>
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<table>
<thead>
<tr>
<th>FATTY ACIDS</th>
<th>FRESH</th>
<th>10% H2O</th>
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<tbody>
<tr>
<td>C14:1 MYRISTOLEIC</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>C16:1 PALMITOLEIC</td>
<td>0.09</td>
<td>0.08</td>
</tr>
<tr>
<td>C18:1 W9 OLEIC</td>
<td>6.30</td>
<td>5.94</td>
</tr>
<tr>
<td>C18:2 W9 LINOLEIC</td>
<td>3.10</td>
<td>2.92</td>
</tr>
<tr>
<td>C18:3 W3 LINOLENIC</td>
<td>0.35</td>
<td>0.33</td>
</tr>
<tr>
<td>C20:4 W6 ARACHIDONIC</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>C22:5 W3 CLUPANODONIC</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>C12:0 LAURIC</td>
<td>0.09</td>
<td>0.08</td>
</tr>
<tr>
<td>C14:0 MYRISTIC</td>
<td>0.44</td>
<td>0.41</td>
</tr>
<tr>
<td>C16:0 PALMITIC</td>
<td>4.08</td>
<td>3.85</td>
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<tr>
<td>C16:0 STEARIC</td>
<td>1.89</td>
<td>1.76</td>
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<table>
<thead>
<tr>
<th>AMINO ACIDS</th>
<th>FRESH</th>
<th>10% H2O</th>
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</thead>
<tbody>
<tr>
<td>ARGinine</td>
<td>0.73</td>
<td>0.69</td>
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<tr>
<td>LYSine</td>
<td>1.46</td>
<td>1.36</td>
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<tr>
<td>METHIONINE</td>
<td>0.57</td>
<td>0.54</td>
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<tr>
<td>CYSTINE</td>
<td>0.45</td>
<td>0.42</td>
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<td>TRYPtopHAN</td>
<td>0.20</td>
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<td>HISTIDINE</td>
<td>0.53</td>
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<tr>
<td>THRONEINE</td>
<td>0.80</td>
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<td>ISOLEUCINE</td>
<td>1.16</td>
<td>1.09</td>
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<tr>
<td>LEUCINE</td>
<td>1.76</td>
<td>1.66</td>
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<tr>
<td>PHENYLALANINE</td>
<td>0.96</td>
<td>0.90</td>
</tr>
<tr>
<td>VALINE</td>
<td>1.39</td>
<td>1.31</td>
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**AMINO ACIDS**

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>FRESH</th>
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<tbody>
<tr>
<td>GLYCINE</td>
<td>0.91</td>
<td>0.86</td>
</tr>
<tr>
<td>ASPARTIC ACID</td>
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<td>1.23</td>
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<tr>
<td>GLUTAMIC ACID</td>
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<td>3.55</td>
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<tr>
<td>PROLINE</td>
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<td>1.49</td>
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<tr>
<td>SERINE</td>
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<td>0.62</td>
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<tr>
<td>ALANINE</td>
<td>0.74</td>
<td>0.70</td>
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**MINERALS & TE'S**

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<thead>
<tr>
<th>Mineral</th>
<th>FRESH</th>
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<tr>
<td>Ca</td>
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<td>0.57</td>
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<tr>
<td>P TOTAL</td>
<td>0.23</td>
<td>0.22</td>
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<tr>
<td>Na</td>
<td>0.16</td>
<td>0.15</td>
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<tr>
<td>Cl</td>
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<tr>
<td>K</td>
<td>0.61</td>
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<td>Mg</td>
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<td>0.08</td>
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<tr>
<td>Fe</td>
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<td>55.14</td>
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<tr>
<td>Cu</td>
<td>6.76</td>
<td>6.26</td>
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<tr>
<td>Mn</td>
<td>13.30</td>
<td>12.54</td>
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<tr>
<td>Zn</td>
<td>71.75</td>
<td>67.62</td>
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<tr>
<td>Co</td>
<td>0.00</td>
<td>0.00</td>
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<tr>
<td>I</td>
<td>254.84</td>
<td>240.19</td>
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<tr>
<td>Se</td>
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**VITAMINS**

<table>
<thead>
<tr>
<th>Vitamin</th>
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<tr>
<td>VITAMIN A</td>
<td>4936.44</td>
<td>4652.63</td>
</tr>
<tr>
<td>VITAMIN D3</td>
<td>7836.60</td>
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<tr>
<td>VITAMIN E</td>
<td>97.31</td>
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<tr>
<td>VITAMIN B1</td>
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<td>VITAMIN B3</td>
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<td>VITAMIN B12</td>
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<td>VITAMIN C</td>
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<td>VITAMIN K</td>
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<tr>
<td>ASCORBIC ACID</td>
<td>36.65</td>
<td>34.54</td>
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</tbody>
</table>

This information is intended as a guide only. For actual data we recommend that analysis work is carried out to confirm the nutrient parameters precisely.
List of References


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73. Kalyanaraman B. Teaching the basics of redox biology to medical and graduate students: Oxidants, antioxidants and disease mechanisms. Redox Biol 2013;1:244-57.


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418. Park HK, Ahima RS. Physiology of leptin: energy homeostasis, neuroendocrine function and metabolism. Metabolism 2015;64(1):24-34.


431. . BIOINFORMATICS.
308

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657. Garrow JS. Energy imbalance in obesity is not "just a hypothesis". *BMJ* 2013;346:f3079.


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