UNIVERSITY OF SOUTHAMPTON

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Human Development and Health

The effect of maternal and postnatal obesity on offspring anxiety and memory and the role of altered HPA axis function and neuroinflammation

By

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ABSTRACT

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THE EFFECT OF MATERNAL AND POSTNATAL OBESITY ON OFFSPRING ANXIETY AND MEMORY AND THE ROLE OF ALTERED HPA AXIS FUNCTION AND NEUROINFLAMMATION

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Poor maternal nutrition during pregnancy is detrimental to fetal development and adversely affects long-term health by increasing the risk of chronic diseases, such as neuropsychiatric disorders. Previous studies show that maternal obesity may influence offspring behaviour such as anxiety/stress in adult life. This may be due to altered development of the hypothalamo-pituitary-adrenal (HPA) axis. This may be exacerbated by obesity in adult life which independently influences behaviour and HPA axis function. The mechanisms behind the effect of obesity to impact neuropsychiatric disorders is unclear, however increased inflammation found in obese individuals may be inducing permanent changes to HPA function. This study investigated the effects of maternal and postnatal obesity on behaviour, HPA axis function in young and mature adult mouse offspring, and assessed neuroinflammation as a potential mechanism.

In this study, female C57BL/6 mice were fed either an obesogenic high-fat diet (HF; 45% kcal fat) or control diet (C; 7% kcal fat) 6 weeks before mating, throughout pregnancy and lactation. Offspring were fed C or HF diet from weaning onwards. Maternal care and pup anxiety were assessed on postnatal day 7 via pup retrieval and ultrasonic vocalisations (USVs) during maternal separation. In 15 and 52 week-old offspring, anxiety was assessed by open field (OF) and elevated plus maze (EPM) and memory was assessed by novel object recognition (NOR). Corticosterone and ACTH concentrations (basal and area under curve [AUC]) were measured during a 30 minute restraint test. Analysis of neuroinflammation was performed via immunohistochemistry and mRNA levels of genes associated with HPA axis function and inflammation.

A maternal obesogenic HF diet was associated with poor maternal care and anxiety in males from 1 week of age, and subtle changes to anxiety persisted into young and mature adulthood. Postnatal obesity was associated with decreased and increased anxiety at 15 and 52 weeks of age respectively, and memory was impaired at 15 but not 52 weeks of age in males. Changes in anxiety and memory were associated with HPA dysregulation and microglial activation in the brain at 15 but not 52 weeks of age in males. In female offspring, changes in anxiety and memory were only observed at 52 weeks of age due to postnatal and maternal obesity respectively. Anxiety, but not memory, in females corresponded to changes in HPA regulation, but not inflammation at this age.

Maternal obesity, in addition to further postnatal obesity, subtly exacerbates some effects of anxiety and the stress response which is seen primarily in male, but not female, offspring at multiple ages. Overall, the effect of maternal obesity is sex-specific and age-dependent. These data are a novel addition to the existing literature on the effects of maternal obesity on HPA axis function and behaviour, particularly due to the additional assessment of further postnatal obesity. Further analysis of the role of inflammation during obesity at different stages of the life course will enhance our understanding of the risk of neuropsychiatric disorders to future generations.

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I confirm that:

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Definitions and Abbreviations

11-βHSD	11-beta hydroxysteroid dehydrogenase system
ACTH	Adrenocorticotropic hormone
ADHD	Attention deficit hyperactivity disorder
ARC	Arctuate nucleus
AU	Arbitrary units
AUC	Area under the curve
BBB	Blood brain barrier
BMI	Body mass index
C	Chow (control) diet
CA	Cornu ammonia
CAR	Cortisol awakening response
CBG	Cortisol binding globulin
CD	Cluster of Differentiation
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
CORT	Corticosterone
COV	Coefficient of Variation
CRFR	Corticotropin-releasing factor receptor
CRH	Corticotropin-releasing hormone
CRP	C-reactive protein
CT	Cycle threshold
DG	Dentate Gyrus

Definitions and Abbreviations

DI	Discrimination index
DOHaD	Developmental Origins of Health and Disease
E	Embryonic day
ELISA	Enzyme-linked immunosorbent assay
EPM	Elevated Plus Maze
FCγR1	Fc gamma receptor 1
FKBP51	FK506 binding protein 51
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Geomean	Geometric mean
GFAP	Glial fibrillary acidic protein
GOI	Gene of interest
GR	Glucocorticoid receptor
HF	High-fat
HKG	Housekeeper gene
HPA	Hypothalamic pituitary axis
Iba-1	Allograft inflammatory factor-1
IF	Immunofluorescence
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
LGA	Large for gestational age
LPS	Lipopolysaccharide
MC2R	Melanocortin 2 receptor
MR	Mineralocorticoid Receptor

MRAP	Melanocortin 2 receptor accessory protein
mRNA	Messenger ribonucleic acid
NEC	No Enzyme Control
NOR	Novel Object Recognition
NTC	No Template Control
OCT	Optimal cutting temperature
OF	Open Field
P	Postnatal day
PVN	Paraventricular nucleus
qPCR	Quantitative polymerase chain reaction
RT	Room temperature
SEM	Standard Error of the Mean
TBS	Tris-buffered Saline
TNF	Tumour Necrosis Factor
VMH	Ventromedial hypothalamus
VZ	Ventricular zone
WHO	World Health Organisation
Wk	Week

Chapter 1 General Introduction

1.1 Overview

There is an increasing prevalence of neuropsychiatric disorders, including anxiety and depression, worldwide. These disorders stem from changes in nervous system and normal brain function. Early life environment, such as appropriate maternal nutrition, is essential for offspring health and wellbeing throughout life. A poor maternal environment is associated with long-term health risks in adult offspring, such as cardiovascular disease, diabetes, and psychiatric disorders. In this thesis I examine maternal obesity as a poor early life environment due to its association with increased morbidity and mortality in both mother and fetus. Maternal obesity is a rapidly growing health concern worldwide and the consequences of prenatal over-nutrition are poorly understood. Obesity itself is characterized by chronic low-grade systemic inflammation, which may be able to cross the placenta and induce fetal developmental programming. The effect of obesity in postnatal life is well documented, with health concerns ranging from diabetes to cancer, however associations with mental disorders such as anxiety are still being investigated.

Hypothalamo-pituitary-adrenal (HPA) axis function is a key regulator of the stress response in an individual and thus anxiety. The HPA axis reacts to stressors by generating a cascade of hormonal signals and increasing glucocorticoid production, which is normally self-regulating due to negative feedback mechanisms. Any alterations in the functioning of this system can cause stress response to be altered, including behavioural modifications. It should also be noted glucocorticoids are potent inhibitors of inflammatory processes. Current research suggests increased neuroinflammation is able to influence HPA axis function and associations between prenatal inflammatory stress and long-term HPA axis programming have been found.

Rodent studies have found associations between prenatal obesity, induced by high-fat diet, and offspring behavioural deficits, neuroinflammation and stress response, however results differ across these studies (potentially due to variable experimental conditions), and the mechanisms linking these outcomes are unclear. Research into the effects of both maternal and postnatal obesity combined and their potential interaction on offspring outcomes is also lacking. This thesis will investigate the impact of high-fat induced maternal obesity on offspring stress, inflammation and behaviour and aims to elucidate mechanisms that may link them. In addition, the effect of postnatal obesogenic high-fat diet will also be studied alone and following maternal high-fat feeding. Outcomes from this study may help to contribute to the growing knowledge of how early life environment can have a long-term impact on offspring health and behaviour.

1.2 Neuropsychiatric Disorders

Neuropsychiatric conditions are mental health disorders that affect cognition and behaviour and are the result of brain dysfunction. There are a range of neuropsychiatric diseases including schizophrenia, depression and anxiety, all of which place a burden on society and impair the quality of life of the those affected, as well as their ability to learn and work. Neuropsychiatric disorders can occur from childhood which then is a predictor of adulthood disorders (1). The prevalence of neuropsychiatric disorders are increasing and there are estimates that approximately 38.2% of the EU population suffers each year from a mental disorder (2). The most frequent disorders are anxiety (14%), insomnia (7%), major depression (6.9%) and attention deficit hyperactivity disorder (ADHD) in the young (5%). In terms of disability, measured by disability-adjusted life years (DALY), neuropsychiatric disorders in the EU were found to contribute 27% of total all cause burden in the adult population, which is a greater proportion compared to other regions worldwide (3). Currently less than one third of all cases receive treatment, which could indicate a lack of self-awareness of disease/seriousness of disease, the stigma attached to mental disorders, as well as lack of specialist medical training to deal with mental health disorders. Indeed in the UK alone a recent survey by the Care Quality Commission (CQC) into mental health services revealed that 39% of NHS trusts and 23% of independent services were rated as requiring improvement in 2017 (4). A better way forward would be prevention entirely, however this first requires understanding the underlying causes of neuropsychiatric disorders.

The focus of this thesis is anxiety disorders, which are amongst the most prevalent mental disorders in the developed world (5). In humans, anxiety can present itself in many ways such as fear, excessive worry, tension, fatigue and apprehension. The Diagnostic and Statistical Manual of Mental Disorders classifies anxiety disorders as: panic disorder, specific phobias, social phobia, generalized anxiety disorder, obsessive compulsive disorder and stress disorders such as post-traumatic stress disorders (6). In animal studies behaviour can be assessed using well-established tests such as the elevated plus maze (EPM). It is well established that stress/anxiety and memory are linked, and an increase in anxiety is able to impair memory and learning (7). The impact of anxiety on public health is significant considering that it increases morbidity, mortality and general quality of life (8, 9).

Currently, the causes and influences of mental health disorders, including anxiety, have not been fully elucidated. Various factors have been found to influence mental disorders such as psychological trauma/stress (10), traumatic brain injury (11), genetic predisposition (12), infection (13), substance abuse (14) and adverse life events (15). There is growing evidence that the

maternal environment also influences neuropsychiatric disorders. Adverse environments include maternal exposure to psychological stress or trauma, infection and substance abuse (such as alcohol and drugs), as well as diet and body composition (16-18).

1.3 Developmental Origins of Health and Disease (DOHaD)

In 1989 a seminal paper was published by Dr David Barker on British adults linking low birth weight to adult male offspring elevated systolic blood pressure and cardiovascular disease risk (19) (change to growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease). This paper was closely followed by another study in the same year by Barker showing an inverse relationship between birth weight and death rate of ischaemic heart disease(20). What followed was a new research field, the Developmental Origins of Health and Disease (DOHaD). Since then, numerous studies in humans have linked changes in fetal development to a response to altered early environment with a wide range of diseases, including osteoporosis (21) and type II diabetes (22) as well as neuropsychiatric disorders such as depression and anxiety (23, 24). Animal models have helped to confirm these relationships and provide insight into the mechanisms involved (25-27).

During fetal development there are critical periods of developmental plasticity, wherein the fetus is able to adjust its growth trajectory or make subtle physiological adaptations to changes in the prenatal environment (28). This adaption may be beneficial for improved chance of survival in early life, however prediction of postnatal environment can be detrimental in the long-term and compromise adult health (29). The programming of adult pathology due to prenatal insults is likely the consequence of reduced functional capacity in key organs, a “thrifty” phenotype, where resources are allocated for critical organs (e.g. the brain) at the expense of other organ systems thus increasing vulnerability of these organs to adverse environmental influences in later life (30). Findings in the past decade suggest a “second hit” hypothesis as an explanation for some adult onset diseases (31-33). This hypothesis suggests that a maternal ‘insult’ may induce alterations in prenatal environment that may not be sufficient to alter the adult phenotype. However, a further ‘insult’ (such as adverse stressors) in postnatal life may act as a ’second hit’ which through activational effects may reveal or amplify the underlying defects culminating in disease states.

A key factor that has previously been shown to influence non-communicable disease, including neuropsychiatric disorders, is maternal nutritional intake. Key studies on the effect of the Dutch Famine of 1944-1945, wherein the Nazi occupation of Amsterdam initiated a 5 month period of famine, demonstrated that a poor diet during pregnancy can influence long-term mental and

general health of their children (34). The supplies of rations during the occupation were 400-800 calories mainly consisting of bread and potatoes and this was supplied to all female residents regardless of social class. Immediately after the occupation food was plentiful again, allowing for insight into the effects of malnutrition at critical periods of pregnancy. As detailed medical records were kept during this time period, it was found that in addition to a greater risk of adult obesity (35), there was also an effect on neural outcomes such as an increase in congenital neural defects, and an increased risk of schizophrenia in adulthood (34) (which was particularly due to malnutrition in early gestation (36)). This study showed the importance of maternal nutrition during pregnancy for long-term mental health.

While early studies like those of the Dutch Famine focussed on undernutrition and the consequences of this on offspring have been well investigated, more attention is starting to be paid to that of overnutrition. The prevalence of obesity has rapidly risen worldwide in recent years. As obesity increases, so too does obesity in pregnancy and this may be affecting long-term health of offspring. In 2007 the developmental over-nutrition hypothesis suggested a link between the increase of obesity during pregnancy and long-term obesity in offspring (37). Currently, there is a growing body of research to suggest that maternal obesity via overnutrition is associated with long-term functional programming of organs and tissues during fetal development, leading to increased disease risk.

1.4 Obesity

1.4.1 Overview

Obesity is currently a global epidemic with rates of obese and overweight people increasing annually. Obesity is characterized as an excess of adipose tissue and generally defined as having a body mass index (BMI) of >30kg/m^2. The World Health Organisation (WHO) estimates that 39% of adults worldwide over the age of 18 are overweight, and of these people 13% are classed as obese (38). The WHO also estimates that over 2.8 million deaths worldwide per year are attributed to being overweight or obese (39). In the UK the figure for obesity in 2016 was as high as 26% of the population (40). The reason for this is commonly attributed to diets high in fats, such as processed 'junk' foods, that have become more readily available in the past few decades and this constant abundance of excess nutrients causes fat accumulation, contributing to rising obesity rates. Currently, being overweight and obese is having a greater impact on public health

than being underweight (41), with overnutrition now evident in developing countries as well as developed ones (42).

Obesity is a major factor in metabolic disease that can cause insulin resistance, cardiovascular disease and type II diabetes among other health problems (43). In fact, obesity is now the leading cause of mortality and morbidity (especially in males) worldwide (44). More recently obesity has been implicated in impaired cognitive function and behaviour (45), all of which lead to diminished quality of life and financial tolls on society.

In humans, obesity has been reported to be associated with a higher incidence of depression (46), anxiety (47) and memory impairments (48) but not all studies are in agreement (49), possibly due to variations in the clinical assessment of these disorders and/or obesity. In rodent models however, it has been shown that obesogenic high-fat feeding is associated with increased anxiety behaviours (50-52), in addition to learning impairments and memory (53) and depression (54, 55). As well as obesogenic high-fat feeding, other models of obesity were found to influence neuropsychiatric disorders; a recent study created a mouse model of obesity by utilising gut microbiota from high-fat fed obese mice (56), which showed an increase in weight, neuroinflammation, anxiety and impaired memory. These data suggest that regardless of the method of weight gain, the key influence on anxiety and neuropsychiatric disorders is obesity itself.

1.4.2 Maternal Obesity

Sufficient nutrition is critical during pregnancy since appropriate nutrients are responsible for offspring cell growth and development. Nutrients are passed to the developing fetus through the placenta and this intake shapes its perception of the external nutritional environment. Any adverse prenatal environment (such as maternal obesity) may generate persistent changes in fetal biological systems, which subsequently increases the risk for developmental disorders in later life, as previously discussed.

The prevalence of maternal obesity is increasing, with approximately 1 in 5 women of child-bearing age obese in the UK (57), and this has led to an increase of over double of first trimester pregnancy complications by obesity (from 7.6% in 1989 to 15.6% in 2007) (58). Maternal obesity has long been associated with short-term health risks during gestation and perinatally to both the baby and the mother, however it has also now been recognised to be associated with long-term health risks during postnatal development and throughout adult offspring life. This highlights the necessity of understanding the effect of maternal obesity on fetal development.

Independent of maternal diabetes, it is well-established that offspring are more likely to be obese if their mothers were obese during pregnancy, and this change in adiposity extends from birth to childhood to adulthood. Being born large for gestational age (LGA) is in fact a predictor for increased BMI at one year of age, and this in turn is a strong predictor for BMI at eight years of age (59). A study of siblings showed that children who were born prior to their mothers receiving bariatric surgery for obesity were more likely than their siblings born post-surgery to have significantly higher body weights at 12 and 21-25 years of age (60), whilst their siblings benefited from improved metabolic profiles.

Current research shows that maternal obesity increases the risk of offspring obesity, cardiometabolic disease and altered immune function (61-64). Maternal obesity is linked to neonatal insulin resistance, and this may persist in childhood (11 years of age) (65) and in young adulthood (20 years of age) (66). It has also been shown that offspring from obese mothers who were born LGA and are also obese in childhood have twice the risk of developing insulin resistance (65). Maternal obesity has also been associated with elevated systolic and diastolic blood pressure at 17 and 32 years of age (67, 68). Due to changes in the cardiometabolic profile of offspring from obese mothers, a recent study by Reynolds *et al.*, has investigated the link between maternal obesity and premature adult mortality rates of 37,709 subjects. It was found that there was 40% increased risk of death from all causes, and a 29% increased risk of death due to cardiovascular disease (69). The intergenerational feedback loop of increasing adiposity has also been linked to increased risk of some cancers (70), with which birth weight is positively associated (71). These outcomes indicate the seriousness of this global obesity epidemic.

In addition to cardiometabolic risks, current data in humans suggest that maternal obesity can increase risk of neuropsychiatric disorders in offspring. Autism and developmental delay (72), ADHD (73), depression, schizophrenia and anxiety (74) are among the multitude of disorders that are shown to be increased following maternal obesity.

Animal studies have also shown associations between maternal obesity and neuropsychiatric disorders in offspring. In mice, 12 month-old offspring from obese (60% high-fat fed) mothers showed an increase in anxiety, however, this effect was not present in 3 month-old offspring and hyperactivity instead was present in males at this age (75). In another study of 3-month old mice, maternal obesity (due to 60% high-fat feeding) induced anxiety phenotypes in offspring, particularly in females, indicating sex differences in outcomes (76). These results were supported by a similar study showing increased anxiety in 3 month-old offspring from obese (60% high-fat diet) mothers in mice (77). Differences in outcomes are observed in the offspring, likely due to differences in experimental conditions. Despite this, increasing animal studies on the effects of

maternal high-fat feeding is demonstrating a link between maternal obesity and offspring behaviours in adulthood (74).

These observed disorders may be due to disruptions of neuroendocrine function and brain development in fetal life, in particular the HPA axis (76, 77). The mediator of this effect may be low-grade inflammation, induced by obesity. This will be discussed in more detail in section 1.8.

Due to the effects of adult obesity and also maternal obesity to separately increase the risk of neuropsychiatric disorders such as anxiety, there could be potential for an interaction between pre- and postnatal obesity together (as per the "second hit" hypothesis). However, there are currently gaps in the knowledge of the mechanisms involved in how maternal obesity influences long-term consequences for offspring mental health, and whether this in addition to offspring obesity may have unfavourable outcomes. To date there is a paucity of studies assessing the long-term effects of both maternal and postnatal obesity together on neuropsychiatric disorders and their potential mechanisms. However, this is an area which requires more research due to the fact that obese mothers are more likely to beget obese offspring, and therefore the long-term implications of a 'second hit' of obesity need clarifying. In order to study this, animal models have previously been used, and well-characterised behaviour studies may elucidate any changes to neuropsychiatric profiles and potential mechanisms.

1.4.3 Animal Models of Obesity

Research using animal models has helped to understand the mechanisms involved in the effects of altered environments on offspring outcomes at specific periods of development. This is aided by the shorter gestation period in, for example, mice (20 days) and sheep (152 days), compared to human gestation (9 months) and the ability to specifically regulate exact nutrition composition, day/night cycles and conception, amongst other factors. However, the use of rodents, which are litter-bearing, means that littersize must be taken into account as a confounding variable on the offspring data.

A range of animal models have been used to investigate the effect of maternal obesity on outcomes in offspring. In rodent studies, as well as inducing obesity via a high-fat diet, there are also mice with genetic predispositions to be obese due to leptin signalling deficiencies (*ob/ob* and *db/db* mice), strains of mice bred for diabetes and rats with mutations in the *fatty* gene (Zucker and Wistar Rats) amongst others (78). Overall, animal models of obesity produce similar effects to those seen in humans, such as development of the metabolic syndrome (79, 80).

The mouse model used in this thesis was developed by Cagampang *et al.* and induces obesity by feeding a 45% saturated high-fat diet, both in the maternal and/or postnatal period (81). High-fat diet is fed to dams before, during and after pregnancy through lactation and subsequent offspring are fed either a control or high-fat diet post-weaning. This model, used for over a decade, has robustly shown the effects of maternal high-fat programming to increase offspring body weight when fed a high-fat diet postnatally, associated disease risks and programming of the metabolic syndrome (82-85).

In addition to using animal models to replicate conditions of obesity, well-validated behavioural tests in animals allow us to assess neuropsychiatric disorders. Gold standards of testing exist for anxiety such as EPM testing and open field (OF) thigmotaxis behaviour (86), as well as memory tests such as novel object recognition (NOR; non-spatial memory) and Morris water maze (spatial memory) (87). These well-established tests allow for quantification and analysis of various types of behaviour.

1.5 Developmental Contribution to Neuropsychiatric Disorders

Neuropsychiatric disorders are associated with a range of changes in the brain which can affect normal function. Prenatal environment (88), exposure to toxins (89), brain defects or injuries (11), and substance abuse (90) are amongst the variety of factors that can influence the normal processes in the brain. There is also increasing evidence for a genetic basis for neuropsychiatric profiles and more recently a role for epigenetic processes, as shown in studies of twins (91).

A role for infectious disease such as neurosyphilis, herpes simplex encephalitis and HIV infection has also been observed (17, 92). In particular, infections of the central nervous system (CNS) are able to damage the fetal brain and cause aberrant behaviour. Damage to the brain includes lesions and neuronal degradation. Problems with motor function, memory and cognition have all been associated with infection. Differential behaviour is linked to the brain region affected, for example damage to the frontal lobes can affect social behaviour and violent tendencies, however damage to the limbic system which involves the hippocampus and hypothalamus can affect memory and language (93).

Increasing evidence suggests neurodevelopment *in utero* as a crucial time for establishing risk and susceptibility to neuropsychiatric disorders in later life. The time-sensitive nature of brain development means that the maternal environment may permanently influence brain

development and lead to altered neuronal development and function affecting long-term risk of neuropsychiatric disorders (94, 95).

1.5.1 Brain Development

The development of the brain is a highly orchestrated process and starts *in utero* at 3 weeks post conception in humans. Brain development starts with neurulation from the ectoderm of the embryo and is a highly regulated process of apoptosis and cell proliferation that occurs in 2 phases of primary and secondary neurulation (96). Whilst primary neurulation is essential in humans, secondary neurulation is generally critical in tailed animals (97).

By the end of the first 3 weeks of human conception, the embryo will have undergone gastrulation and thus the neural progenitor cells will have differentiated and be positioned along the rostral-caudal midline of the upper layer of the 3-layered embryo. The neural progenitor cells are found in the neural plate region of the embryo. Neural tube formation starts with the appearance of 2 ridges on either side of the neural plate, with the neural progenitor cells between these 2 ridges. The ridges then rise, fold inwards and fuse to become the hollow neural tube, a process that takes several days (98). The centre of the neural tube is fused first, and then this proceeds in both the rostral and caudal directions. Full fusion failure leads to neural tube defects (99). Neurulation in humans takes place between 21 and 28 days post conception and in mice neurulation occurs 8.5 to 10.5 days post conception (100). After complete formation of the neural tube, the neural progenitor cells form a single layer of cells that lines the centre of the neural tube immediately adjacent to its hollow centre, in addition to the neural stem cells that will proceed to become neurons and glia (astrocytes and oligodendrocytes) of the CNS.

The shape of the neural tube cavity changes as the brain becomes larger and more complex forming the ventricular system of the brain. The “ventricular zone” (VZ) is the region where neural progenitor cells will become ventricles. Neural progenitor cells found in the rostral region of the neural tube will grow to form the brain and ones found in the caudal region will become the hindbrain and spinal column (101).

Between 4-8 weeks post conception in humans, embryonic patterning occurs wherein by the end of the embryonic period there is a primitive “map” of eventual nervous system organisation which sets the stage for later development (102). During the fetal period of development there is change to the morphology of the developing brain due to structural folding and separating of the 2 brain hemispheres longitudinally. Neural production begins at 42 days post conception in the embryonic period which extends through mid-gestation (103). Grey matter structures are formed from different populations of neurons in areas such as the spinal column, and after production

neurons migrate away from the VZ (104). Neurons in the cortex region produce neurotrophic and neurotransmitter factors and extending the dendritic and axonal processes that form fiber pathways of the brain neural networks. These major fiber pathways serve to make up the white matter found in the brain. Myelin sheathing of the axons of neurons enables efficient data transmission and due to the white fatty nature of myelin this gives rise to the term “white matter” (101). In the fetal period of brain development the main progressions are due to neuron production, migration and differentiation. Therefore, appropriate maternal environment is critical during this period.

1.5.2 Development of the Hypothalamus

The hypothalamus is a region of the brain that regulates fundamental aspects of physiological homeostasis and behaviour. The region is highly conserved across vertebrate species, from fish to mammals. The hypothalamus is located in the forebrain and has integral development and functional connections with the pituitary gland which releases hormones into the general circulation (105).

In vertebrates during neural tube formation, a hypothalamic primordium is induced. Hypothalamic induction then requires signalling from morphogens secreted by surrounding tissues and this creates a positional identity in the neural plate. The hypothalamus is induced and patterned with specific regions of the tissue being defined by transcription factors (106). These regions are generally consistent across vertebrate species. During patterning, local environmental signals induce the subdivisions of the hypothalamus; preoptic, anterior, tuberal, mammillary regions. Further subdivisions occur to organise the hypothalamus into multiple functional nuclei: anterior periventricular nucleus (apV), arctuate nucleus (ARC), medial mammillary nucleus (MMN), ventromedial hypothalamus (VMH), premamillary nucleus (PMN), suprachiasmatic nucleus (SCN), supraoptic nucleus (SON) and the paraventricular nucleus (PVN) (107).

Hypothalamic histogenesis occurs when dividing neural progenitor cells residing in the ventricular zone, produce neuronal and glial precursors which then migrate into the parenchyma. Functional connections are made as these differentiating neurons migrate to their final location. Radial glia, astrocytes and oligodendrocytes are also generated (106).

Because of the importance of the hypothalamus in the regulation of physiological homeostasis, it has been suggested that impairments of hypothalamic development during perinatal life may result in lifelong metabolic dysregulation (108, 109). As mentioned previously, during brain development there are critical periods when brain maturation and function can be altered by maternal environment. The functional hypothalamus can be split into two developmental

processes: the determination of cell numbers, which involves neurogenesis, neuron migration and cell death, and the formation of functional circuits, which include axon growth and synaptogenesis (110). In mice the majority of hypothalamic neurons are produced between embryonic (E) 12 and E14 and between E12 and E17 in rats (111). In humans, literature suggests that early hypothalamic neurogenesis occurs around weeks 9 and 10 of gestation (112, 113), which is a comparable stage of gestation. Hypothalamic development also includes proper migration of neurons from their sites of origin to their final positions in the mature hypothalamus. This developmental process primarily occurs during late gestation in rodents (114). As the rodent hypothalamus is immature at birth, it continues to develop during the first 2 weeks of postnatal life. During this period, neurons send axonal projections to their target sites and form functional synapses. Experiments using axonal labelling techniques have shown differential periods of establishment between hypothalamic nuclei with PVN being fully established at postnatal day (P)6 and the ARC up to P16 (115). Synapses are formed after neuronal projections are established, and in rodents, synapses mature gradually from birth to adulthood. This period of circuit formation differs greatly to humans whose hypothalamic circuit formation occurs primarily during fetal life and begin to form as early as 21 weeks of gestation (113). Adverse maternal environments, such as obesity and related inflammation, have recently been shown to cause alterations to hypothalamic development, particularly during circuit formation (116, 117). Furthermore, changes to the development of circuit formation in the hypothalamus, such as alterations of transcription factors and neuronal differentiation, are suggested to induce neurodevelopmental disorders that disrupt both physiological and psychological homeostasis (as reviewed by Biran *et al.* (118)).

1.5.3 Development of the Hippocampus

The hippocampus is part of the limbic system and plays an important role in short and long-term memory and spatial navigation.

The hippocampus forms in the dorso-medial region of the telencephalon adjacent to the cortical hem (CH) (119). At the start of development at approximately E12.5 in mice (Figure 1.1A), the hippocampus structure is called the hippocampus primordium which consists of Cajal-Retzius cells (orange) and radial glial cells (119). These two cell types proliferate within the dentate neuroepithelium (DNE), producing embryonic neural stem cells. Migration of these stem cells later occurs in the sub granular zone (SGZ) and the dentate gyrus (DG). DG development occurs between E13-15 in mice at approx. E14.5 (Figure 1.1B) as dentate precursors of the primary matrix (dark blue circles) that are located in the ventricular zone (VZ) migrate towards the pial side of the cortex forming the secondary matrix. In the VZ, radial glial precursors (dark blue) will

give rise to DG granule neurons (119). Most of these neurons are formed in the prenatal and early postnatal period (120). The hippocampal fissure is formed at E17.5 (Figure 1.1C) and migration of dentate precursor cells occur there, forming the tertiary matrix (light blue). At this stage the glial scaffold (not shown) extends from the CH to the hippocampal fissure and pial surface, and dentate precursor cells migrate using the scaffold to the pial surface. This scaffold vanishes nearing birth and a second scaffold created by radial glia develops. The radial glia cell bodies are based in the SGZ found in the DG (120). From the HNE, hippocampal neurons (red triangles) are created and migrate along radial glial cells towards their location in the hippocampal fields (CA1 and CA3 are shown). The blades of the DG form at birth (Figure 1.1D). Granule neurons in the DG (red triangles) appear first in the upper blade, below the hippocampal fissure and Cajal-Retzius cells reach the pial side and induces formation of the lower blade of the DG. Precursor cells in the primary and secondary matrix disappear and only the tertiary matrix continues to actively divide and produce granule neurons in the postnatal DG development.

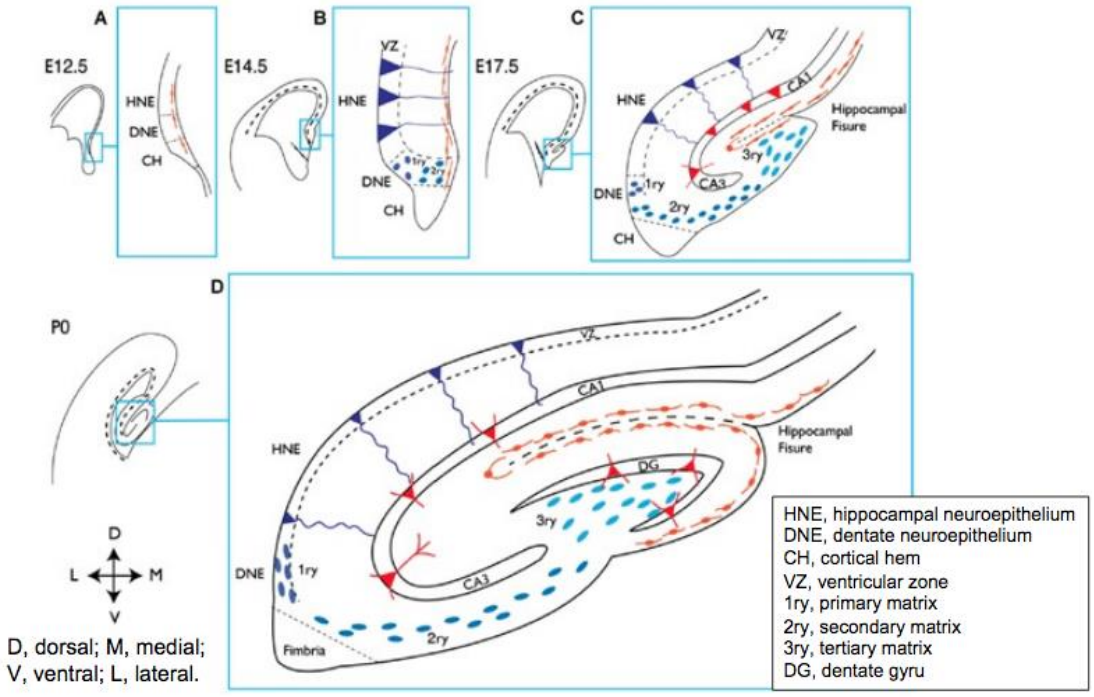

Figure 1.1 – **Development of the hippocampus**. Adapted from Urban *et al.* 2014.Developmental stages at E12.5 (A), E14.5 (B), E17.5 (C) and at birth P0 (D). Cajal-Retzius cells (orange), dentate precursors of the primary matric (dark blue), dentate precursors of the tertiary matrix (light blue), hippocampal neurons in (C) and granule neurones in the DG (D) (red triangles).

The hippocampus is divided into 2 main parts: The Ammon's horn and the DG. The Ammon's horn has a primary neuronal cell layer and is composed of glutamatergic excitatory pyramidal neurons called the pyramidal cell layer (PCL) (121). The PCL is composed of the regions CA1 and CA3 with

the CA2 being the transitory region between them (122). Pyramidal cell precursors from the neuroepithelium migrate to the ammonic plate over several days following maturation and this is guided by radial glia. Neurogenesis of pyramidal cells peak in the final week of gestation in mice (123). GABAergic interneurons migrate into the hippocampus and develop functional synapses prior to the excitatory neurons (124). The axons of CA3 pyramidal neurons synapse with that of CA1 pyramidal neurons and the dendrites of CA3 pyramidal cells continue to mature postnatally forming spines which in turn receive synaptic input from the axons of DG granule neurons. The primary cell layer of the DG is made of these granule cells and 85% are formed postnatally (125).

In addition to the hypothalamus, there are critical windows of hippocampal plasticity throughout the fetal and early postnatal periods. In mice, hippocampal development occurs during late gestation and the first 2 weeks postnatally (126), and in humans DG development begins during the third trimester and continues into adolescence (127). Due to the hippocampal involvement in learning and memory, any alterations to development can lead to poor cognition outcomes. Various adverse maternal insults such as early life stress, poor nutrition and immune activation have all been shown to lead to long-term alterations in hippocampal-related cognitive functions, at least partly via changes in neurogenesis structure and circuit functioning (as reviewed by Hoeijmakers *et al.* (128)) .

1.6 HPA Axis

1.6.1 Overview

The HPA axis is an essential system involved in a range of homeostatic functions via the secretion of glucocorticoids. Cortisol is the major circulating glucocorticoid in humans. The HPA axis regulates stress-related hormones: corticotrophin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and glucocorticoids (Figure 1.2) and acts in response to various stressors (such as inflammation, emotions and energy storage and expenditure) to up-regulate these hormones (129, 130). Glucocorticoids promote glucose mobilisation (gluconeogenesis), insulin secretion and behavioural alterations such as anxiety and “fight or flight” amongst other functions. Dysregulation of the HPA axis affects a variety of functions in the body, including appetite (131), behaviour and cognition (132, 133), circadian rhythm (134), metabolism (131) and inflammation (130).

The hypothalamus links the nervous system to the endocrine system via the pituitary gland. Central activity in the hypothalamus occurs in the paraventricular nucleus (PVN), which is the key

regulator of the stress response (129). It is the PVN that synthesises and secretes CRH and vasopressin in response to stress, sickness, activity and levels of circulating cortisol in the bloodstream. CRH and vasopressin release is also influenced by the circadian rhythm (sleep-wake cycle) of the individual. CRH and vasopressin then act on the anterior pituitary, which releases ACTH into the circulation and in turn causes the zona fasciculata region of the adrenal cortex to produce glucocorticoids; cortisol (in humans) or corticosterone (in rodents). Glucocorticoid synthesis is initiated by transport of cholesterol to the inner mitochondrial membrane by steroidogenic acute regulatory protein (StAR). A series of enzyme-catalysed conversion steps then cause glucocorticoids to be synthesised. As well as cortisol, the adrenal cortex also produces aldosterone and some sex hormones.

The glucocorticoids, which enter the general circulation, exert negative feedback on the hypothalamus and pituitary gland, thus self-regulating their own production (Figure 1.2). Cortisol concentrations during physiological or psychological stressors can be used as an indicator of HPA axis responsivity. Performing stressful tests such as the Trier Social Stress test in humans (135) or the restraint stress test in rodents (136) and collecting cortisol before, during and after testing can allow for calculating area under the curve (AUC) measurements to assess overall stress responsivity.

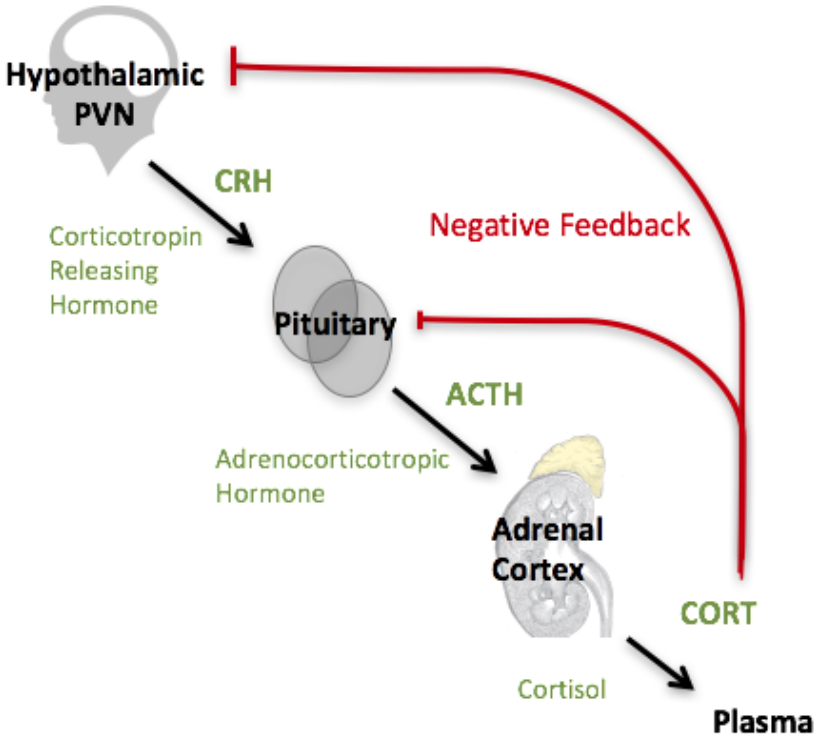

Figure 1.2 - **The HPA axis**

There is a long history of research detailing sex differences observed throughout numerous components of the HPA axis, its function and response to stress and this may underlie the basis for sex disparities in disease development. Overall the extensive biological factors underlying sex difference in stress are complex, but it has been shown that gonadal steroids play a major modulatory role in HPA axis regulation in both humans and animals alike. Estradiol in particular is able to exert effects on HPA function, including increasing adrenal gland sensitivity to circulating ACTH (137), enhancing CRH gene transcription in the hypothalamus (138) and increasing sensitivity to glucocorticoid negative feedback (139). The effect of gonadal steroids starts in early development and alters brain circuit organisation and morphology that permanently modifies adult stress responses and behaviour (140). Overall the main difference across studies and species shows that females initiate the HPA axis more rapidly and secrete a higher output of stress hormones (140). Unfortunately, despite this knowledge of sexual dimorphism, the vast majority of HPA research is performed exclusively on male subjects, and few reports use females or provide direct comparisons between sexes.

1.6.2 Glucocorticoids

1.6.2.1 Glucocorticoid Action

Cortisol acts by binding to the glucocorticoid receptor (GR), which is found in the cytoplasm of most cells in the body, but predominantly in hypothalamic neurons and pituitary glands (141). Being lipophilic, glucocorticoids pass easily through the cell membrane. In addition, glucocorticoids can also bind to mineralocorticoid receptors (MR). MRs are predominantly found in the hippocampus, but they are also expressed in hypothalamic sites (142). Upon ligand binding the receptors are activated and the receptor-ligand complex translocates from cell cytoplasm to the nucleus where it binds to glucocorticoid response elements in the promoter region of target genes, altering gene transcription. This binding activates anti-inflammatory genes in the nucleus and/or repression of pro-inflammatory genes in the cytoplasm. Glucocorticoids are also able to act directly via membrane-located receptors to alter membrane lipids and cytoplasmic proteins, and glucocorticoid-induced apoptosis also occurs to reduce inflammation in the body (143). The GR is the primary mediator of feedback regulation in the HPA stress response, and MR also plays an important role in glucocorticoid-mediated negative feedback of the HPA axis. MR is postulated to mediate ‘proactive’ feedback involved in the maintenance of basal HPA activity mainly at the nadir of circadian rhythm (144, 145). Disruption of the GR within the PVN of the hypothalamus has been shown to cause hyperactivity of the HPA axis and an increase in weight, but not behaviour in mice (146). Another study of GR deletion in mice also shows clear sex differences in HPA dysfunction, again with no effect on behaviour (147). These studies show that disruption of

the GR can lead to dysfunction of the whole HPA pathway, however further investigation is needed to assess its potential role in behaviour. MR knockout mice have also shown elevated anxiety and HPA axis hyperactivity via increased CRH PVN, ACTH pituitary and glucocorticoid levels (148, 149). These mice also displayed decreased granule cell density in the hippocampus suggesting that MR may play a role in neurogenesis. Conversely, increased MR activity in the forebrain is related to decreased anxiety (150) and some improvements in spatial memory (151).

1.6.2.2 Glucocorticoid Metabolism

Cortisol is bound by cortisol-binding globulin (CBG) in free circulation. Cortisol is metabolized by the 11-beta hydroxysteroid dehydrogenase system (11-βHSD) which consists of 2 enzymes: 11-βHSD1 and 11-β HSD2 (Figure 1.3). 11-βHSD1 utilises nicotinamide adenine dinucleotide phosphate (NADPH) to convert inactive cortisone into active cortisol and 11-βHSD2 utilises NAD+ to reverse this conversion. Cortisol is also metabolised irreversibly by A-ring reductase enzymes (5α- and 5β -reductases) in the liver. Metabolic clearance rate is influenced by the degree of regeneration of cortisol from cortisone via 11-βHSD1 in liver and fat (152). These conversions are all catalysed by the 3α-HSD enzyme. Cortisol metabolites and free circulating cortisol are excreted in urine via the renal system.

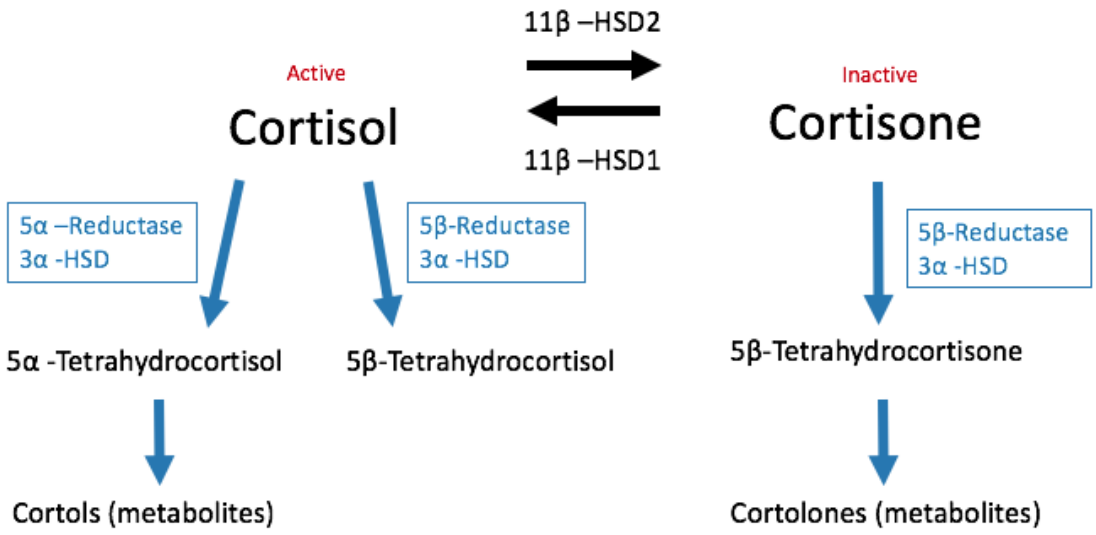

Figure 1.3 - **Cortisol Metabolism by the 11- βHSD system ,5α- and 5β reductases and 3α-HSD enzyme**

1.6.2.3 Circadian Rhythm of Glucocorticoid Secretion

Cortisol release follows a circadian rhythm. A circadian rhythm is an approximately 24-hour cycle of physiological processes present in all living organisms including animals, plants, fungi and cyanobacteria (153). It is generally thought to arise from an innate and genetic timekeeping system aptly nicknamed the "biological clock". Environmental cues called "*zeitgeibers*" (from the German "time giver") such as light and temperature, which change with the seasons, cause the circadian rhythm to adjust to the surroundings of the organism. This allows the organism to anticipate and prepare for specific and consistent environmental changes. As well as physiological processes, behaviour and biochemical processes are altered also. In mammals, the suprachiasmatic nucleus (SCN) of the anterior hypothalamus is the centre of this regulatory function which drives the secretion of hormones (154).

Cortisol secretion follows a diurnal pattern, with a peak in the morning and nadir in the evening in humans. In rodents that are nocturnal, corticosterone concentrations are at their nadir in the morning and peak in the evening. This high morning cortisol in humans prepares the body for increased metabolic demands during the day time (generally when humans are most active) after the overnight period of sleep. In humans, salivary cortisol circadian rhythm can be detected at around one month of age (155). Any alterations in cortisol secretion can induce an onset of dysregulation of physiological processes such as disrupting carbohydrate and lipid metabolism, immune response, behaviour and cognitive function (156-158). There is also growing evidence to suggest that cortisol rhythmic activity can significantly affect human health and disease (159, 160).

1.6.2.4 Sampling of Cortisol

It is crucial to understand that corticosterone (in rodents) or cortisol (in humans) in isolation cannot accurately reflect the HPA axis stress response. For example, adrenal sensitivity can mask the activation of the hypothalamus and pituitary regions of the HPA axis thereby causing a skewed perception of the accurate stress response. Due to both free active and CBG-bound cortisol being measured in plasma analyses, this may mask how much of the active cortisol is present, as an increase in CBG-bound cortisol would cause an underestimation of the HPA axis stress response.

Given the circadian rhythm of cortisol and the large number of potential influences on cortisol secretion (such as physiological and psychological stressors, light exposure and food consumption), it is important to sample cortisol at a time point of least variation (161, 162). Long-term cortisol can be measured through levels detected in hair which can be an indicator of

concentrations over long periods of time (163). Conversely, short-term cortisol concentrations can be measured by sampling urine, saliva or blood plasma/serum. Total cortisol is measured in blood and urine, whereas salivary cortisol is measured as “free” or unbound. Using these measurements, cortisol activity can be assessed, such as cortisol reactivity, daily output, long-term output and cortisol awakening response (CAR). To assess the diurnal variation in cortisol, daily total cortisol concentration can be measured by taking blood, saliva or urine cumulatively throughout the day.

The CAR is the cortisol level that peaks in the early morning (Figure 1.4) and has been found to be robust, as social factors such as age, use of oral contraceptives, smoking and sleep duration do not have a considerable impact on the CAR in humans (164). Irregularities of the CAR have been linked to behavioural alterations (165) and physical health (166).

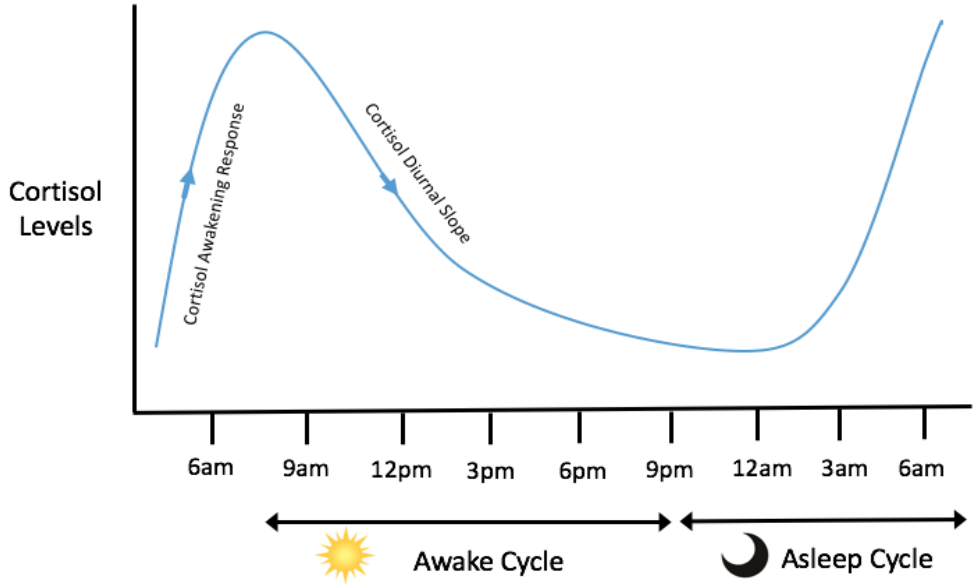

Figure 1.4 - **Cortisol levels over a 24-hour day cycle showing diurnal variation in cortisol and CAR**

1.6.3 The Role of the Hippocampus in HPA axis Function

The limbic system is a set of interconnected brain structures located in the telencephalon and diencephalon. As well as the hypothalamus, the primary structures within the limbic system include the amygdala, hippocampus, thalamus, basal ganglia, and cingulate gyrus. This system regulates autonomic and endocrine function, and supports a variety of functions including behaviour, emotion and memory. These limbic brain structures have also been shown to interact with HPA activation. These structures indirectly act with the PVN through CRH neurons via intermediary relay neurons in other regions including hypothalamic nuclei and neurons in the immediate vicinity of the PVN (167).

The hippocampus is involved with terminating the HPA axis response to stress. Hippocampal damage can delay termination of glucocorticoid release, leading to hypersecretion (168), demonstrating the role of the hippocampus in negative feedback inhibition of the HPA axis. The hippocampus contains a high density of GR and MR (169) and deletions of GR in this region result in prolonged HPA axis responses (170). In addition, decreased levels of GR in the hippocampus have been associated with HPA hyperactivity in depression (145), and decreased MR have been implicated in anxiety behaviour and impaired cognition (171). The ventral region of the hippocampus is important for stress regulation (172), in particular the ventral subiculum which initiates stress regulatory output (173). Damage (lesions) to this area can induce glucocorticoid hypersecretion after OF testing or restraint stress, showing increased anxiety in the former (174). Damage to the ventral subiculum has also been shown to enhance depletion of CRH immunoreactivity over time under prolonged stress, suggesting that this may be a mechanism prolonging HPA axis activation. During aging, GR expression in the hippocampus is reduced and this may contribute to the loss of glucocorticoid negative feedback implicated in aging (175). There is a substantial population of CRH-producing cells and CRH-1 receptors (CRFR1) within the pyramidal cell layer of the hippocampus (176). While acute hippocampal CRH administration appears to improve performance in spatial memory (177) and memory retention in passive avoidance and fear conditioning tests (178, 179), overexpression of hippocampal CRH has been shown to impair performance during spatial memory testing (180, 181) which is supported by CRFR1 blockade/knockout experiments in rodents (182, 183). In addition, chronic CRH overexpression over weeks is able to alter structural changes in hippocampal neurons including lower total dendritic length and poor dendritic arborisation (184), indicating long-term changes to hippocampal structure and function which is linked to cognitive impairments, primarily memory.

1.6.4 Ontogeny of HPA axis Function

In humans, HPA axis related hormone activity is detected as early as between 8-12 weeks of gestation in fetal development (185) when adrenal blastema differentiate and acquire steroidogenic characteristics (186). Between 10-20 weeks of gestation, cortisol in the cord blood is greater in the umbilical artery than the umbilical vein which indicates this steroid is produced by the fetus (187).

During pregnancy both the fetal hypothalamus and placenta secrete CRH which regulates growth of pituitary corticotrophs, adrenocortical differentiation and steroidogenic maturation of the fetal HPA axis (188). CRH is also a potent vasodilator of fetoplacental circulation (185) and the progressive increase in concentration in both fetal and maternal circulation in late gestation suggests a significant role in modulating the timing of parturition (188). Placental CRH is identical

to fetal CRH and stimulates fetal pituitary ACTH, however contrary to normal HPA function, adrenal glucocorticoid works to stimulate placental CRH secretion, leading to an increase in CRH, ACTH and cortisol towards the end of gestation (188). This exponential increase of cortisol towards the end of gestation is responsible for normal brain and neuroendocrine development, as well as maturation of lungs and other organs (189). An adverse maternal environment, such as stress, can increase this positive HPA feedback mechanism and thus induce preterm labour and this is supported by observations that babies born in early third trimester have increased CRH concentration levels (190). ACTH is produced by anterior pituitary corticotrophs and this hormone controls fetal adrenocortical growth, differentiation and steroidogenesis (185). During the 2nd and 3rd trimester in humans, the fetal adrenal cortex becomes enlarged and shows rapid growth and steroidogenesis activity in the "fetal zone", a specialised cortical compartment. The fetal zone exists during fetal life and produces dehydroepiandrosterone sulphate (DHEA-S) which is the main steroid product of the fetal adrenal gland during gestation, and cortisol is also produced.

The expression of GR and MR in the developing brain is species-specific. Animals that birth mature young (such as humans) have maximal brain growth and HPA maturation *in utero*, however in species that give birth to immature young (such as rodents) much of the brain development also takes place postnatally (191). In rodents, for example, a critical period of HPA axis development happens postnatally, called the stress hypo-responsive period (P4-P14), which likely occurs prenatally in humans (192). Diaz *et al.* showed, using a mouse model, that GR mRNA is expressed in the fetal tissues, placenta, fetal membranes and fetal brain regions; hypothalamus, hippocampus and pituitary from midgestation (E12.5), but MR mRNA expression occurs only in late gestation (E15.5) (193). In fetal rat brain GR and MR have shown to be expressed at low levels during gestation but increase rapidly after birth (193). GR mRNA is present in the human embryo at 8-10 weeks of gestation in the gut, muscle, spinal cord, sex cords and adrenals among other tissues (194, 195). In human fetal brain both GR and MR have been shown to be expressed in hippocampus at 24 weeks of gestation onwards and GR mRNA expression levels are lower than that of MR mRNA (196).

1.7 The Role of the HPA axis in Anxiety and Memory

The HPA axis is the major neuroendocrine mediator of the response to stress and therefore plays a role in neuropsychiatric disorders, including anxiety and in cognitive function, as well as memory. Anxiety is linked with activation of the HPA axis (197, 198), as commonly shown by

upregulated diurnal cortisol secretion (199-201). HPA axis dysfunction via blunted responsiveness is also associated with anxiety in rodents (202). In general, HPA activation is usually a short-term adaptive response, and behavioural and stressful memory impairments are short-lived (203). However long-term exposure to HPA dysfunction is linked to a range of neuropsychiatric disorders. In humans, chronic HPA hyperactivity, as shown by hypercortisolaemia, has been noted in a range of anxiety disorders including panic disorders (204, 205), generalized anxiety disorder (199, 206) and in social anxiety disorders when faced with a socially relevant stressor (207, 208). In addition, literature suggests that aging increases basal HPA axis function and stress responsiveness (209).

HPA activation during a stressful response is also able to mediate memory via changes to the hippocampus. While acute stress has been reported to have both enhancing and detrimental effects on general memory, chronic HPA activation mainly impairs memory (53, 210, 211). There are numerous studies implicating elevated glucocorticoid exposure in impaired memory in both animal and human studies. Several protocols to measure the effect of HPA activation on memory have been used, however the most widely used (as assessed by systematic review (212)) involves "chronic mild stress" in which animals are exposed to unpredictable stressors over a period of time from days to weeks. In both young and aged rodent studies alike, chronic stress, associated with HPA activation (in particular elevated basal corticosterone levels), has been shown to impair spatial memory (213-215), recognition memory (216, 217), and chronic corticosterone ingestion (8 weeks) has also shown to impair spatial memory performance (218). In addition, circulating corticosterone levels at the time of memory tests have been shown to be linked to spatial memory performance and memory retrieval (219). In humans, both stress tests and cortisol treatment has been shown to impair performance in a declarative memory (conscious recollection of learned information) task in adults (220, 221) and stress-induced declarative memory impairment has been shown to also affect elderly subjects (222, 223). In humans, hyperactive HPA activity linked to neuropsychiatric disorders in general have been implicated in hippocampal dysfunction, which in turn give rise to memory deficits (224).

Changes to memory via elevated corticosterone are mediated through hippocampal morphological and molecular changes, reduced neurogenesis, impaired synaptic plasticity (225) and dendritic atrophy of hippocampal region CA3 (214, 226). In addition to measures of corticosterone, other HPA outputs have shown to impair memory including elevated CRH. CRH treatment in P10 rat pups have shown to impair short-term recognition memory and spatial memory testing from 3 to 10 months of age which was associated with CA3 neuronal atrophy, further indicating a role for this hippocampal region (223). As the hippocampus has a high density of GR and MRs, dendritic atrophy, mediated by HPA activation, may affect expression, and chronic

stress and associated HPA activity has indeed been linked to a downregulation of GR and MR mRNA (227, 228) which may be linked to poor memory test performance (229, 230).

1.7.1 The Effect of Obesity on HPA axis Function

1.7.1.1 Body Weight and Cortisol

Obesity is linked with altered HPA axis function and changes in basal cortisol levels have been associated with differences in body weight. Hypocortisolism has been associated with a reduction in weight such as in Addison's disease (231) and after adrenalectomy (232), the latter of which prevent obesity in rodent models (233). On the other hand, hypercortisolism is associated with weight gain, such as in Cushing's disease (234) which increases the amount of adipose tissue by promoting differentiation of pre-adipocytes into mature adipocytes and increasing lipoprotein lipase activity (235). Elevated cortisol can increase food intake (236), therefore chronically elevated glucocorticoids may further stimulate appetite and promote obesity (237).

1.7.1.2 Obesity and the HPA-axis

Numerous studies have shown how obesity can directly affect the HPA axis. However, although it is widely studied, a recent comprehensive systematic review by Rodriguez *et al.* found that the effects were not consistent (238). Circulating basal cortisol in humans and animals have been shown previously to be elevated in obese individuals (239-242) although may sometimes appear normal due to increased renal cortisol clearance rate (243), as confirmed by elevated urinary cortisol excretion (244, 245). In contrast, some studies report hypocortisolism in obese individuals (246) and some studies suggest cortisol is unaffected by obesity (247). Higher BMI has also been linked to increased cortisol concentrations in hair (248).

Obesity has also been associated with changes in the circadian rhythm of circulating cortisol. Elevated evening cortisol levels in human males (249) have been observed in obesity, as well as a flatter decline of the cortisol diurnal slope throughout the day (247), which has been associated with elevated risk of cardiovascular disease, type II diabetes and stroke (250). Studies describing the relationship between obesity and CAR are mixed. Some reports suggest that obesity can cause a blunted CAR (247, 251, 252), with others suggesting an elevated CAR (253, 254) or no relationship at all (255). However, a systematic review of these CAR studies suggest that a blunted response is most likely to be associated with obesity (238).

The inconsistencies in the relationship between obesity and cortisol may reflect differences in methodology. In animal studies this may be due to variations in mouse strain, consistency of diet,

housing conditions or evaluation time points (256), and in human studies they may be due to variations in cortisol sampling techniques.

Cortisol metabolism may also be altered in obesity. In both obese human and rodent studies, it has been shown that increased 11β-HSD1 activity, which converts inactive cortisone into active cortisol, may be linked to hypercortisolism due to its reported upregulation in adipose tissue (244, 257, 258). However, it has also been reported that hepatic 11β-HSD1 activity may be compromised, as elevated cortisone metabolite excretion was reported in both obese men (245) and women (257) compared to controls. It has been suggested that the increased adipocyte cortisol regeneration may contribute to lower central cortisol secretion, and lower hepatic regeneration may be causing compensatory mechanism of HPA axis activation in obesity thus promoting metabolic syndrome and obese phenotype (238).

In terms of stress responsivity, obesogenic high-fat feeding affects the HPA response to stress tests by increasing corticosterone (259) and ACTH output (260) in rats. There is also a reduced ability to restore basal corticosterone following stress in rats, suggesting a possible 'resistance' to corticosterone negative-feedback (261). This results in increased systemic corticosterone; a concept presented by a recent study in which high-fat feeding increased HPA axis activity via elevated circulating corticosterone levels and induced stressed behaviour (262). Glucocorticoid feedback resistance has also been shown in obese human males (263). High-fat fed obese mice also show elevated basal corticosterone levels (242).

1.7.1.3 Obesity and HPA-related Neuropsychiatric Disorders

Although HPA dysfunction has been associated with obesity, the relationship with neuropsychiatric disorders is inconsistent. Studies of diet-induced obesity in rodents have shown that a 60% high-fat diet maintained for 22 weeks in male mice display increased anxiety behaviour on an EPM but no change in basal corticosterone (264). 60% high-fat feeding for 8 weeks has also been shown to increase OF test anxiety behaviour linked to a reduction in GR and MR mRNA expression in the hippocampus in female rats (51). However, another study of 45% high-fat obesogenic diet feeding for 12 weeks in male rats reported increased basal plasma corticosterone alongside decreased anxious EPM behaviour (265). Fewer studies look at the relationship between obesity, HPA function and memory, however one study in obese rats (58% high-fat fed for 12-15 weeks) that exhibited elevated basal plasma corticosterone showed impaired spatial memory and these effects were apparent in both sexes (266). This memory deficit may be linked to the reduced hippocampal GR and MR observed in the previous study (51) and further research into this area must be done to determine the effect of diet induced obesity on HPA-related memory deficits.

Taken together, there is considerable evidence that obesity can have an impact on HPA axis function in humans, which is confirmed in animal models. However, the impact of obesity on neuropsychiatric disorders is less clear and requires further research.

1.7.2 The Effect of Maternal Obesity on Offspring HPA axis Function

There is growing evidence that HPA axis function may be influenced by aspects of the maternal environment, for example by maternal stress/depression/mood disorders in humans (267-269) and rodents (270). Given the rising incidence of obesity, there is considerable interest in the effect of obesity during pregnancy, both for the mother and for her offspring, and the potential ability to cause developmental programming. In humans, increased levels of maternal triglycerides and cholesterol throughout pregnancy have been associated with increased cortisol reactivity in offspring aged 3-5 years old, suggesting maternal lipid transfer may be a factor in programming HPA axis function (271). In addition, prenatal exposure to maternal obesity is a predictor of adverse neuropsychiatric outcomes in children (272).

Obesity before and during pregnancy (via 50% increase in food intake compared to control fed) has been associated with elevated basal cortisol and ACTH in 19.5 month old adult sheep offspring (273) (the first animal model demonstration of altered HPA axis function in adult offspring from mothers fed a high-fat diet throughout pregnancy and lactation). A 58% high-fat obesogenic diet before and during pregnancy and lactation has also been shown to have differential effects in young and mature adult male mice. At 3 months of age mice show reduced anxiety on the EPM test and this is coupled with reduced GR mRNA expression in the ARC and VMH regions of the hypothalamus and increased FKBP51 mRNA expression, a gene linked to GR sensitivity and stress-related conditions (see section 1.8.5.1), in the PVN region of the hypothalamus. At 12 months however, there was increased anxiety measured by EPM testing and this was coupled with increased GR in the PVN, ARC and VMH regions of the hypothalamus (75). FKBP51 mRNA expression stayed elevated from control mice at this age, and although there were changes to the GR, there was no effect of altered corticosterone during restraint stress at either age. This particular study shows the significance of offspring age as a factor to take into consideration when measuring the effects of maternal obesity and may account for differential results observed between studies.

Another study of perinatal obesogenic high-fat feeding in rats also shows early life developmental programming. A study by Sasaki *et al.* showed that 60% high-fat feeding before and during pregnancy and lactation affects offspring in young adulthood (3 months of age) by increased anxiety behaviour in both male (in the OF test) and female (in the EPM test) offspring, decreased

basal corticosterone in both sexes, and reduced ability to regulate basal corticosterone following restraint stress in both sexes also (76). This alteration of the HPA axis is supported by another study showing increased basal ACTH production in adult offspring, indicating a hyperactive HPA axis, from mothers fed a high-fat diet during lactation (274). These studies show that HPA axis function in adulthood may be influenced by early life diet.

Maternal obesity may also be able to modulate HPA function in fetal life itself. A mouse model of maternal obesity via high-fat and high sucrose (HFHS) intake (45% fat and 17% sucrose) before and during pregnancy and lactation reports higher plasma corticosterone levels in male, but not female, offspring on gestational day 17 (275). This effect correlated with later findings on offspring at 9 weeks of age where both sexes of offspring from mothers fed the HFHS diet showed elevated circulating corticosterone. This altered HPA did not affect behaviour at this age (275). Further studies on the effects of maternal obesity on fetal HPA function are required to confirm the validity of this outcome.

The effect of maternal obesogenic high-fat feeding currently has varied results on long-term offspring memory deficits. Maternal obesity (60% high-fat) before and during pregnancy has been shown to affect hippocampal development at fetal gestational day 17 by decreased apoptosis in the Ammons horn, decreased neuronal differentiation in the DG and by region-specific changes in proliferation of neural precursors (276). Due to the hippocampus' key involvement in memory, early alteration of hippocampal development may affect long-term memory processes. Indeed Bilbo *et al.* reported that maternal 60% fat diet showed an increase in hippocampal inflammation (via microglial activation and cytokine expression) from birth to adult (3 month) offspring following lipopolysaccharide (LPS) treatment, and also basally, indicating early life programming of these offspring (277). However, this effect was coupled with improved memory in male and female offspring during short-term water maze memory trials and no effect on long-term memory. Other similar rat studies utilising the same fat diet and perinatal feeding time course as the previous study showed no cognitive deficit in the water maze in male offspring from high-fat fed dams (278) or basal alteration of both spatial and non-spatial memory in both sexes (279), despite the latter showing increased hippocampal inflammation and increased anxiety in these same mice (279). Further investigation into the role of prenatal inflammation on offspring memory is required (see section 1.8.4).

Further studies linking behavioural changes in rodents from obese mothers and HPA axis function are required as there are gaps in current knowledge on what age these behavioural and HPA axis changes occur in offspring, the range of severity of potential HPA dysfunction and the mechanisms driving this. Current knowledge of the effects of prenatal overnutrition, and the

interaction with further postnatal high-fat feeding, on offspring HPA is also limited and investigation of this early life 'programming' must be performed in order to understand the mechanisms involved in this long-term change.

1.7.3 The Effect of Maternal Obesity on Maternal HPA axis Function and Neuropsychiatric Disorders

As mentioned previously, poor maternal behaviour reported in animal studies is able to permanently alter HPA axis function in offspring, thus implying the importance of neonatal maternal environment. Poor maternal care has also been linked to other aspects of offspring development and disease risk such as: accelerated pubertal onset (280), higher risk of obesity in young adulthood (281) and midlife adult heart disease and hypertension (282). Poor maternal care with high BMI has also been suggested as a risk factor for schizophrenia in offspring, thereby indicating that both of these maternal factors may be linked and implicated in neuropsychiatric disorders (283). Obesity is linked to increased levels of anxiety and/or depression in animals (52) and humans (284). It is therefore possible that obesity could alter maternal behaviour towards her offspring detrimentally (262, 285). Indeed, a US meta-analysis associated obesity during human pregnancy with elevated antenatal and postpartum depression symptoms (43% and 30% increase from non-obese control subjects respectively) (286). Unfortunately, there are limited studies on the effects of pregravid obesity and obesity during pregnancy on maternal HPA function in humans. However, one study in humans showed that pregravid obesity was associated with elevated evening cortisol levels during late pregnancy (35+ weeks) (287). This elevation in cortisol may link in with the increased maternal psychiatric disorders reported post-pregnancy.

In animal studies, maternal care during the lactation period has been shown to affect offspring stress response in adulthood (288) and emerging literature suggests a role for obesity in poor maternal care. In a recent study by Connor *et al.* rats were fed a 45% high-fat diet during pregnancy and lactation and this was associated with reduced maternal care via licking and grooming behaviour between P3 and P8 (289). A 58% high-fat diet in mice before and during pregnancy and lactation also suggested some poorer maternal behaviour via pup retrieval tests (increased time sniffing pups and self-grooming before retrieving), cannibalistic traits and aggression toward unfamiliar social stimulus via social avoidance testing (262). This poorer behaviour was associated with increased maternal corticosterone at gestational day 16 (262). Interestingly, a recent study has suggested that neonatal mice exposed to a high-fat diet *in utero* are also able to influence the behaviour of their nursing dam, indicating a bi-directional effect of obesity on maternal behaviour. Using a 45% maternal high-fat diet, pups were cross fostered and maternal behaviour, following pup retrieval testing, showed that pups exposed to a high-fat diet

in utero impaired maternal behaviour by reducing the time the mother spent nest building and being in contact with pups (290). Maternal behaviour was most impaired when dams exposed to a high-fat diet were paired with offspring exposed to a high-fat diet suggesting both have an effect on maternal behaviour, and this group also showed a non-significant impairment in pup retrieval time (290). These findings suggest that changes to maternal behaviours previously associated with the high-fat feeding may in part be mediated through the offspring. Overall, the current literature suggests a role for maternal obesity on maternal care and behaviour however there are a paucity of studies assessing these effects on long-term offspring HPA function and neuropsychiatric disorders as a whole, and further research is required to understand the mechanisms behind these changes.

1.8 The Role of Inflammation

1.8.1 Overview

Inflammation is the body's first response to cell and tissue damage by infection, noxious stimuli such as chemicals or physical injury. Activation of the innate immune system by acute rapid inflammation is a short-term response that results in healing. Immune cells, such as macrophages and leukocytes, are recruited to the damaged region and inflammatory mediators, such as cytokines, chemokines and growth factors, work to remove the stimulus and repair tissue (291). Cytokines are chemical messengers that travel away from the cells that release them to alter the functioning of other cells. Chemokines also leave the cell and attract other cells into the region. Together, cytokines and chemokines cause increased blood flow to the area and the entry of further immune cells in order to destroy the 'invader' and prevent spread of infection. The process of inflammation is also able to induce 'sickness behaviour' in order to increase survival. Sickness behaviours include a reduction of activity, dietary intake, social interaction and difficulties in forming new memories (292) and these changes reduce energy output in order to fight infection. Acute inflammation has been widely researched, however, less is known about the effects of chronic inflammation. Chronic inflammation is a maladaptive response that involves active inflammation and tissue damage. This persistent inflammation is associated with long-term human disease risk including allergy (293), cancer (294) and cardiovascular disease (295).

The brain is a typically immune-privileged site in the body with a blood brain barrier (BBB) preventing entry of peripheral immune cells as well as infectious agents. This ensures the brain is fully protected. In the brain, microglia form the resident innate immune cells, which are activated

during acute inflammation. However, the brain is mainly made up of astrocytes that maintain neuronal condition and are involved in the composition of the BBB. Astrocytes can become activated in response to neuronal signals or activated microglia following damage to the brain, and release growth factors (296). Both microglia and astrocytes are able to produce cytokines and chemokines in order to modulate the inflammatory response. However sustained upregulation (chronic inflammation) may cause damage to tissue, via neuronal cytotoxicity, including the BBB. Common cytokines released during insult to the brain include interleukin-1 beta (IL-1β), tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6).

1.8.2 HPA axis Activation during Inflammation

The HPA axis responds to changes in the body's internal environment in response to stressors such as inflammation (297, 298). During inflammation, peripheral inflammatory mediators are released and these signal to the brain via humoral and neuronal pathways to trigger central HPA axis activity at the PVN of the hypothalamus. The HPA axis is able to be activated by a number of cytokines including IL-1β, IL-6 and TNF-α (299). The HPA axis cascade is then activated leading to increased release of circulating glucocorticoids. One of the main functions of glucocorticoids is immunosuppression. In the cytoplasm of immune cells glucocorticoids bind to GRs and this results in transactivation of anti-inflammatory genes and transrepression of inflammatory genes through interaction with transcription factors activator protein-1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB). These actions result in a variety of processes that reduce inflammation across the body such as inhibition of activation and mobilisation of immune cells, as well as production of AP-1 and NFkB dependant cytokines (300). Glucocorticoids also induce apoptosis of monocytes, lymphocytes, eosinophils and endothelial cells (143). These interactions show a bi-directional effect of inflammation to activate HPA and for HPA activation to reduce inflammation and that a well-functioning HPA axis plays an important homeostatic role in immunological function.

1.8.3 Inflammation in Obesity

Obesity results in an inflammatory state in the body, particularly in metabolic and adipose tissues, which is reversible with weight loss (301). This chronic inflammation is thought to come about by adipocyte dysfunction. Adipocytes are the main cells in adipose tissue and store energy as fat, but are now also known to influence endocrine processes and play multiple metabolic roles via the secretion of 'adipokines' such as adiponectin and leptin. In lean individuals, adipocytes are small in size and are able to release hormones that modulate body fat mass. However, as an individual increases in weight to become obese, these adipocytes enlarge and control mechanisms become

dysregulated, causing macrophages to accumulate in the adipose tissue and inflammation to occur (302).

Pro-inflammatory cytokine TNF-α was first discovered to be elevated in the adipose tissue of obese mice (303) and humans (304) over two decades ago. Since then it has become well established that obesity is associated with low-grade chronic inflammation. A range of inflammatory mediators (as well as TNF-α) such as IL-1β, chemokine ligand 2 (CCL2), IL-6 and C-reactive protein (CRP) are consistently increased and link obesity to an increased risk of metabolic dysfunction (305, 306). Adipose tissue is not the only target of overexpression of inflammation in obesogenic high-fat models; liver (307), pancreas (308), muscle (309) and brain (310) have also all been reported to show increased inflammatory marker expression. This indicates that the effects of obesity are prevalent throughout the body and could influence a wide variety of homeostatic functions.

Accumulating evidence suggests that as well as low-grade systemic inflammation, obesity is also associated with inflammation in the brain. Inflammation in the hypothalamic and hippocampal regions of the brain, which influence behaviour and cognition, could lead to alterations in anxiety and memory. It is known that obesity is associated with increased neuropsychological disorders such as anxiety (311) and depression (46) and this could potentially be associated with hypothalamic inflammation (312). Inflammation of the hypothalamus has previously been shown to alter feeding patterns by inducing insulin resistance and propagating obesity, with studies showing an obesogenic high-fat diet alone could cause this hypothalamic inflammation via pro-inflammatory infiltration and activation of microglia (310, 312, 313), and a reduction of BBB integrity (314). Obesity is also thought to have a negative impact on memory in both rodents (315) and humans (48). In a mouse model of obesity, the pro-inflammatory cytokine IL-1β was suggested to impair hippocampal function and thus mediate memory deficits (316).

1.8.4 Effect of Maternal Obesity on Offspring Development and Inflammation

1.8.4.1 Inflammation in Maternal Obesity

The low-grade inflammation observed in obese individuals could be mediating effects of maternal obesity. During pregnancy, functional inflammation occurs to facilitate and protect implantation, the placenta and the fetus. In obese pregnancies however, due to the chronic elevated inflammatory state of obese individuals, this can lead to perturbations in this inflammatory state and consequently it has been shown that CRP and IL-6 circulating levels are indeed increased in maternal plasma compared to mothers of normal BMI (317). During maternal infection or illness in pregnancy there is an increase in circulating inflammatory cytokines delivered to the fetus that

are able to induce an inflammatory response in the fetal brain during critical periods of development (318). Inflammation in the fetal brain has also been identified as a significant risk factor for some neuropsychiatric disorders including autism and schizophrenia (319, 320). In humans, offspring from obese mothers demonstrate elevated inflammatory circulating CRP levels (321) and fetal neuroinflammation has been shown to be upregulated in a sheep model of maternal obesity (322). It has also been shown that chronic maternal LPS dosage and obesogenic high-fat feeding (before and during pregnancy and lactation) have similar programming effects in adult offspring when faced with a high-fat diet in later life (19 weeks) (323). These programming effects resulted in an increase in adiposity and weight gain which was more pronounced in females, and also maternal LPS + later postnatal high-fat elicited an increase in male offspring inflammation (as measured by blood leukocyte count), the effect of which was a trend in male offspring from obese mothers (323). Currently though, there is limited data on the effects of maternal obesity, and the associated chronic inflammation, on the offspring brain in particular.

1.8.4.2 Maternal LPS-treatment and Offspring Neuroinflammation

Circulating fetal inflammation may create an adverse environment for offspring brain development by crossing the BBB and increasing neuroinflammation across multiple regions of the brain (117, 277, 324). In order to evaluate the fetal brain response to maternal inflammation, rodent models of maternal LPS injections have found that IL-6, IL-10 and TNF-α mRNA expression is upregulated in the choro-amnion and placenta and that this corresponded with changes in the fetal brain (325). Both epidemiological and experimental studies in animals have demonstrated that maternal inflammation can damage the developing brain (326, 327). This may be caused by pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α released by microglia and astrocytes which have been shown to directly damage oligodendrocytes and neurons. Cerebral injection of IL-1β into the neonatal rat at P5 has been shown to induce neuronal death and delays myelination (328), TNF-α is able to induce cell death in mature oligodendrocytes and apoptosis in developing oligodendrocytes (329, 330) and intrauterine injection with LPS at E15 in mice has shown a reduction in brain weight, hypo-myelination, and brain lesions in grey and white matter at P14 (331). Another study of maternal intraperitoneal inflammatory injection with IL-6 at E12.5 showed deficits in behaviours linked to neuropsychiatric disorders, increased anxiety and deficits in social interaction in adult offspring, and neutralization of IL-6 reverses these behaviours (332).

1.8.4.3 Maternal Obesity and Offspring Neuroinflammation

The effects of maternal obesity on offspring inflammation have generally been studied only in the past decade. The study by Bilbo *et al.* assessing mouse offspring from 60% high-fat fed mothers has shown increased basal microglial allograft inflammatory factor 1 (Iba-1; marker of microglial

activation) activation in the 13 week-old adult offspring hippocampus following LPS injection (277). This Iba-1 activation was associated with increased anxiety and improved memory. In another study investigating the effects of maternal and postnatal 60% high-fat diet in rats, Iba-1 was increased in the cortex of offspring from obese mothers, although a stronger effect was seen in offspring fed a postnatal high-fat diet (278). In this same study a maternal high-fat diet increased IL-6 in the offspring cortex, and these results were associated with a slight cognitive decline. Non-human primate offspring from obese mothers (35% high-fat fed before and during pregnancy) show increased circulating and hypothalamic cytokine IL-1β expression and activated microglia in the early third trimester (117). Sex differences have also been observed in rodent offspring, female offspring from obese mothers (60% high-fat before and during pregnancy and lactation) increased pro-inflammatory cytokines and microglial activation alongside decreased cognitive function including anxiety at one month of age (324), whereas male offspring from this study showed hyperactivity. In this study, dietary intervention onto the control diet (10% fat) during lactation ameliorated some of the effects of inflammation and social deficits.

Although the mechanisms behind how maternal inflammation influences fetal neuroinflammation currently remain unclear, increasing evidence shows that there is a link. Compared to lean mothers, obesity during pregnancy has been shown to display elevated low-grade inflammation and growing evidence indicates that this in turn may be able to also influence fetal inflammation in the brain which could lead to impairments in brain development and impact neuropsychiatric disorders. Overall, the existing literature suggests that offspring from obese mothers have evidence of elevated fetal neuroinflammation that persists into adulthood. Such effects in the brain may be linked to neuropsychiatric disorders, however further research must be performed to confirm this effect and its severity on long-term health, and behavioural/HPA implications are as of yet still unclear. In addition, the links between maternal and postnatal high-fat feeding on neuroinflammation are little investigated. Due to evidence that offspring from obese mothers are prone to become obese themselves, the effects of both maternal and postnatal obesity must be explored, and the mechanisms causing offspring neuroinflammation also.

1.8.5 Effects of Obesity on the Placenta

The human placenta is a villus hemomonochorial organ that is the barrier between mother and fetus that mediates selective transport of oxygen and nutrients, waste exchange and hormone production. This transport is facilitated by maternal and fetal vascular proximity and exchange takes place through the trophoblast cells that serve as the barrier between these systems. In short, transfer can be divided into 3 stages: delivery via maternal blood, transfer across trophoblast tissue and uptake by fetal circulation. Term placenta receives approximately 70% of

uterine blood flow. For efficient health and growth of the fetus the placenta must be healthy; infant size at birth is generally thought to be determined by fetal nutrient supply (333), which is in turn determined by placental size, morphology and capacity for transport (334).

The placentas of obese women are significantly heavier at birth and this has a strong correlation with neonatal birth weight and fat mass (335, 336). Maternal metabolic environment influences early placental growth and gene expression, and this influences later placental function. In comparison to women with average BMI's, term placentas of obese women are characterized by an increase in pro-inflammatory mediators and macrophages, as well as lipid content (337, 338). Pro-inflammatory cytokines IL-1β, TNF-α and IL-6, as well as CRP, which are known to increase in obese individuals, were increased in the placentas of obese women causing a chronic inflammation of the placenta (339). Zaretsky *et al.* reported that IL-6 can be transported across the placenta into fetal circulation (340). This is in agreement with other studies showing elevated IL-6 in both maternal circulation during obesity (317) and in umbilical cord blood of offspring from obese mothers (341). Longitudinal studies of maternal obesity also showed consistently elevated maternal circulating CRP (317, 342) and CRP has also been shown to be increased in umbilical cord blood alongside other pro-inflammatory cytokines IL-1β and TNF-α (341). Although IL-6 has been shown to cross the placenta, this has not been shown for other inflammatory markers, regardless of elevated levels in maternal circulation and the placenta.

During normal pregnancy the placenta secretes an array of cytokines including IL-6, IL-1β and TNF-α and increases both local and systemic levels which may be important in determining fetal allograft fate (343). This cytokine secretion likely increases maternal circulating levels and plays a role in placental development and function across gestation. In a mouse model of obesity (45% high-fat) cytokine activation was reported to be increased at gestational day 17.5 but not 15.5 which correlated with circulating cytokine levels in the fetus at this time point also (344). Cytokine changes in response to maternal obesity were also shown to be sexually dimorphic with female fetus' showing a reduction in cytokine mRNA expression at gestational day 15.5 whereas there was no effect in males at this age (344). This suggests that the placenta may, to some extent, be able to protect the developing fetus from the effects of maternal and placental elevated inflammation. This study may also suggest that due to the correlation in time between elevated placental and circulating fetal cytokines there may be a method of transfer across the placenta.

1.8.6 The Effects of Inflammation on Glucocorticoid Action

Persistent chronic inflammation, like during obesity, may be a result of glucocorticoid resistance. Failure of glucocorticoids to inhibit inflammation and neuroendocrine responses to stress may

result in disease risk (345). The reason for glucocorticoid resistance in inflammatory disorders is currently unknown, however some studies suggest that chronic inflammation may be associated with reduced glucocorticoid sensitivity. Cytokines have been shown to influence GR signalling pathways, disrupting glucocorticoid action (346), although results are not always clear. However a variety of cytokines are shown to consistently impair glucocorticoid signalling via decreased GR translocation and decreased activation of GR inducible enzymes (346, 347).

In addition to cytokines, other immunoregulatory pathways may also alter GR function and translocation, these include: Mitogen activated protein kinase (MAPK), NF-kB and Januskinase (JAK) – signal transducers and activators of transcription (STAT) (348).

1.8.6.1 The Role of FKBP51 in Neuropsychiatric Disorders

FK506 Binding Protein 51 (FKBP51) is a gene encoding a protein that is part of the immunophilin protein family. Glucocorticoids act by binding to HSP90-GR complexes, initiating phosphorylation of GR Ser^{203} and exchange of FKBP51 for FKBP52 in the Glucocorticoid/GR-chaperone containing complex (Figure 1.5). Translocation of the complex to the nucleus then occurs where it acts as a transcription factor (349). Binding of the GR by FKBP51 prevents nuclear translocation, whereas binding by FKBP52 facilitates nuclear translocation. Increased glucocorticoids thus increase FKBP51 expression proportional to the glucocorticoid-GR complex, which makes levels of endogenous FKBP51 a good biomarker of glucocorticoid responsivity and resistance. In mouse models, FKBP51 has been reported to be elevated under conditions of stress (350, 351) and glucocorticoid administration (352), which supports this. FKBP51 is thought to interact with MR in a similar fashion to GR as a co-chaperone protein (353-355) that increases with overexpression of MR (356), although the FKBP5/GR interaction is the most thoroughly examined. Increased levels of FKBP51 are associated with a reduction of GR activity (357) by diminishing its glucocorticoid binding affinity and therefore reducing its transcriptional activity (349, 358). FKBP51 may therefore have a role in neurological diseases related to HPA activity and immunoregulation. Indeed, studies have shown overexpression is associated with anxiety disorders and decreased stress coping (359), and FKBP51 knockout mice are shown to have reduced anxiety behaviours (360), but knockout female mice have shown no effects on hippocampal dependant spatial memory (361).

FKBP51 may be a mediator of inflammatory effects on glucocorticoid action. FKBP51 expression has recently been shown *in vitro* to significantly reduce under sustained IL-1β inflammatory conditions coupled with glucocorticoid treatment at various concentrations. Glucocorticoid treatment alone increased FKBP51 expression as would be expected (362). In addition, another study showed that mice subject to social defeat stress test also showed elevated IL-1β and

decreased FKBP51 expression in *ex vivo* enriched microglial cultures, further suggesting a link between inflammation and FKBP51 (363). These results show that chronic inflammatory conditions may be able to decrease glucocorticoid responsiveness and promote resistance, and is supported by previous research showing that GR function may be influenced by other cytokines such as IL-1α (364), IL2 and IL4 (365) and IL-13 (366).

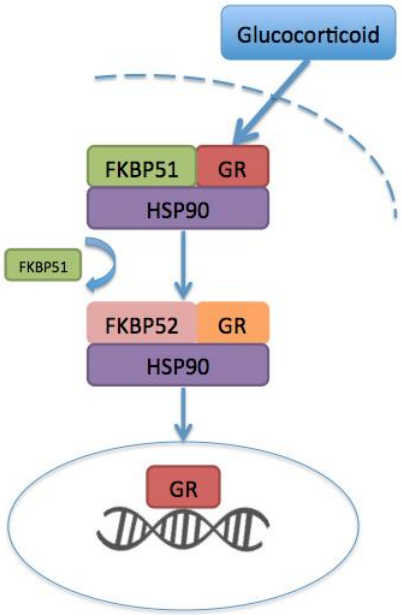

Figure 1.5 - **Glucocorticoid-GR complex**. FKBP51 binds to the HSP90-GR complex. Once glucocorticoids bind to this complex, FKBP51 is exchanged for FKBP52, allowing for GR translocation to the nucleus and exert action as a transcription factor.

1.9 Summary

With the rising prevalence of neuropsychiatric disorders, in particular anxiety, it is important to understand the underlying causes behind these diseases. Obesity is currently a worldwide epidemic, including amongst pregnant women, so it is important to understand how maternal obesity will affect future generation's disease risk and behaviour, particularly in relation to neuropsychiatric disorders. Increasing research suggests both maternal and postnatal obesity are linked to neuropsychiatric disorders and altered HPA axis function which may be an underlying cause, however the mechanisms linking these factors are unclear. Previous studies show that increased inflammation from obese women may be able to cross the placenta and affect fetal brain development *in utero*. Increased inflammation is able to alter HPA function, therefore offspring from obese mothers may have altered HPA function that may in turn affect behaviours, such as anxiety and memory (Figure 1.6). In addition, prolonged obesogenic high-fat feeding of offspring into their adult life may further influence HPA function, affecting behaviour and cognition. Limited information is currently known about the effects of both maternal and later postnatal obesity combined on offspring HPA function and behaviour, and the mechanisms involved.

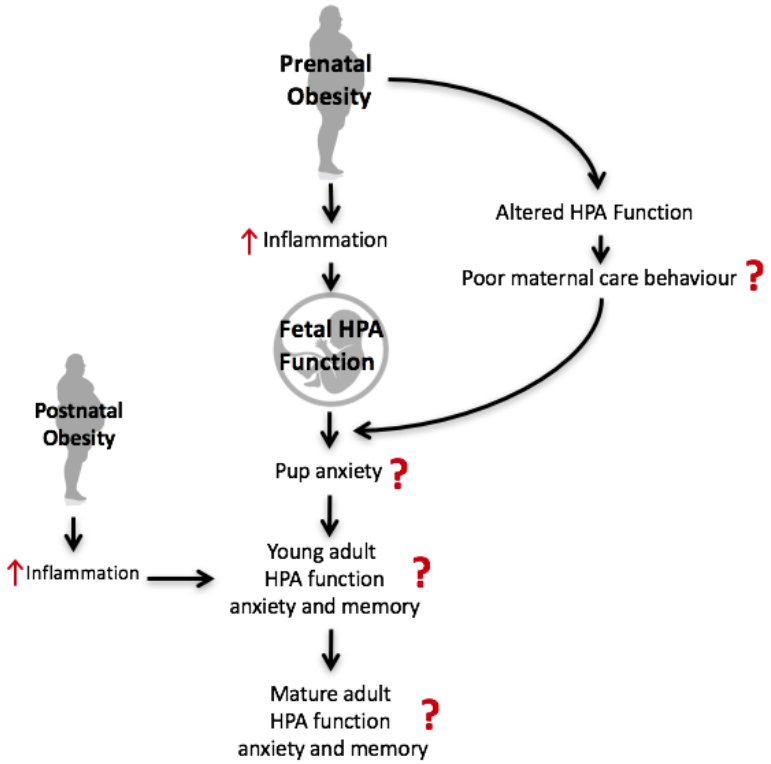

Figure 1.6 - **Schematic Diagram of Flow of Logic**

1.10 Hypothesis and Aims

Thesis Hypothesis: Maternal obesity induced by high-fat diet will impair short and long-term offspring HPA function via a mechanism of increased neuroinflammation, which could increase anxiety behaviour and impair memory. These effects will be further modified by postnatal obesity.

Aim: Early life environment may have a long-term impact on offspring health and behaviour. The aim of this study was to investigate the impact of high-fat induced maternal obesity on offspring stress and behaviour and assess whether neuroinflammation may be a mechanism for any potential changes. In addition, the effect of postnatal obesogenic high-fat diet will also be studied alone and following maternal high-fat feeding.

Objectives

1: To investigate the effect of maternal and postnatal obesogenic high-fat feeding on maternal care at offspring postnatal day 7 and on offspring behaviour, including anxiety and memory, at postnatal day 7, and 15 and 52 weeks of age.

2: To investigate the effect of maternal and postnatal obesogenic high-fat feeding on HPA axis function in 15 and 52 week-old offspring.

3: To investigate the effect of maternal and postnatal obesogenic high-fat feeding on brain and circulating inflammation in 15 and 52 week-old offspring.

Chapter 2 General Methods

2.1 Animals

Experimental protocols were performed in accordance with the UK Home Office Animal (Scientific Procedures) Act 1986 under UK Home Office Project Licenses held by Dr Felino Cagampang (PPL30/2968 and PFE11A5B5).

2.1.1 Lifecourse high-fat study - experimental design

The experimental design is presented in Figure 2.1. A control standard chow diet (C; 7% saturated fat; standard RM1 laboratory chow [Special Diet Services, UK]) or high-fat diet (HF; 45% saturated fat; 824053; Special Diet Services, UK) was fed to 8 week old non-virgin C57Bl/6 dams (proven breeders, previously maintained on control diet) for at least 6 weeks prior to conception, during pregnancy and lactation. All of the resultant offspring were used in the study. Offspring were divided evenly from each litter to then receive either a C or HF diet from the time of weaning onwards, resulting in 4 groups of offspring: C/C, C/HF, HF/C and HF/HF (Figure 2.1). Diet formulations can be found in Appendix A. All mice were housed in a 12-hour light/dark cycle, with the light cycle during the daytime (0700 h -1900 h).

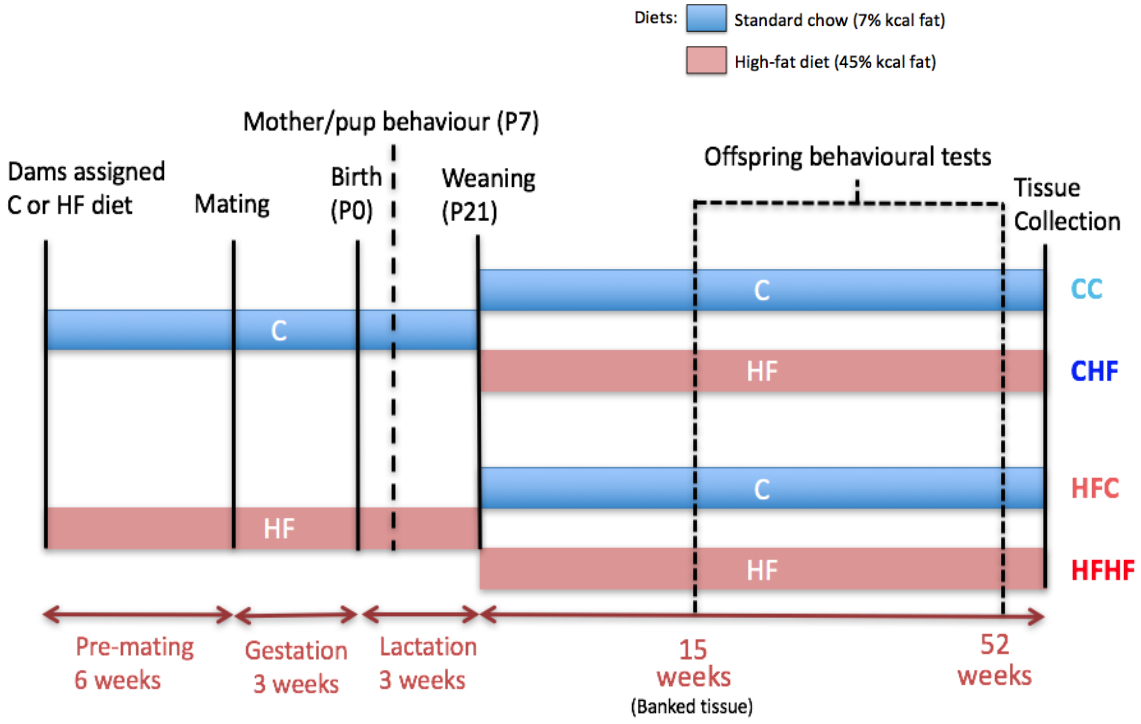

Figure 2.1 - **Experimental design**. C, control; HF, high-fat.

2.1.1.1 Cohorts A-C

Data presented in this thesis was obtained from several cohorts of animals, all using the same HF-feeding regime as shown in Figure 2.1. One cohort (C) was performed entirely by myself and followed animals to 52 weeks of age. Data were also obtained from banked tissues from animals performed previously (cohorts A-B) that followed offspring to 15 or 30 weeks of age. Further details about each cohort are described below. Details of tests performed and data obtained from each cohort can be found in Table 2.1 and numbers of animals per group for each data set are presented in Table 2.2.

Cohort A: Offspring were sacrificed at 30 weeks of age and tissue collected by Dr Felino Cagampang in 2012 (funded by Diabetes UK). No behavioural tests were conducted on these animals. (Mothers, n = 8 [C, n = 4, HF, n = 4]; Offspring n = 11[Male; CC, n = 3, CHF, n = 3, HFC, n = 2, HFH, n = 3)

Cohort B: Offspring were sacrificed at 15 weeks of age at time ZT8 (~1500 h) and tissue collected by Dr Felino Cagampang in 2010 (funded by BBSRC). No behavioural tests were conducted on these animals.

Cohort C: This cohort was performed by myself during my candidature. (Mothers, n = 14 [C, n = 5, HF, n = 9]; Offspring n = 86 [Female; CC, n = 9, CHF, n = 7, HFC, n = 11, HFHF, n = 10, Male; CC, n = 13, CHF, n = 13, HFC, n = 12, HFHF, n = 11)

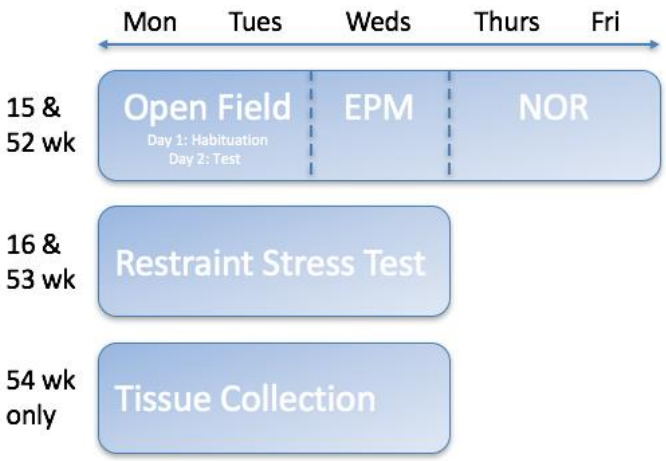

Figure 2.2 - **Behavioural testing and tissue collection schedule.**

For all cohorts, dams were weighed prior to experimental start and weekly from then on. Dams were placed on their diets for a minimum of 6 weeks prior to conception to ensure HF-fed dams reached a prerequisite 15% minimum increase in weight before mating. Although in previous cohorts the HF diet increased dam body weight by at least 35% in approximately 6-8 weeks, this was more difficult to achieve in cohort C and the weight increase threshold was reduced to 15%.

The mean time to reach the weight threshold was 8 weeks (range 6 – 10). Twenty dams started the HF protocol however 11 dams were excluded from the study: 6 dams did not gain weight after 6 weeks on the HF diet, 2 dams did not achieve a pregnancy despite a 2 week mating period, 1 dam had a rectal prolapse and 2 dams cannibalised their litter. A minimum of 15% weight increase was achieved in 9 dams fed the HF diet. All offspring were weighed weekly from weaning (day 21) until 16 weeks of age, after which offspring were weighed fortnightly.

Table 2.1 - **Data obtained from each mouse cohort.**

	Pup behav	**15 wk behav**	**15 wk gluc tol**	**15 wk molec**	**15 wk blood**	**30 wk immuno**	**52 wk behav**	**52 wk molec**	**52 wk blood**
Cohort A	X	X	✓	X	X	✓	X	X	X
Cohort B	X	X	✓	✓	✓	X	X	X	X
Cohort C	✓	✓	X	X	✓	X	✓	✓	✓

Table 2.2 - **Number of mice in each experimental group for each data set. For full experimental details see below.**

DATA SET	**COH-ORT**	**CC**		**CHF**		**HFC**		**HFHF**	
		M	F	M	F	M	F	M	F
Pup Anxiety	C	11	9	X	X	13	13	X	X
Maternal Behaviour (pups used)	C	6	4	X	X	8	8	X	X
15 wk OF	C	13	9	13	7	12	11	11	9
15 wk EPM	C	12	8	13	7	12	10	11	10
15 wk NOR	C	12	9	13	7	11	10	10	9
15 wk mRNA analysis	B	4	5	6	4	5	4	5	3
15 wk Cort ELISA	C	8	6	6	6	6	6	6	6
30 week Immuno	A	2-3	X	2-3	X	2-3	X	2-3	X
52 wk OF	C	10	8	12	4	12	9	7	8
52 wk EPM	C	11	9	12	5	12	9	7	8
52 wk NOR	C	11	9	12	5	11	8	7	8
52 wk mRNA analysis	C	7-8	8	7	4-5	7-8	7-9	6-7	8
52 wk Cort ELISA	C	7	6	6	5	6	6	6	6
52 wk ACTH ELISA	C	7	6	6	5	6	6	6	6
52 wk IL-6 ELISA	C	9	7	8	5	6	6	7	7

2.1.2 Offspring Tissue Collection and Processing

For Cohorts A-B: Mice were fasted overnight from 1700 h prior to tissue collection. Tissue collection occurred between the hours of 13 00 – 1700 h. For Cohorts A-B mice were anesthetised with halothane and under deep anaesthesia cardiac puncture was performed to obtain whole blood into a lithium heparin (LiHep) tube. This was centrifuged at 3000 rpm for 10 minutes at 4°C to obtain plasma which was stored at -80°C until further use. Mice were then killed by cervical dislocation and the whole brain was extracted from the mouse skull.

Cohort A: Whole brain was placed in a cryotube and snap frozen in liquid nitrogen and stored at -80°C until sectioned for immunohistochemistry (IHC).

Brain was placed into Optimal Cutting Temperature Compound (OCT; Sakura, USA) and anchored to a metal stand with the front of the brain facing outwards. After placing in the cryostat at -16°C and orientating the brain so that slicing would give a cross-section of the brain, sections were cut until just before the hypothalamic region was reached. Ten sections 12 µm thick were consecutively cut with hypothalamus and hippocampus present (Figure 2.3). These were collected on an uncoated microscope slide and immediately placed over dry ice and stored at -20°C until used for IHC (see Section 2.3).

Cohort B: Whole brain was placed on clean sterile aluminium foil (precooled on dry ice) and placed on dry ice with the cortex facing down on the surface of the foil. The brain was allowed to slowly freeze before wrapping with foil and transferred directly to -80°C for future cryostat sectioning and micropunching out the PVN.

Cohort C: Mice were not fasted overnight prior to tissue collection. Tissue collection occurred between the hours of 1300 h – 1700 h. Mice were sacrificed via cervical dislocation and cardiac puncture was performed while the heart was still beating to obtain whole blood which was collected into a LiHep tube (Sarstedt, Germany). Oestrus stage of female mice was assessed (see section 2.1.2.3). This was centrifuged at 3000 rpm for 10 minutes at 4°C and the plasma was stored at -80°C until further use (see Section 2.5.3). Whole brain, pituitary, adrenal glands, spleen and adipose tissue (inguinal, mesenteric and gonadal) were collected in cryotubes and snap frozen in liquid nitrogen and then stored at -80°C for future work. Only brain tissue was used for the current study.

For Cohort B-C: OCT was used to anchor whole brain to a metal stand, with the front of the brain facing outwards. After placing in the cryostat at -16°C and orientating the brain so that slicing would give a cross-section of the brain, sections were cut until just before the PVN region was reached. Three sections 200 µm thick were consecutively cut which span the entire PVN region of

the brain. These were collected on an uncoated microscope slide and immediately placed over dry ice. A 1 mm micropunch (Harris Uni-core) was used to separately punch out the PVN and CA3 area (Figure 2.3) in each of the 3 sections. Samples were stored at -80°C until further use for analysis of HPA-related and inflammatory mRNA levels (Section 2.4.1.1).

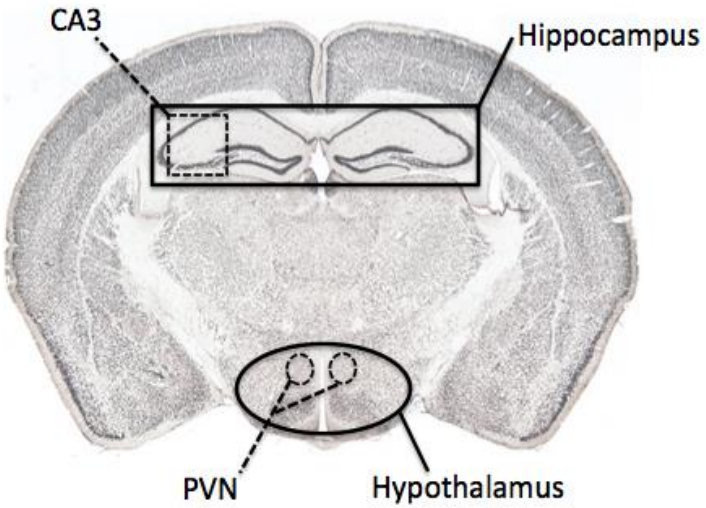

Figure 2.3 - **Cross-section of mouse brain showing positioning of tissues of interest; hippocampus containing CA3 and hypothalamus containing PVN.**

2.1.2.1 Cohort C: The effect of maternal and postnatal HF diet on 52 week female oestrus cycle

The oestrus stage at sacrifice was assessed by using the vaginal cytology method. In brief, a pipette was used to flush the vaginal cavity with water in order to take up cells, and the solution was placed onto a uncoated microscope slide. Using a standard microscope set to 10x magnification, cell types were assessed and cycle stage noted. There are 4 stages of the mouse cycle: proestrus, estrus, metestrus and diestrus.

Table 2.3 - **Oestrus stage at sacrifice in Cohort C.**

52WK FEMALE OESTRUS CYCLE STAGE	PROESTRUS	OESTRUS	METESTRUS	DIESTRUS
CC	0	1	4	4
CHF	0	2	3	0
HFC	0	2	2	3
HFHF	2	2	3	1

2.1.3 Lipopolysaccharide (LPS)-treated mice

LPS-treated mice were used as a control measure to assess a widely used mouse model of inflammation which could be compared to my data (367, 368). C57BL/6 female mice between 4-5 months of age (n = 2) were injected with 200 µl of LPS at 10µg/ml concentration into the intraperitoneal cavity. Animals showed sickness behaviour suggesting brain inflammation. Mice were sacrificed 4 to 6 hours after treatment and perfused. Whole brain, pituitary and adrenals were collected and snap frozen in liquid nitrogen and stored at -80˚C. PVN and CA3 brain regions were micropunched the as for Cohorts B-C in section 2.1.2.

2.2 Behavioural Tests

Maternal care behaviour was assessed by a pup retrieval test 7 days after birth (P7). Offspring behavioural testing included measurement of ultra-sonic vocalisations (USVs) at postnatal day 7 and OF, EPM, NOR and restraint stress tests at 15 and 52 weeks of age.

2.2.1 Maternal Behaviour – Pup Retrieval Test

In previously reported pup retrieval protocols (262, 369, 370), all pups or several pups from a litter are entered into the home cage at the same time. In order to measure both maternal care and pup USVs from individual mother-pup pairs, I used a modified protocol in which pups were entered into the home cage one at a time.

Experimental Procedure: At P7, mothers and pups were transported to the test room in their home cage and left to acclimatise for 30 minutes. Pups were then separated into a new cage and the mother in the home cage placed in a separate room. After 40 minutes the mother in the home cage was brought back into the test room. A pup was selected randomly from the litter and placed back into the home cage with its mother at the furthest side opposite the nest, ensuring the mother was in her nest. The following measurements were taken:

- ***Investigation Latency –*** time taken for the mother to initially locate the pup.
- ***Interaction*** – time taken between the mother finding the pup and picking up the pup.
- ***Nest Latency*** – time between the mother picking the pup up to successfully bringing it back to the home nest.

If 120 seconds passed without successful pup retrieval to the nest, this was noted as a failed test. The first pup was then removed to another cage and a second pup chosen at random from the litter was then placed into the home cage, when the mother was in the nest.

The Pup Retrieval test was performed 20-40 minutes after USV test (see section 2.2.2.1). All pups in each litter underwent this test for consistency to ensure all later behaviour was not attributed to changes in pup testing. Only two pups were included in maternal average results however, as this was the largest litter some mothers had and also prevented maternal test learning behaviour.

Data Analysis: Investigation Latency, Interaction and Nest Latency of only the first two pups tested were averaged per mother and results compared from control and HF-fed mothers.

2.2.2 Offspring Behaviour

Offspring behaviour was measured at P7 (pup), 15 weeks (young adult) and 52 weeks (mature adult) of age in order to assess whether maternal HF and/or postnatal diet alters offspring anxiety and memory. In adult animals, 4 behavioural tests were performed (see below). The order of experiments was always the same and took place over a 2 week period, starting at each of 15 and 52 weeks. The protocol is presented in Figure 2.2. NOR tests took place on either the Thursday or Friday of week 1. The restraint stress test for each animal took place on one day in week 53. At the completion of all experiments at 53 weeks, animals were killed for tissue collection at 54 weeks (see Figure 2.2). A week was required to complete the restraint stress tests and tissue collection from all animals from each batch. From here on in, the age of experiments will be simply referred to as 15 or 52 weeks.

2.2.2.1 Ultra-Sonic Vocalisations (USVs) during Maternal Separation

This test assessed pup anxiety and was performed at P7 as C57Bl/6 pups are known to call between 3-12 days of age (371). In general, mouse pups call ultrasonically at approximately 50-80 KHz (372) with C57Bl/6 mice most often calling at 70 kHz (369, 373), which cannot be detected by human ears. USVs can be detected using hardware such as bat detectors, which measure the mouse vocalisations and modify the noise into a frequency detectable by humans. The protocol was adapted from a previous study (372).

Experimental Procedure: At P7, mothers with their litter of pups were transported to the test room in their home cage and left to acclimatise for 30 minutes. Pups were then separated into a fresh cage and the mother in the home cage placed in a separate room. Pups then remained separated from their mother for 10 minutes. A single pup (with as little handling as possible) was then weighed and placed in a shallow box placed on a heating pad and filled with 1 cm of wood shavings. USVs were recorded for 4 minutes with a bat detector (Bat4, Magenta, UK) set at 70 kHz placed ~5 cm above the pups and recorded in waveform audio file format (.wav) files using Audacity software (Audacity 2.1.0, USA). 2 males and 2 females were recorded per litter and selected at random.

Data Analysis: USVs were analysed using sound analysis software SAS Lab Lite (Avisoft Bioacoustics, Germany). Sound files were visualised using SAS Lab Lite (Figure 2.4) and quantified by the number of individual call USVs and also 'call clusters', defined as a grouping of calls made within 1 second of each other, which were considered as a single call outburst, visible between 60-80 kHz during the 4 minutes of recording.

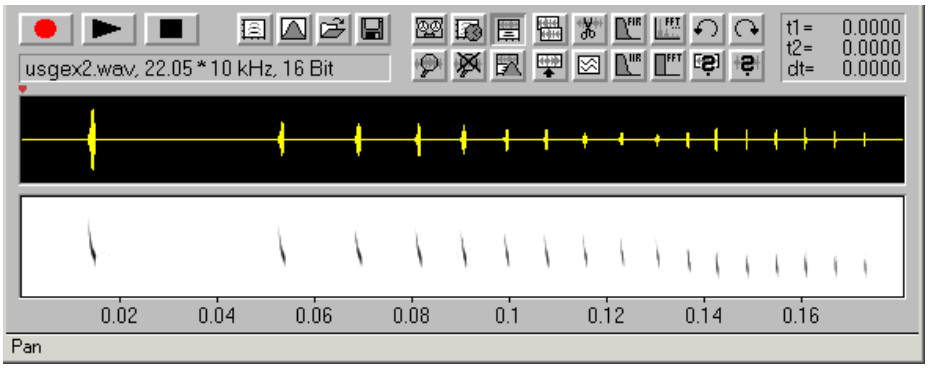

Figure 2.4 - **Representative example of USVs from a 7 day-old pup during maternal separation, shown visually using software SAS Lab Lite.**

2.2.2.2 Open Field (OF) Test

The OF test measures exploratory and locomotor activity in mice and is also used to assess anxiety behaviours (374). In order to minimise anxiety induced by handling, routine cleaning of mouse cages took place 3 days before testing (375). The test was performed twice on the same day (in the morning then in the afternoon) and the results averaged to minimise any potential diurnal variations (376). Experimental conditions were kept as silent as possible and the mice were unable to see the experimenter during test period.

Apparatus: The OF box was a 38 x 38 cm enclosure with a white floor and clear walls with 4 metal walls outside the clear walls. Each wall comprised of 16 evenly spaced lasers on 2 levels, allowing for tracking of mouse horizontally around the box, as well as vertically. Four OF boxes sit side by side so that 4 mice can be tested concurrently.

Test Habituation: One day prior to OF testing, mice were habituated to the test environment. Mice were transported to the test room in their home cages and left to acclimatise for 30 minutes. The mice were then placed in the OF test box and permitted to explore for 5 minutes, mimicking the actual test. The mice were then returned to their home cages and the OF test box disinfected and odours eliminated with Hibiscrub ready for the next mouse.

Experimental Procedure: Mice were placed in the test room in their home cages and left to acclimatise for 30 minutes. The OF software, Activity Monitor 6 (Med Associates Inc, USA), was set up to record mouse movements around each test box. Individual mice from consecutive home cages were then placed in the top right corner of OF test boxes and the test was run for 5 minutes. Tested mice were placed in a separate cage until all mice from each home cage were tested. The number of faeces and spots of urine were recorded from each mouse's test. Hibiscrub was used to disinfect and eliminate odours between testing.

Data Analysis: The Activity Monitor software creates a box around the mouse during the OF test, defined by the number of beams on the X and Y-Axis (4 x 4). When the animal is moving within the box (i.e. grooming, rearing etc.) the beam breaks are called '*stereotypic events*' (Figure 2.5A). If the animal moves outside of the box and breaks 3 beams, in a time that is less than the resting delay (500 ms), this is defined as an 'ambulatory event' (Figure 2.5B).

For each OF test, the Activity Monitor software produces a data file with the following measurements:

- *Stereotypic Counts* – The distance travelled making stereotypic events.
- *Time Stereotypic* – The time spent making stereotypic events.
- *Time Resting* – Time after an ambulatory episode when mouse stays inside the set area for duration of resting delay (500 ms).
- *Ambulatory Count* – The rectilinear distance travelled making ambulatory events (Figure 2.5C).
- *Ambulatory Time* – The time spent whilst ambulatory.
- *Ambulatory Episode* – The number of episodes of time stopping and starting ambulation.
- *Distance Travelled* – The distance travelled 'as the crow flies' (Figure 2.5C).
- *Vertical Count* – The number of times the mouse rears (rearing is defined as X or Y-axis and Z-axis beams being broken concurrently).
- *Jump Counts* - A period of time when there are no X or Y-axis beam breaks. (Limitation - this may reflect mouse standing against wall and beams going through legs).
- *Average Velocity* - The average velocity during ambulatory episodes (cm/s).

OF Activity Monitor software was also used to analyse distance travelled, time resting and entries into the centre vs outer area of the OF box.

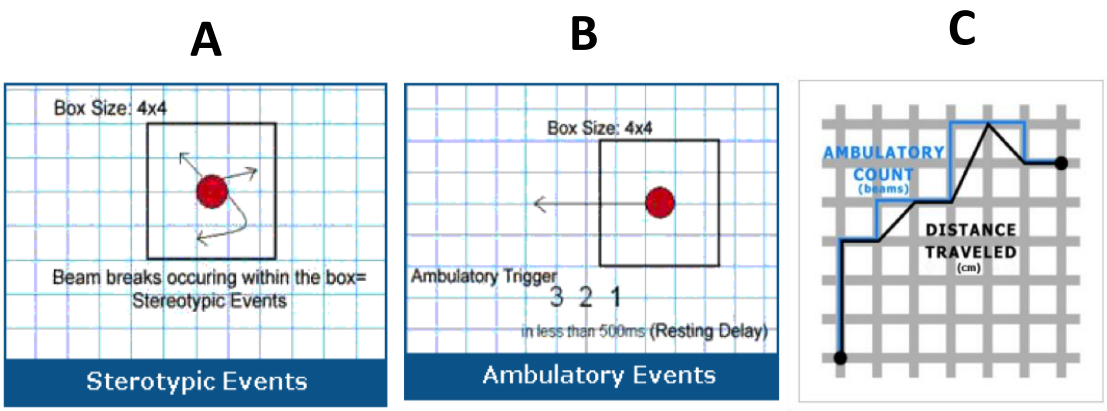

Figure 2.5 - **Schematic diagram of stereotypic events (A), ambulatory event (B), distance travelled and ambulatory counts (C) during the OF test.**

2.2.2.3 Elevated Plus Maze (EPM) Test

The EPM is a well-validated test assessing anxiety (377). Rodents typically prefer to spend time in darker, safer areas, i.e. the closed/darker arms of the maze; therefore, the time spent in the exposed open arms shows less anxiety-like behaviour. Mice habituate to the EPM quickly so acclimatisation is not performed for this test as it would bias results. This protocol was used as previously described (378).

Apparatus: The apparatus is a Perspex (Poly(methyl methacrylate)) elevated platform comprising of 2 open arms and 2 closed arms (each 50 cm long and 10 cm wide). The closed arms have walls that are 40 cm high (Figure 2.6). The EPM was elevated by 50 cm from the ground on an MDF (medium density fibreboard) base. Paper was placed over the closed arms to ensure a darkened area and lines drawn on the platform centre area to separate each arm and the centre square, with a non-toxic odourless red marker pen, for easier visualisation. Lines were also drawn in the middle of each open arm (creating a far right and far left section) in order to assess how far mice were willing to explore the outer reaches of the open space. A camera was placed above the centre junction on a clear acrylic platform and used to record the trial.

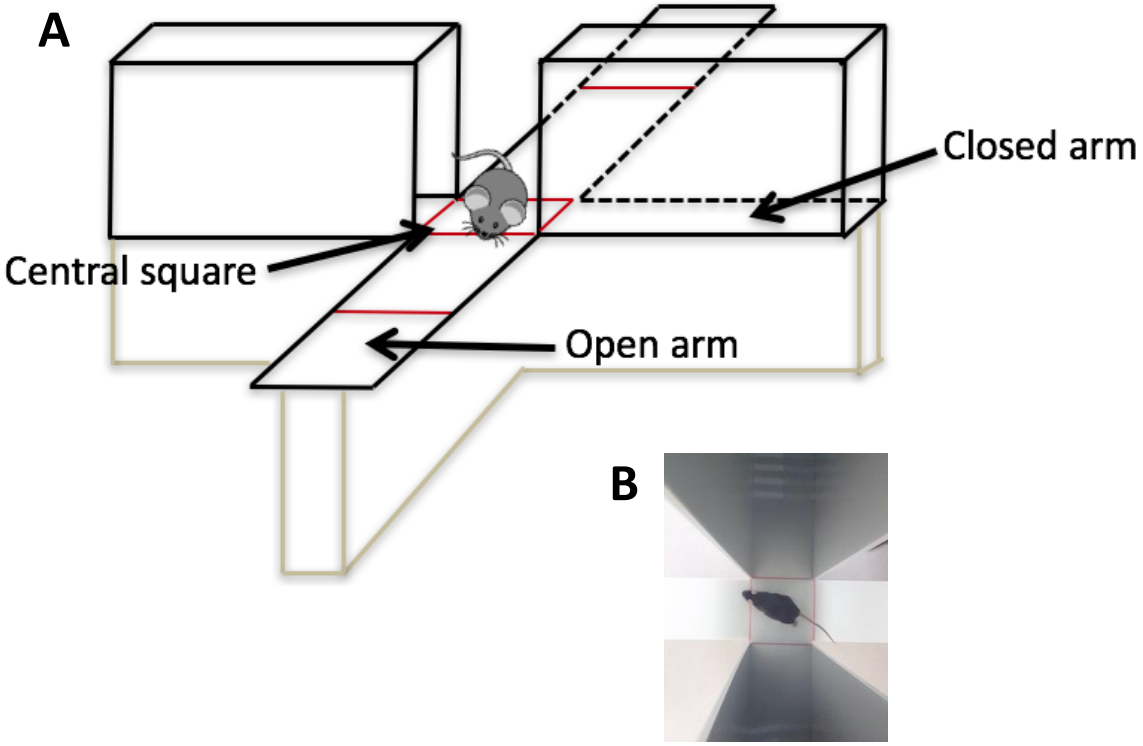

Figure 2.6 - **EPM apparatus design (A) and view from recording apparatus (B) of mouse movement during EPM testing.**

Experimental Procedure: No previous habituation was required for this experiment. Mice were transported to the test room in their home cages and left to acclimate for 30 minutes. The EPM was cleaned with Hibiscrub and left to dry. Lines were drawn into the EPM as explained previously. Mice were placed individually in the centre junction of EPM facing opposite the experimenter towards an open arm and video recording was started. After the experimenter left the room, the test was run for 5 minutes and the number of entries into the far right and left open arm sections recorded by observing through the window of the experimentation room. After the test, mice were returned to fresh separate cages and the EPM was disinfected with Hibiscrub. Mice were returned to home cages after all mice for that day were tested.

Data Analysis: Using playback software (DivX, San Diego, USA), the video recordings were viewed and time spent in each arm was calculated. Entry into a set open or closed area was defined by the placement of the mouse's hind legs into that area (379).

2.2.2.4 Novel Object Recognition (NOR) test

The NOR test assesses non-spatial recognition memory in rodents. This memory test was adapted from previous protocols (380, 381). The premise for this test is that mice naturally investigate unfamiliar (novel) objects in their environment and therefore mice are expected to spend more time with a new object when it is presented, indicating intact normal non-spatial recognition memory.

Apparatus: OF test boxes were used for the NOR test, taking advantage of the fact that habituation to the box had previously occurred during OF testing. The objects used were 50 ml corning flasks filled with sand and a red Lego® sculpture of similar size and shape to the flasks. Both objects had a small upper surface, to prevent mice from sitting on top of them, as observed in preliminary trials testing a range of possible objects for the test. For each subsequent mouse experiment the position of the novel object was changed (top left or bottom right) to ensure no bias to novel object placement. Movement of the mice during the trial was recorded with video cameras on stands approximately 30 cm above the testing area. Hibiscrub was used to clean the objects and the box between trials (132).

Experimental Procedure: Mice were transported to the testing room and left to acclimatise for 30 minutes in their home cages. *Acquisition Trial*: Two identical corning flasks were placed in the box diagonally and a mouse placed into the box facing the top right corner (equidistant from both objects). The trial was recorded for 4 minutes using a camera situated approximately 30-40cm above the OF box with the experimenter hidden from view. *Inter-Trial Period:* After 4 minutes the mouse was removed and placed in a new cage for one minute, the testing box was cleaned and both corning flasks were removed. A new corning flask (identical to that used in the acquisition trial) and a Lego® sculpture were placed diagonally into the box in the same location as the previous objects. *Retention Trial*: After one minute out of the testing box, the mouse was placed back into the box facing the top right corner as before. Another 4 minutes were recorded (Figure 2.7). After the trial, the mouse was placed back in a separate cage until all mice were tested.

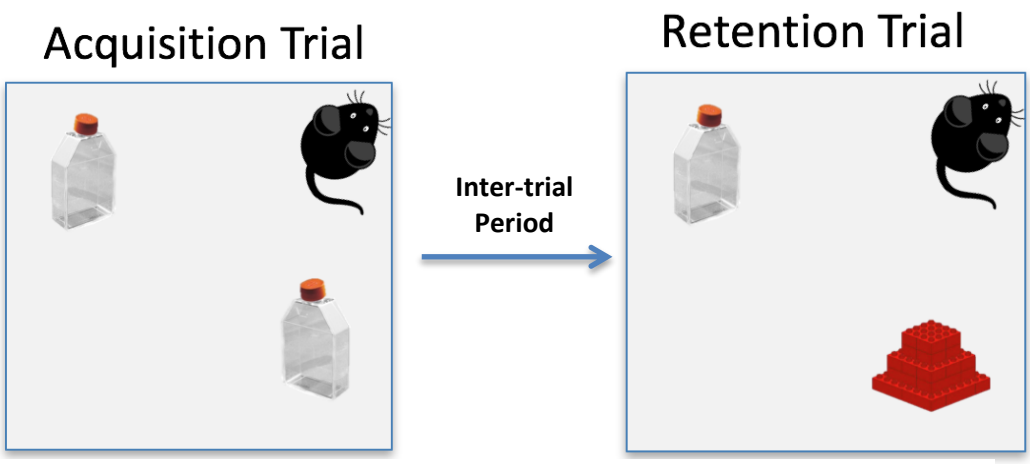

Figure 2.7 - **Novel object recognition test setup of acquisition and retention trials.**

Data Analysis: Exploration of an object was defined by the mouse smelling, licking or touching the object, with the front paws closely towards the object. Other behaviours such as sitting on the object were not accepted and this interaction was not counted. The total time spent exploring each object was calculated.

The following parameters were calculated:

- Total time spent exploring both identical objects in the acquisition trial (s)
- Total time spent exploring both objects in the retention trial (s)
- Total time spent exploring objects (acquisition + retention trial) (s)
- Discrimination Index (DI) – calculated from just the retention trial

$$DI = \frac{\text{exploration time of novel object} - \text{exploration time of familiar object}}{\text{Total exploration time of objects}}$$

A lower discrimination index indicates a poorer ability to discriminate between the object, thus a poorer recognition memory.

2.2.2.5 Restraint Stress Test

The restraint stress test assesses stress responsiveness by measuring plasma corticosterone concentrations prior to, during and after a psychological stressful event. This protocol was based on previous study (382).

Apparatus: Acrylic restraint tubes (VetLab Supplies Ltd, Pulborough, UK) were used for the restraint stress test. The restraint tubes were clear red in order to create a darkened environment for the mice but to allow observation of the mouse to ensure its health and wellbeing. Hibiscrub was used to disinfect and remove odour from restraint tubes between testing each mouse.

Experimental Procedure: Tests were performed between 0900 h and 1200 h. Mice in their home cages were placed in the testing room for 30 minutes to acclimatise. Five minutes before testing,

topical anaesthetic (5% lidocaine, Glaxosmithkline, UK) was applied to the mouse's tail. Each mouse was placed into a restraint tube and a tail snip of <1 mm was performed. Immediately after being placed in the restraint tube (0 minutes), blood was taken (~50 µl) into a lithium-heparin coated Microvette® tube (Starstedt, Germany) via tail 'milking'. The mouse remained in the restraint tube for 30 minutes, with further blood samples at 15 minutes and 30 minutes from the start. The mouse was then released from the restraint tube and put into a fresh cage, with careful monitoring of recovery. At 60 minutes from initial restraint, the mouse was briefly placed back into the restraint tube to take a final blood sample. Mice were then placed back into home cages and further monitored for 30 minutes. Blood samples were then centrifuged at 2000 RPM for 5 minutes and separated plasma was pipetted into a new Eppendorf tube and stored at -80°C until use.

Data Analysis: Corticosterone concentrations were assessed by a corticosterone enzyme-linked-immunoassay kit (Enzo Life Sciences, USA) (see Section 2.5.1).

2.3 Immunohistochemistry

IHC was performed to measure the presences of inflammatory markers in brain sections. These included CD68 and CD11b which are standard markers of microglial activation, as well as GFAP which is a marker of astroglial activation. 30 week-old non-perfused (previously snap-frozen) whole brains from mouse Cohort A were used for immunohistochemical analysis.

2.3.1 Immunohistochemical Staining

Sections of brain cut by cryostat were dried and post-fixed in absolute alcohol for 15 minutes at 4°C. They were then washed 3 times for 5 minutes each in phosphate-buffered saline (PBS) in a rocking machine. Slides were then quenched for endogenous peroxidise activity using 1% H_2O_2 for 10 minutes at room temperature (RT). They were then washed for 5 minutes with fresh PBS twice. Sections were then incubated in 2% Bovine Serum Albumin (BSA, Fisher Scientific, Loughborough, UK) at RT and 1% serum for one hour at RT to block non-specific binding and then incubated in primary antibody (or goat serum for control tissue) overnight at 4°C. The following day, sections were washed for 10 minutes in PBS on an orbital rotator machine three times. They were incubated with secondary antibody for 1 hour. After incubation they were washed in PBS again for 5 minutes three times in total. For DAB staining the sections were then incubated in Avidin Biotin Complex (Vector Labs, Peterborough, UK) for 30 minutes at RT. Sections were washed in PBS for 10 minutes three times. Staining was detected by using DAB as the chromogen, catalysed by

0.015% H_2O_2. Slides were incubated for 30 seconds in the DAB solution and then immediately washed in PBS to prevent DAB from overstaining. Haematoxylin was used as counterstain before dehydration and coverslipping.

2.3.2 Immunofluorescent Staining

Sections of brain cut via cryostat were dried and post fixed in absolute alcohol for 15 minutes at 4°C. They were then washed 3 times for 5 minutes each in PBS in a rocking machine. Sections were then incubated in 2% BSA at RT and 1% serum for one hour at RT to block non-specific binding and then incubated in primary antibody (or goat serum for control tissue) overnight at 4°C. The following day, sections were washed for 10 minutes in PBS on an orbital rotator machine three times. They were incubated with secondary antibody for 1 hour. After incubation they were washed in PBS again for 5 minutes three times in total. Sections were then incubated for ten minutes in 1 ug/ml Hoescht 33258 (Sigma Aldrich, Poole, UK) to stain nuclei and cover slipped using Prolong gold anti-fade reagent™ with DAPI (Invitrogen, Paisley, UK). Fluorescence images were taken using a Leica DM5000 microscope (Leica, UK). Details of antibodies are shown in Table 2.4.

Table 2.4 - **Details of primary and secondary antibodies used for immunofluorescence.**

Primary Antibody	Antibody Details	Catalogue #	Distributor	Concentration
CD11b	Rat mAB	MCA711	AbD Serotec	2 ug/ml (1:500)
CD68	Rat mAB	MCA1957	AbD Serotec	2 ug/ml (1:500)
GFAP	Rabbit mAB	P14136	Sigma Aldrich	2 ug/ml (1:500)

Secondary Antibody	Antibody Details	Catalogue #	Distributor	Dilution
Rabbit anti-rat IgG	Biotinylated	BA-4001	Vector Laboratories	(1:500)
Donkey anti-rat IgG	Alexafluor®488	A21208	Invitrogen, UK	(1:500)
Donkey anti-rabbit IgG	Alexafluor®488	A21206	Invitrogen, UK	(1:500)

2.3.3 Immunofluorescent Analysis

Immunofluorescent analysis was performed using LAS AF Lite (Leica Microsystems, UK) and ImageJ (Image Processing and Analysis in Java) software. Using a Leica DM5000 3 channel fluorescence camera, 10x magnified images of hypothalamic or hippocampal area for each separate sample were captured, ensuring the same area of interest was taken for all samples from all treatment groups. All images were anonymised to reduce bias during the image analysis process. LAS AF Lite software was then used to separate these images into colour channels (Dapi

blue; nuclei, Tx2 red; blood vessels, GFP FITC green; inflammatory marker) and saved as a .tif file. The GFP FITC green colour channel was used to analyse markers of inflammation. Each sample's GFP FITC colour separated image was then imported into ImageJ. Using the 'threshold' function, the image was set to a constant value based on each inflammatory marker assessed. Minor background noise in the image was removed using the 'despeckle' function. The image was then set to analyse the following: area, area fraction and mean grey value. The results then gave the % area value and mean total area of staining of the inflammatory marker of interest. Both outcomes corresponded to each other for all samples and mean total area of staining was used to express staining in graphs (Chapter 5).

2.4 mRNA Levels

2.4.1 RNA Isolation of Brain PVN/CA3 Tissue (Cohorts B and C)

RNA was extracted from micropunched PVN and CA3 brain tissue as per instructions obtained from Dr Felino Cagampang who has refined RNA extraction from small quantities of brain tissue. The tissue was taken from Cohort B at 15 weeks of age and from Cohort C at 52 weeks of age.

Tissue was minced on ice using a kimble pestle in 200 µl Trizol until tissue was dissolved. 50 µl chloroform was added, shaken vigorously for 15 seconds and incubated at RT for 15 minutes. Samples were then centrifuged at 12,000 rpm for 10 minutes at 4°C. The upper aqueous phase was transferred into a new eppendorf and 100 µl of isopropanol and 20 µl glycogen (2 µg/µl working solution) were added to the sample. This was shaken and stored overnight at -20°C. The following day frozen tubes were centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatant was removed, the pellet washed in 200 µl 80% ethanol and centrifuged again at 12,000 rpm for 10 minutes at 4°C. As much ethanol was removed as possible from the samples and the RNA pellet left to dry for 45 minutes at RT. The RNA pellet was finally eluted in 30 µl of RNase-free water and gently pipetted into solution. RNA samples were kept on ice until they were stored at -80°C.

2.4.2 RNA quality analysis

2.4.2.1 RNA Yield and Purity

Determination of RNA quality and quantity was performed using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Delaware, USA). 1.5 µl of RNA sample was analysed by measuring UV-visible absorbance (220 nm-750 nm). The NanoDrop displayed concentration in

ng/µl and purity as two ratios at 260 nm and 280 nm, and 260 nm and 230 nm. A satisfactory purity reading was taken as 2.0 ± 0.5.

2.4.2.2 RNA Quality

RNA quality was also assessed by Agarose gel electrophoresis. Good quality mammalian RNA should show 2 clear bands (28S and 18S ribosomal RNA). RNA samples were prepared by mixing 2 µl (or 1 µl of RNA sample if the NanoDrop showed the RNA was of high concentration and quality) of the sample with 5 µl RNAse free water, 1 µl RNA loading buffer and 2 µl de-ionized formamide. All 10 µl was added to each gel well.

Agarose gel (2%) was made by dissolving Agarose (Sigma Aldrich, UK) in 1x Tris/Borate EDTA buffer (TBE; Thermofisher, UK) and heating in a microwave until boiling point was just reached and the solution was clear. This solution was allowed to cool and GelRed (0.03 µg/ml) added. Gels were then cast in a gel tank, any bubbles removed with a pipette tip and a gel comb added. Once the gel had set, gel electrophoresis was performed in an electrophoresis tank containing 1x TBE buffer and run at 130 V for 20 minutes. Gels were imaged using GeneSnap (Syngene, Synoptics Ltd., Cambridge, UK). The presence of two clear bands (28S and 18S) show that RNA is undamaged (Figure 2.8).

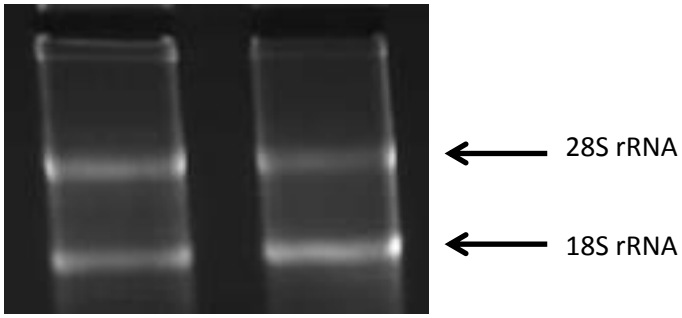

Figure 2.8 - **Representative image of an Agarose gel showing 18S and 28S rRNA banding in mouse hippocampal tissue.**

2.4.3 cDNA synthesis

To create cDNA from RNA, reverse transcription (RT) PCR was performed using TaqMan® Reverse Transcription Reagents (ThermoFisher Scientific, Basingstoke, UK). A reaction mixure was created by adding 2 µl 10x RT Buffer, 4.4 µl $MgCl_2$ (25 mM), 4 µl dNTPs Mix, 1 µl random hexamers, 0.4 µl RNAse inhibitor and 0.5 µl of Multiscribe Reverse Transcriptase, per reaction. This mastermix was made up with reverse transcriptase enzyme added last. 12.3 µl of mastermix was added to each PCR tube. The appropriate amount of RNase-free water was added, and 400 ng RNA added last to make 20 µl in total per well. Each sample was gently mixed and pulse centrifuged briefly to spin

down the solution. Samples were transferred to a thermal cycler and ran at 25°C for 10 min, 48°C for 30 min, 95°C for 5 min and 4°C infinity. Once samples were reverse transcribed, they were diluted 1 in 5 with RNAse-free water and stored at -20°C until used for qPCR.

As well as the RNA samples, standards and controls were also put through RT PCR. Pooling 1-2 µl from each RNA sample together into one tube created a 'standard'. This standard was 1:2 serially diluted with nucleic acid-free water following RT PCR for qPCR assay. Two 'non enzyme controls' (NECs) were created by replacing reverse transcriptase with RNase-free water to test for genomic DNA contamination during RNA extraction. A 'no-template control' (NTC) was created by replacing RNA sample with RNase-free water, thus checking for nucleic acid contamination in RT PCR mastermix. 'Coefficient of variation' (CV) controls were created by using pooled RNA samples (as in standard) to create 6 identical samples to assess sample variability across each plate.

cDNA synthesis of all PVN and all CA3 samples at both ages and sexes were created separately as real-time qPCR was performed on PVN and CA3 separately. Positive controls of LPS-stimulated (4h) mouse brain tissue and plasma (n = 2) were used to assess inflammatory gene of interest (GOI) qPCR results too.

2.4.4 Housekeeper gene selection for PVN and CA3 tissue

The Primerdesign geNorm™ Reference Gene Selection Kit (Primerdesign, UK) with Double-Dye (hydrolysis) probe for mouse was used to determine the optimal, most stable housekeeper genes (HKGs) to use in the study. A 6-gene geNorm was performed using ACTB, SDHA, CYC, PGK1, ATP5B, and 18S HKGs. These were tested on a random subset of samples; three samples per diet group per sex per age per brain region. All samples were plated out in duplicate and all 6 HKGs were tested using the 384-well plate qPCR protocol below. The crossing point (cp) values were analysed using qbase+ 3.0 software (Biogazelle, Belgium), and from this analysis, the software generated the average expression stability value (M) and the pairwise variation value (V). The M value shows how stable the HKGs are, with a lower M value representing a more stable HKG, as shown in Figure 2.9. The V value determines the optimum number of reference genes to use. The graph in Figure 2.10 shows the effect of including an extra HKG and how the normalisation factor changes with the addition of another HKG. The optimal number of reference genes is when the V score drops below 0.15, indicating that there is no significant effect of adding another HKG. Therefore the data in Figure 2.10 show that between 2-4 HKGs are optimal to use for normalisation of target genes. In this study I chose 3 HKGs and details of these can be found in Table 2.5.

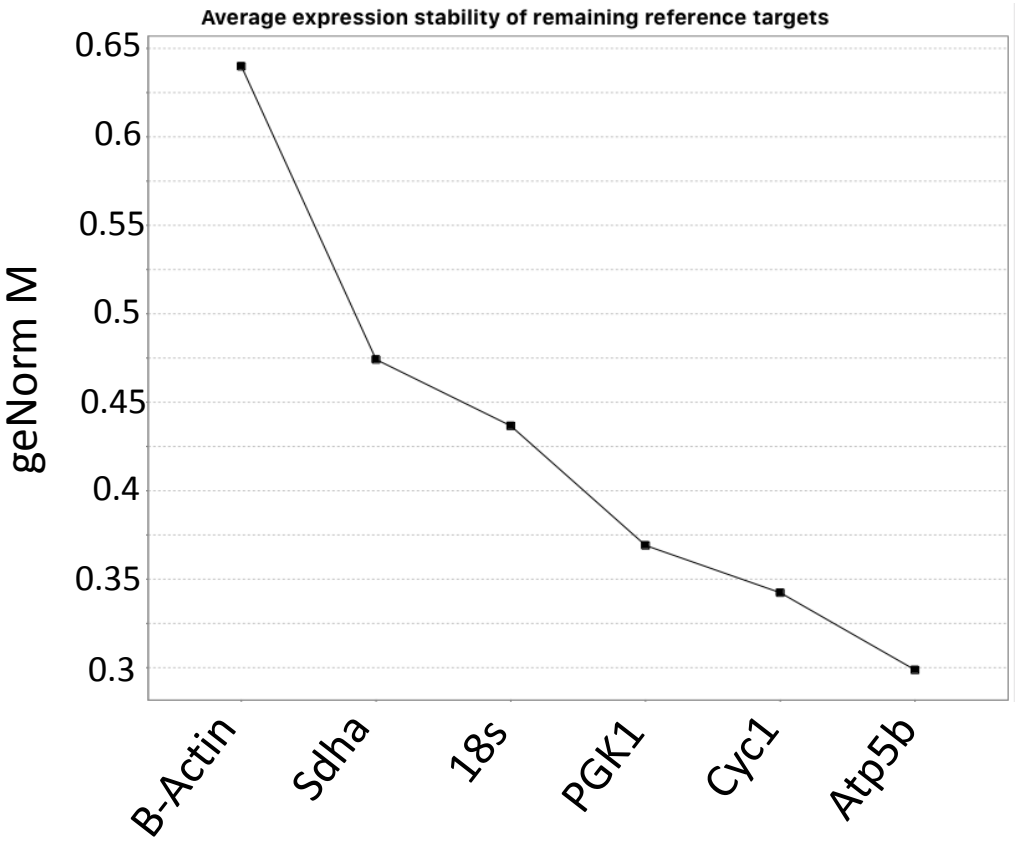

Figure 2.9 - **geNorm analysis M Values of HKGs.** PGK1, CYC1 and ATP5B have the lowest M score and are therefore the most stable.

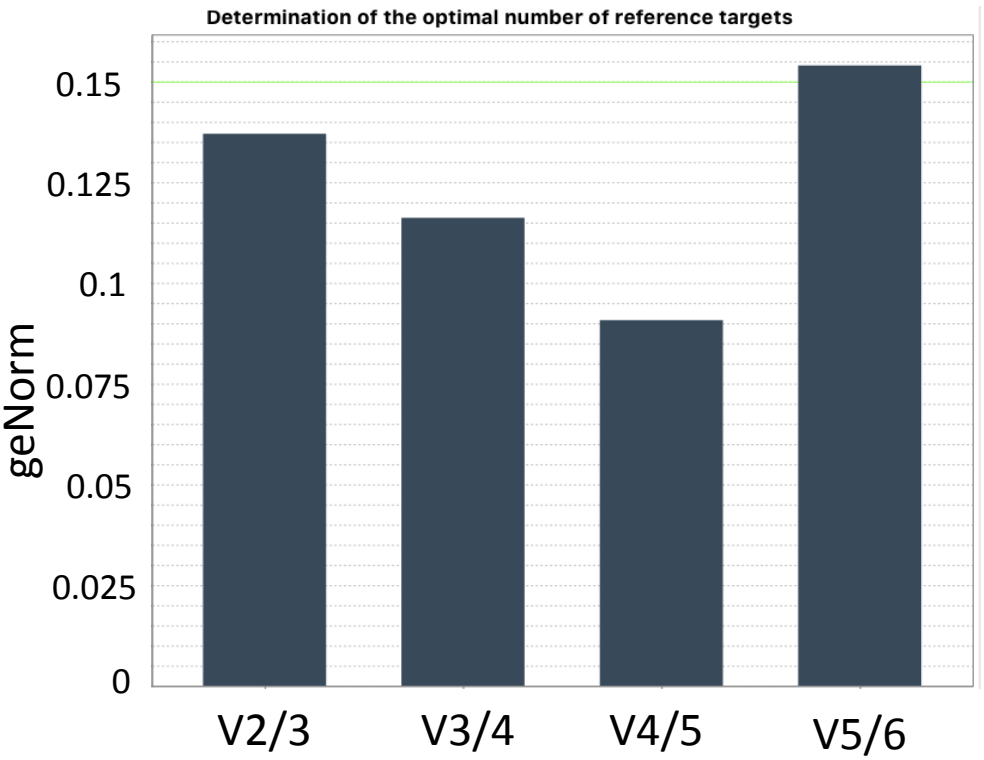

Figure 2.10 - **geNorm analysis V score.** The use of two-four HKGs have a V score of below 0.15 and therefore the optimal number to use for normalisation of target genes.

Table 2.5 - **Primer sequences**. Primer sequences (5' to 3') used to measure mRNA levels of genes of interest by qRT-PCR and associated probe number.

GENE	Accession No.	Company	Primer	Sequence 5'-3'
CYC1	NM_025567.2	PrimerDesign	------	------
ATP5B	NM_016774.3	PrimerDesign	------	------
PGK1	NM_008828.3	Sigma Aldrich	Forward Reverse	5'-GTCGTGATGAGGGTGGACT -3' 5'-TTTGATGCTTGGAACAGCAG -3'

2.4.5 GOI Primer Design

Most primers used in this study were previously validated by Prof Jessica Teeling's group (data unpublished) however FKBP51 and MR were designed and validated by me using the design specifications shown in Table 2.6. Primers were assessed on NCBI Primer Blast tool and ordered from Sigma-Aldrich Custom DNA Oligos (Poole, UK). Only primers that spanned an exon-exon boundary were chosen, in order to avoid amplification of DNA. Primer specificity was assessed via qPCR melt-curve analysis and running the qPCR product on a 2% Agarose gel (see Section 2.4.2). Specificity was determined by whether a single melt curve was observed during qPCR, and on the gel if a single band of correct primer DNA size was detected.

Table 2.6 - **Primer design specifications.**

	MINIMUM	OPTIMUM	MAXIMUM
PRIMER SIZE	10	20	30
PRIMER TM	55	60	70
PRIMER GC%	40	55	60
PRODUCT SIZE	100	200	300
SELF COMPLEMENTARITY	1	2	6
3' SELF COMPLIMENTARITY	1	2	6

2.4.6 Quantitative real-time PCR (qPCR)

mRNA expression levels of genes of interest were assessed by qPCR and normalised to the geometric mean of the 3 chosen HKGs (PGK1, Cyc1 and Atp5b). SYBR Green (BioRad, UK) was used to perform qPCR.

384-well plate protocol: Mastermix was first made up with reagent quantities depending on brand of primer used. PrimerDesign primer protocol: 0.6 µl Primer (10 um), 6 µl SYBR Green and 0.4 µl of RNAse-free water, per reaction. Sigma Primer Protocol: 0.4 µl Forward Primer (10 um), 0.4 µl Reverse Primer (10 um), 6 µl SYBR Green and 0.2 µl of RNA-se free water, per reaction.

All samples were split for gene analysis by brain region; all ages, sexes and diet groups in PVN or CA3 samples underwent real-time qPCR on the same plate. The standards and controls created in section 2.4.3 and LPS-treated mouse samples were also placed on each plate.

Using a Thermofisher Matrix™ Electronic Multichannel Pipette, 7 µl of appropriate mastermix was added to each well. Using the same pipette, 5 µl of each cDNA sample (4 ng/µl dilution), controls and standards was also added to each well. A plate sealer was tightly secured over wells, vortexed and centrifuged briefly. Plates were then placed in a Roche LC480 qPCR machine, and Roche software used to set up the plate.

qPCR cycling conditions: Polymerase activation occurred at 95°C for 10 minutes. The product amplification occurred at 45 cycles of 95°C for 10 seconds, 60°C for 30 seconds and 72°C for 1 second (data collection step). After these 45 cycles, there was a cooling step of 40°C for 30 seconds, and then qPCR plate was cooled to 4°C until collection upon which it was stored at 4°C until further use.

2.4.6.1 qPCR analysis

All PVN & CA3 (Cohort B and C): A standard curve was created from standards serially diluted 1:2 with nucleic acid-free water as stated in Section 2.4.3. This 7-point standard curve was used to determine cDNA concentration of genes of interest. The log concentration was plotted against the CT (threshold cycle) value per standard, which should generate a straight line. The CT value is the cycle at which fluorescence reaches maximum threshold of detection. The cDNA concentration of each sample was determined by using its CT value and the standard curve parameters in the following equation:

$$x = 10^{((c-y)/m)}$$

x = cDNA concentration, ***c*** = CT value, ***y*** = y intercept and ***m*** = line gradient

The parameters for Y and M are shown in Figure 2.11.

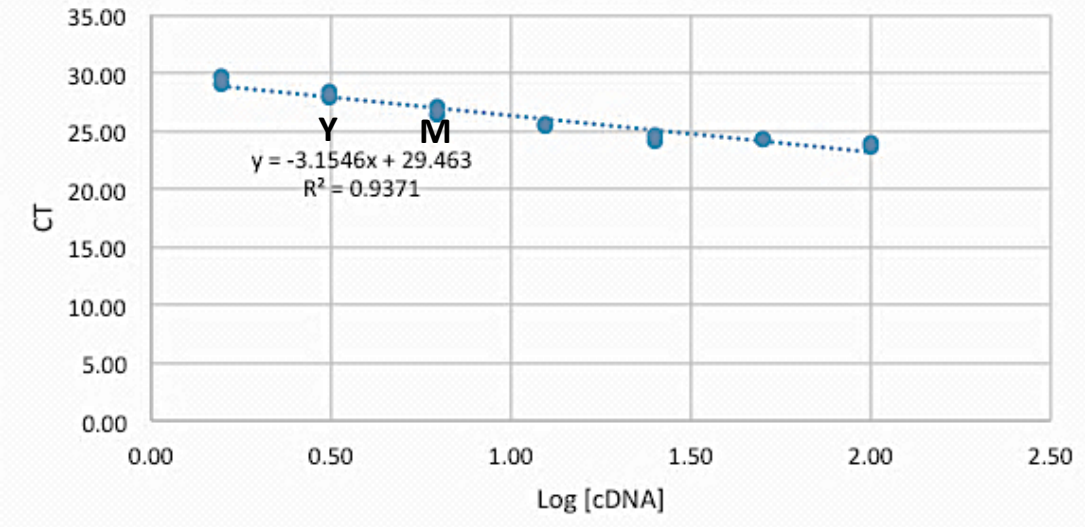

Figure 2.11 - **Representative standard curve for calculation of cDNA concentrations.**

cDNA concentrations were then normalised to the geometric mean (geomean) of the HKG's by dividing cDNA concentration of the GOI by cDNA concentration of the geomean. cDNA concentration of coefficient of variation (COV) controls was assessed for variation between these six samples. This was done using the equation **COV = (Stnd Dev/mean) x 100.**

Intra-assay values were also calculated the above COV equation**.** Values from all genes were under 10%. NEC and negative control values were found to give no or negligible values. Using a *t*-test, the means of LPS-treated samples were compared against the mean of each CC value per age per sex per plate for inflammatory and FKBP51 genes.

2.5 Immunoassays

Enzyme-Linked-Immunosorbent-Assay's (ELISA) were used to measure HPA-axis hormones during the restraint stress test: corticosterone was measured in plasma samples from both 15 and 52 week-old mice and ACTH was measured in plasma samples from 52 week-old mice. The inflammatory marker IL-6 was also measured in samples taken at post mortem at 52 weeks. All plasma samples used were obtained from Cohort C mice.

2.5.1 Corticosterone ELISA

Corticosterone concentrations were determined using the Enzo Life Sciences Corticosterone ELISA kit as per the kit instructions. Corticosterone standards were created at the following

concentrations from the provided stock solution: 20,000, 4000, 800, 160 and 32 pg/ml. Additional pooled sample standards (6 wells per plate) were used to assess intra-assay coefficient of variation on each of the 10 ELISA plates used to measure corticosterone. Plasma samples were prepared by adding 7.5 µl of sample to 7.5 µl of 1:100 Steroid Displacement Reagent (SDR). This was then vortexed, incubated at RT for 6 minutes and then 285 µl of Assay Buffer 15 was added to the sample and vortexed again. 100 µl of samples and standards were pipetted into appropriate wells of the 96-well ELISA plate included in the kit. 100 µl of Assay Buffer 15 was pipetted into appropriate wells to assess non-specific-binding (NSB) and blank results. A further 50 µl of Assay Buffer 15 was pipetted into NSB wells. 50 µl of blue conjugate was added to samples, standards and NSB wells. 50 µl of yellow antibody was added to samples and standards only. The plate was then covered with a plate sealer and incubated at RT on an orbital plate shaker set at 500 rpm for two hours. The wells were then emptied by pouring off the solution and washed 3 times with 1:20 Wash Buffer. All of the final wash buffer was aspirated from wells and 5 µl of blue conjugate added to a Total-Activity (TA) well. 200 µl pNpp Substrate Solution was added to every well. The plate was then incubated at RT for one hour without shaking. 50 µl of Stop Solution was added to every well and the plate was read immediately at optical density 405 nm. Results were corrected for the values obtained in the blank wells.

2.5.1.1 Calculation of Results

The average net Optical Density (OD) bound for each standard and sample was calculated by subtracting the average NSB OD from the average OD bound:

Average Net OD = Average Bound OD – Average NSB OD

The binding of each pair of standard wells was calculated as a percentage of the maximum binding wells (Bo), using the following formula:

Percent Bound = (Net OD/Net Bo) × 100

The percentage bound versus corticosterone concentration of the standards was plotted and a straight line was fitted. The concentration of corticosterone in the samples was determined by interpolation from the standard curve.

Using the pooled sample controls, coefficient of variation was calculated using the following equation **Coefficient of Variation = (Standard Deviation / Mean) * 100.** The average intra-assay COV was 0.98% showing accurate pipetting. The interassay COV value was 1.82% showing consistency in corticosterone results between plates.

2.5.2 ACTH ELISA

ACTH concentrations were determined using the Enzo Life Sciences ACTH ELISA kit as per the kit instructions. ACTH standards were created from the calibrators provided at the following concentrations: 477, 182, 56.5, 19.2 and 7 pg/ml. COV was not calculated for ACTH due to only 2 plates being used. 200 µl of sample, standards and controls were pipetted into designated wells of a Streptavidin coated 96-well plate. 25µl Biotinylated Antibody and 25 µl of Enzyme Labelled Antibody was added to the same wells. The plate was then covered with aluminium foil and incubated at RT on an orbital plate shaker set at 170 rpm for 4.5 hours. The plate was then aspirated and washed 5 times using a working dilution of 1:20 Wash Buffer. 150 µl of TMB Substrate was added to each well. The plate was then again covered with aluminium foil and incubated at RT on an orbital plate shaker set at 170 rpm for 35 minutes. 100 µl Stop Solution was added to each well and the plate read immediately at optical densities 450 nm and 405 nm. The second reading is designed to extend the analytical validity of the highest calibration curve to the value represented by the highest standard calibrator.

2.5.2.1 Calculation of Results

By using the final absorbance values obtained, a dose response calibration curve was constructed using 4 parameter logistics online ELISA analysis software (Leading Technology Group, Australia). Samples and controls were read using the 450nm results for ACTH concentrations up to 150 pg/ml. ACTH concentrations above 150 pg/ml was interpolated using the 405 nm calibrator results.

2.5.3 IL-6 ELISA

IL-6 in plasma was measured using a DuoSet® ELISA Development System (R&D Systems, Abingdon, UK) according to the manufacturer's instructions with minor modifications.

100 µl capture antibody (working dilution 2 µg/ml in PBS) was pipetted into each well of a 96-well MaxiSorp™ (Nunc, Fisher Scientific, Loughborough, UK). The plate was sealed and incubated at RT overnight. The next day the plate was inverted and blotted on clean paper towels and each well was then aspirated and washed with Wash Buffer (0.05% Tween 20 in PBS). This step was performed 3 times in total. The plate was then blocked for a-specific binding by pipetting 300 µl Reagent Diluent (1% BSA in PBS) into each well and incubated at RT for one hour. The plate was then washed three times as above, ready for sample addition. 100 µl of sample (diluted 1:1) and standards (serially double diluted starting at 1 ng/ml) in Reagent Diluent was added per well. The plate was sealed and incubated at RT on an orbital plate shaker set at 170 rpm for 2 hours. The

plate was then washed three times as above and 100 µl Detection Antibody (150 ng/ml diluted in Reagent Diluent) was pipetted into each well. The plate was sealed and incubated at RT for two hours. The plate was then washed again three times as above. 100 µl Streptavidin-HRP (1:40 dilution with Reagent Diluent) was added to each well and the plate placed in darkness and incubated at RT for 20 minutes. 50 µl Stop Solution (2 N H_2SO_4: R&D Systems, USA) was added to each well and the plate gently tapped to ensure thorough mixing. The plate was immediately read at optical density 450 nm.

2.5.3.1 Calculation of Results

Concentrations were determined by interpolating optical density measurements from a 4 parameter standard curve, using online ELISA analysis software (Leading Technology Group, Australia).

2.6 Statistical Analysis

Offspring data were analysed using a custom-created mixed effects model with the main factors of maternal diet, postnatal diet and sex (except 15 week relative mRNA expression data which was statistically analysed by two-way analysis of variance (ANOVA) due to each offspring sample being from a different mother, and having standardized littersizes, and therefore did not require mixed model analysis). A two-way ANOVA was not used because this assumes all mice are independent of each other and cannot factor in the litter of origin of each offspring (mother) or the littersize of each mouse. As mice from the same litter/mother will be more similar than mice from other litters/mothers, the mixed model factors in these dam effects. Littersize varied considerably amongst dams (n = 2-11) and since all offspring from each dam were used, it was important to factor in this variable. Data analysed using the mixed effects model is presented as the corrected 'predicted' group means ± standard error of the mean (SEM). SEM values were derived from the maximal models containing the 'fixed' effects of littersize and diet group. Statistical analysis was performed on non-transformed data or z-transformed data, depending on whether data were normally distributed. This was assessed via data skewness (Skewness < 1 for non-transformed data and skewness > 1 for z transformed data). Data were z-transformed in order to standardise variables where necessary and allow predicted data to be directly compared.

In the NOR test, an independent *t*-test was used to compare the time spent with the left vs right object within each diet group and sex (Chapter 3). Maternal pup retrieval was also analysed using an independent *t*-test, by comparing maternal C or HF diet groups (Chapter 3). LPS-treated mouse data from each gene and brain region (per qPCR plate) was compared to the CC group of each sex

and age on the same qPCR plate using a *t*-test to perform pairwise multiple comparisons i.e. all GOI PVN CC groups were compared to LPS values from the same plate (Chapter 4 and 5). All data analysed by *t*-test is represented as mean ± SEM. Correlation data were analysed on Graphpad Prism.

SPSS (version 22) was used for all statistical analysis of data, except correlations which were performed in GraphPad Prism. All graphs were created in GraphPad Prism. For Chapter 3, data were presented as both sexes combined unless a sex effect was found wherein the results were split by sex. For Chapter 4 all data were presented split by sex as the HPA axis is well-known to display differential sex effects, and most results statistically displayed sex differences anyway. For Chapter 5 due to the frequent sex differences displayed in Chapter 4, 15 week cytokine (TNF-α , IL-1β, IL-6) data, which had no sex effects and low sample numbers, were displayed as both sexes combined but also split by sex to look at trends in the data in each sex.

Power calculations could not be performed before the onset of this thesis in order to determine appropriate experimental group sizes for my cohort of mice (Cohort C) due to the absence of suitable pilot data from previous studies. However, using the first data obtained in 15 week old offspring from OF and NOR testing and from GR in PVN from Cohort B 15 weeks, aiming for a statistical power of 80%, the recommended sample size suggested was n = 7-8 per group per sex. For my mouse Cohort C, I aimed for n = 10 offspring per sex per age.
The actual animal numbers achieved are listed in section 2.1.1.1, showing that sample sizes in my study were sufficiently powered. In Chapter 4 and 5 sample sizes (n = 3-6) used for 15 week mRNA level analysis were dictated by the availability of animal tissues collected in a previous study by Dr Felino Cagampang (Cohort A).

Chapter 3 The effect of maternal and postnatal HF diet on offspring behaviour

3.1 Introduction

Neurological disorders such as anxiety, depression and cognitive problems are an increasing problem around the world. In this chapter, the link between maternal and/or postnatal obesity and potential consequent effects on anxiety and memory will be addressed. There is increasing evidence to suggest that maternal nutrition affects neurodevelopment and has long-term effects on mental health in adult life (383, 384). In addition, maternal undernutrition has been shown to influence cognitive function (385). Obesity is now common in pregnant women with over half of women of childbearing age being overweight or obese in the UK alone. Obesity during pregnancy not only increases pregnancy complications, but can also affect the overall health of the offspring long-term (386). Maternal obesity gives rise to increased weight gain and cardio-metabolic risk (62) in offspring, as well as being associated with increased neuropsychiatric disorders (74).

In human studies, increased BMI before pregnancy has been associated with negative emotionality (387) and decreased cognitive and language scores (388) in children. Maternal obesity also increases the likelihood of low and high birth weight (389), both of which are associated with anxiety in adolescents (390). The relationship between maternal obesity in humans and cognitive disorders is more difficult to ascertain due to difficulties diagnosing children and implementing long-term studies. However, animal studies allow us to investigate the effects of maternal obesity under controlled testing conditions and using well validated behavioural tests. Maternal obesity has been shown to affect maternal care in rodents (289), which in turn has been linked to programming of anxiety phenotypes in adult rodent offspring (391). In humans, maternal care is also associated with offspring behaviour outcome. For example, post-partum depression has been linked to mental health problems in adolescence (392) and maternal stress has been linked to increased anxiety and negative social interactions in children (393). Thus, the effects on offspring of maternal obesity may be indirect via maternal care, as well as through direct effects on offspring neurodevelopment.

Animal experiments commonly investigate the effects of obesity during pregnancy by the feeding of HF diets. Most studies use rats and mice, with HF ranging from 30-78% of total energy from fat and ‘moderate’ obesity described as between 10-25% greater body weight than age matched

control animals (394). In mice it has been shown that a maternal 60% HF diet is able to induce anxiety and impair spatial learning in 3-month-old offspring during a Morris water maze test and EPM (277). This study showed that these outcomes were sex-specific, affecting male offspring more than females. Male offspring fed a maternal HF diet displayed anxiety via faster swimming (increased motivation to escape) during the Morris water maze test and also spent significantly longer inside the closed arms of the EPM, which the mice perceive as a "safe" area. Another study of maternal 60% HF diet demonstrated increased anxiety in 1 month-old female offspring via reduced distance travelled in the centre of OF test and hyperactivity via increased distance travelled in the OF test in male offspring (324). However, effects of sex are not observed in all studies. Sasaki *et al.* found that both 3 month-old male and female offspring fed a maternal 60% HF diet displayed anxiety during OF test and EPM, respectively. Maternal obesity has also been linked to reduced cognitive function. Spatial and non-spatial memory was found to be affected by maternal obesogenic HF-feeding. One study showed that male offspring fed a maternal 60% HF diet impaired memory during non-spatial NOR testing at 12 weeks of age (395). Spatial memory via repeated Morris Water Maze trials in 20-week-old rats was also impaired by a 60% HF maternal diet (278). This study also included postnatal HF-feeding and the strongest impairment was seen in offspring fed a HF diet in both maternal and postnatal life. These studies indicate that there is a link between maternal HF diet and cognitive impairment, although further investigation is necessary to determine if outcomes are age or sex-specific.

In humans, a systematic review across populations and ages linked obesity in adult life to increased anxiety (47), and memory deficits have also been associated with obesity (396). In animal models, assessing postnatal obesogenic HF-feeding alone, anxiety has been reported to be elevated. In a mouse model using a 45% fat diet, 16 weeks of feeding induced anxiety phenotypes, as shown by a reduction of centre time and entries in an OF test (50). This study also showed the onset of anxiety may be time-specific as the majority of these phenotypes were not seen at 12 weeks of feeding, but arose 4 weeks later. A similar study by Andre *et al.* showed a similar outcome. Male mice fed a 49% fat diet showed increased anxiety, as shown by reduced time spent in the open arms of an EPM after 18 weeks of feeding, but not 9 weeks (52). The percentage of dietary fat may also play a role in observed anxiety behaviours, as one study using male mice fed 58% fat showed distinct anxiety phenotypes during OF and EPM after 12 weeks of feeding (54). Animal studies also show effects of HF diet-induced obesity on memory. In male mice fed a 60% fat diet for 20 weeks, spatial memory was significantly impaired in object recognition memory test (similar to NOR) and object location memory test (397). However, in aged 15 month-old male mice also fed a 60% fat diet no difference was seen compared to control diet (398). Overall these studies show that in controlled animal models of diet-induced obesity,

anxiety phenotypes and memory impairments occur, although this appears to be time sensitive and may be dependent on percentage fat intake/diet content. Since many studies only use males, more information is required about the potential sex differences in the effect of obesity on neurological disorders.

There are very few studies that have investigated the potential interaction between exposure to obesogenic maternal and postnatal HF diets on behavioural outcomes. The maternal exposure to a HF diet may predispose to neurological disorders and/or increase susceptibility to the effects of subsequent dietary challenges later in life (399). Therefore, the study described in the following chapter investigates the effects of maternal and postnatal obesity, their potential interactions on behavioural outcomes and whether males and females are affected differently.

3.2 Hypothesis and Aims

Hypothesis: Maternal HF diet will increase offspring anxiety and impair memory, via direct effects on neurodevelopment of the offspring and indirectly via inducing poor maternal care. These effects will be exacerbated by a postnatal HF diet.

Aims: To investigate the effects of maternal and/or postnatal HF diet on

1) Maternal care behaviour
2) Offspring anxiety at 1, 15 and 52 weeks of age
3) Offspring memory at 15 and 52 weeks of age.

3.3 Methods

3.3.1 Cohort C: HF mouse model

Non-virgin female C57BL/6 mice were fed either a control (C, 7% kcal fat) or high-fat (HF, 45%kcal fat) diet between 6-11 weeks (depending on length of time to reach minimum compulsory 15% weight gain) before mating and during pregnancy and lactation (C, *n* = 5; HF, *n* = 8). Offspring were then fed a C or HF diet post-weaning from 3 weeks of age creating 4 dietary groups: CC, CHF, HFC and HFHF, as detailed in section 2.1.1 and in appendix A.

3.3.2 Maternal Care and Pup Ultrasonic Vocalisations

Experiments were performed as previously described in sections 2.2.2.

3.3.2.1 Pup Retrieval Test

At 7 days of age (P7), pups were separated from their mothers and placed into a fresh cage for 40 minutes. Mothers in the home cage were placed into a separate room. After this time the mother and home cage were brought back into the testing room and pups randomly selected and individually placed into the home cage (t_0). The time taken for the mother to retrieve the pup back to the home nest was recorded in three periods of time.

- ***Investigation Latency –*** time taken from t_0 to initially locate the pup.
- ***Interaction*** – time taken between finding and picking up the pup.
- ***Nest Latency*** – time between picking the pup up to retrieving successfully back to the home nest.

Failure to retrieve pups after 120 seconds was also noted.

3.3.2.2 Pup Ultrasonic Vocalisations

At P7, after ten minutes of maternal separation (see above), pups were placed into a small box filled with bedding from their home nest, which was placed over a heat mat to ensure temperature was approximately equal to home nest temperature; 30-33°C (measured with a thermometer). Two randomly selected male and female pups from each litter were then recorded with a bat detector (Bat4; Magenta, UK) using sound recording software (Audacity 2.1.0, USA) for four minutes and replaced back into their litter after testing.

3.3.3 Adult Offspring Behavioural Tests

Experiments were performed as previously described in sections 2.2.2.2, 2.2.2.3 and 2.2.2.4. All adult behavioural testing occurred at 15 and 52 weeks of age. Mice were placed in the testing

room for 30 minutes prior to testing to acclimatise before each experiment took place. Tested mice were placed in a separate cage until all mice from each cage had undergone testing to avoid interacting with untested mice.

3.3.3.1 Open Field

Mice were habituated to the OF environment one day before testing as described in section 2.2.2.2. Mice were placed into the OF box and their movements recorded via specialist software (Activity Monitor 6, Med Associates, USA) for five minutes. Experimental conditions required silence and the inability for mice to see experimenters during trial period. OF tests were conducted once in the morning (9.00h – 11.00h) and once in the afternoon (14.00h – 16.00h). The results were averaged to minimise experimental variability and changes due to diurnal rhythm.

3.3.3.2 Elevated Plus maze

Mice were not habituated to this test, as a naive reaction was required. Mice were placed on the centre square of the maze facing towards the west open arm. A camera placed above the centre square on a clear plastic mount recorded the number of entries and time spent in each arm for five minutes. Experimenters left the room while the test was being performed in order to prevent influencing the areas the mice would choose to enter, but scored entries into the far reaches of the open arms by observing through a door window.

3.3.3.3 Novel Object Recognition

NOR was performed in the same box as the OF maze and therefore mice did not require further habituation to this testing area. Mice were placed inside a box facing the top right corner, 2 identical objects (corning flasks filled with sand) were placed top left and bottom right of the box. Mice were allowed four minutes exploring this environment; this time period is referred to as the **acquisition trial**. Mice were then removed and placed in a separate cage for one minute whilst the objects are removed, the testing area and objects cleaned with hibiscrub, and one familiar object (corning flask filled with sand) and one novel object (red Lego® tower) were placed back in the box; this time period is referred to as the **inter-trial period**. Mice were then placed back in the box and left to explore the environment for a further four minutes; this period is referred to as the **retention trial**.

Cameras set up above the area recorded videos of acquisition and retention trial for later analysis (details in section 2.2.2.4). Interaction time between the mouse and the objects were scored. Interaction included sniffing and exploring with the mouse's feet pointing toward the object.

3.3.4 Statistical Analysis

A mixed effects model was used to analyse offspring data (see section 2.6 for details). This statistical model was used as it factored in the effects of littersize and which dam each animal originally came from. Therefore, all results are independent of dam and littersize.

Data presented in graphs are the predicted means and SEM derived from maximal models (including diet group and littersize) applied to the observed data. Depending on whether the data were normally distributed, data were either not transformed or Z-transformed before mixed model statistical analysis. This ensured dependant variables were standardised allowing the effect estimates to be compared directly. Maternal dam/pup retrieval raw observed data (unadjusted) was statistically analysed using *t*-test. Linear regression analysis was performed using GraphPad Prism. All other analyses were performed using SPSS version 24.

Data were presented as both sexes combined unless a sex effect was found wherein the results were split by sex.

3.4 Results

3.4.1 The effect of HF diet on maternal weight gain

There was no difference in the weight of dams prior to start of the experimental diets (Figure 3.1C). However, there was a difference immediately prior to conception with HF-fed dams significantly increased ($P < 0.0001$) in body weight from control dams (Figure 3.1D). The average time it took for dams to reach minimum weight gain was 8 weeks (range of 6-10 weeks, minimum time 6 weeks). Since dams took different times to reach the threshold, weight gain is presented for the 6 weeks prior to conception (Figure 3.1A). Both groups appeared to gain weight during pregnancy at a similar same rate between C and HF-fed dams (Figure 3.1B). Dams fed a HF diet were still significantly heavier ($P < 0.01$) than C-fed dams at weaning (P21) (Figure 3.1E) and they also had significantly smaller average littersizes ($P < 0.05$, Figure 3.1F).

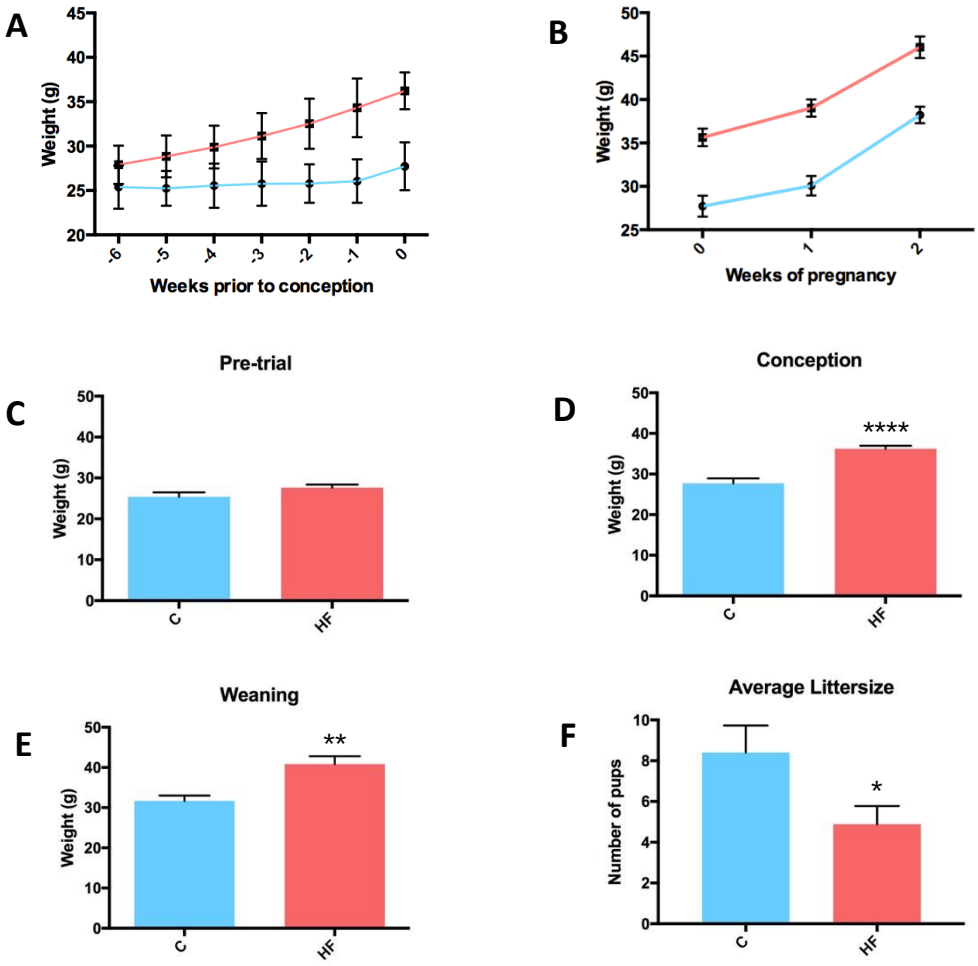

Figure 3.1 - **Maternal weight gain before, during and post- pregnancy and average littersize.** Effect of control (blue; n = 5) and HF (pink; n = 9) diet on dams the 6 weeks prior to conception (A), during pregnancy (B), pre-trial (C), at conception (D), at weaning (E; 3 weeks post pregnancy) and average littersize (F). **** $P < 0.0001$, ** $P < 0.01$ maternal HF diet vs. C. (C – F). Data are displayed as mean ± SEM and statistically analysed by an independent *t*-test.

3.4.2 The effect of maternal HF diet on pup postnatal day 7 and 3 week-old body weight

At both P7 and 3 weeks of age, male offspring weighed significantly more than female offspring ($P < 0.0001$ for both). However, there was no significant effect of maternal diet on weights in either sex at P7 (Figure 3.2A and B). At 3 weeks of age there was a trend for female offspring from HF-fed dams to have greater body weight than offspring from C-fed dams ($P < 0.1$, Figure 3.2D), but there was no difference in weight between offspring when dividing them into their subsequent 4 experimental groups (data not shown). There was a significant negative relationship between pup weight at P7 and dam weight at conception ($P < 0.05$, $R^2 = 0.071$, Figure 3.2E).

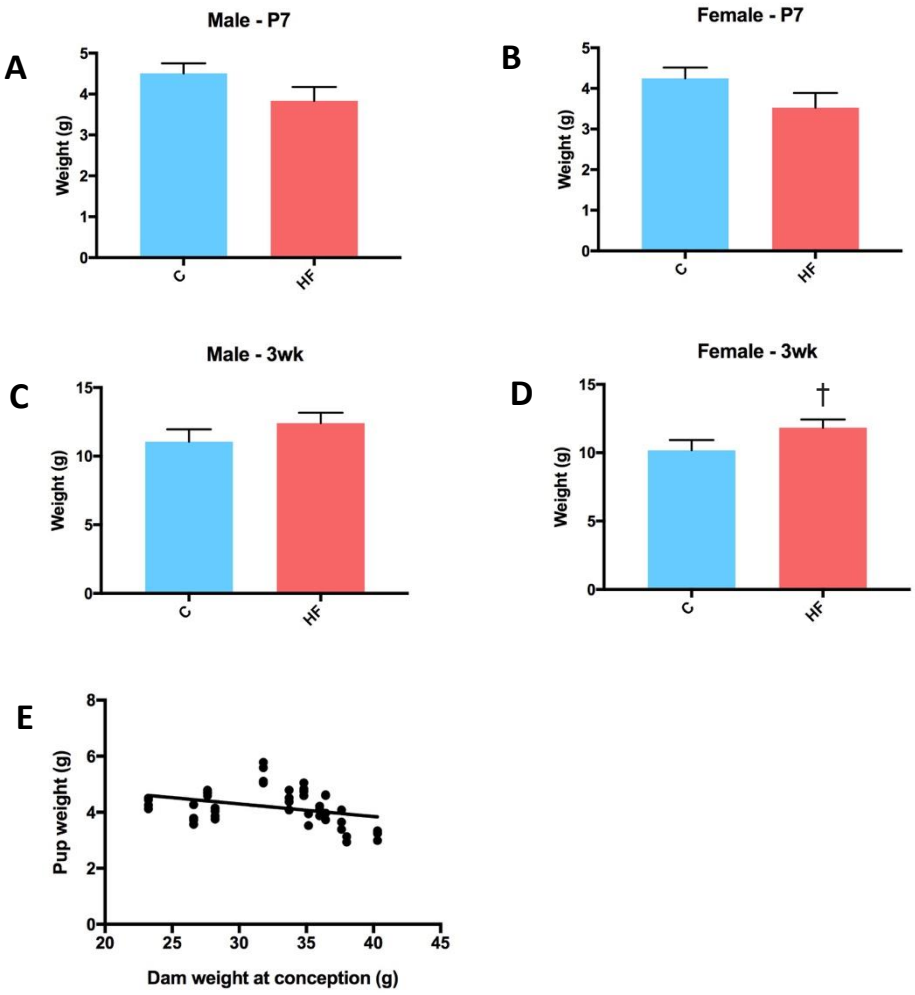

Figure 3.2 – **Offspring body weights at postnatal day 7 and 3 weeks.** Effect of maternal control (blue; n = 11 for males, n = 9 for females) and HF (red; n = 13 for males, n = 13 for females) diet on male (A, C) and female (B, D) offspring; (E) the relationship between dam weight at conception and pup weight at postnatal day 7 correlation ($R^2 = 0.071$). †, $P < 0.1$. Data are displayed as predicted means ± SEM from maximal models.

3.4.3 The effect of maternal and postnatal HF diet on adult offspring weight

Offspring weights between 3 weeks (weaning) and 15 weeks are shown in Figure 3.3A and B. At 15 weeks of age in both males and females there was a significant effect of postnatal HF diet to increase weight (both sexes; $P < 0.0001$, Figure 3.3C and D) and a trend for maternal HF diet to increase weight also (male; $P = 0.077$, females; $P = 0.068$, Figure 3.3C and D). In both male and female offspring, maternal and postnatal HF diet combined (HFHF) induced the largest weight difference from CC (both sexes; $P < 0.0001$), followed by postnatal only HF diet (CHF) (males; $P < 0.0001$, females; $P = 0.003$) shown in Figure 3.3C and D. In the HFHF group there was an interaction such that the maternal and postnatal HF diet group combined resulted in a greater weight that either of these factors alone (males; $P < 0.0001$ Figure 3.3C, females; $P < 0.01$ Figure 3.3D). Weights of postnatal C-fed mice (CC and HFC) were not different from each other in either male or female offspring. Male offspring at 15 weeks were significantly heavier than females overall ($P < 0.0001$).

Offspring weights between 15 weeks and 52 weeks are shown in Figure 3.4A and B. At 52 weeks, in male offspring only, maternal HF diet tended to increase weight ($P = 0.064$), but in both sexes there was an effect of postnatal HF diet to increase weight (Figure 3.4C and D, both sexes; $P < 0.0001$). Male offspring at 52 weeks were significantly heavier than females overall ($P < 0.01$).

In terms of weight gain over periods of time, there was a strong effect of postnatal HF diet to increase weight gain significantly ($P < 0.0001$) in both sexes at 3-15 weeks (Figure 3.5A and B), in females at 15-52 weeks ($P < 0.0001$, Figure 3.5D), and both sexes for total weight gain between 3-52 weeks ($P < 0.0001$, Figure 3.5E and F). This effect was a trend ($P = 0.087$) in male offspring between 15-52 weeks and there was no difference between the HFC group to either the CHF or HFHF groups (unlike all other graphs which showed significance between these groups) indicating during this time period the HFC group gained weight at a similar rate to the postnatal HF groups.

Overall, male offspring weight gain was significantly higher than females between 3-15 weeks ($P < 0.0001$, Figure 3.5A and B) and total weight gain between 3-52 weeks ($P < 0.05$, Figure 3.5E and F). However, female offspring weight gain was increased between 15-52 weeks due to the postnatal HF diet groups ($P < 0.0001$, Figure 3.5C and D).

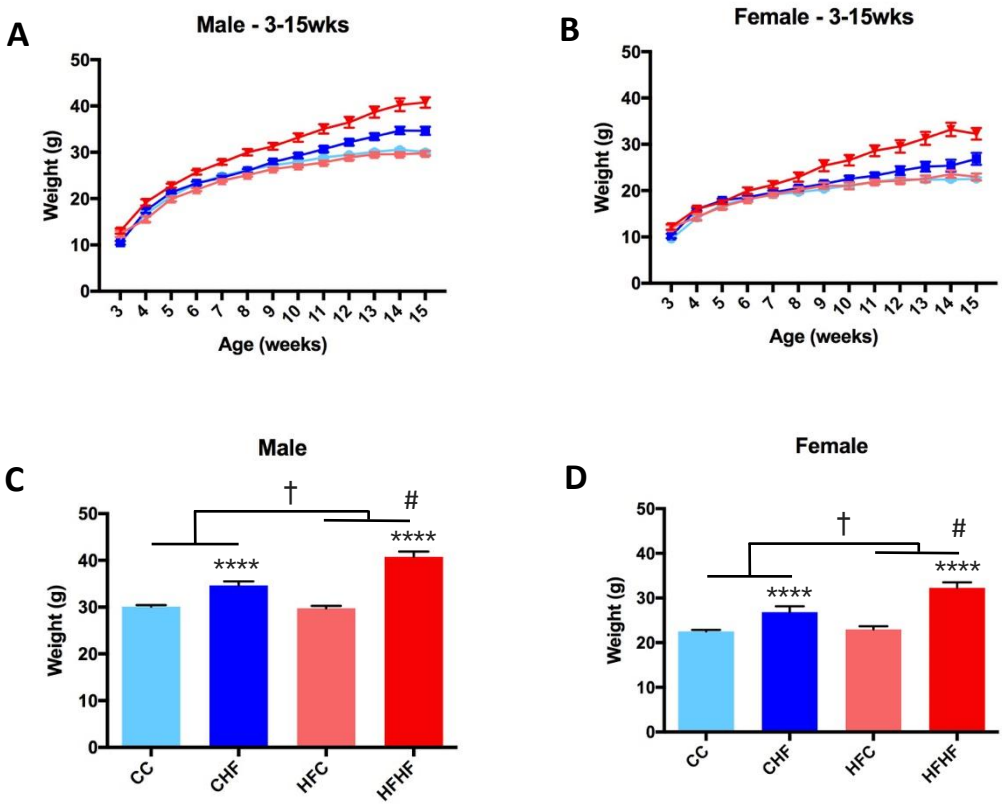

Figure 3.3 – **15 Week offspring body weights**. The effect of maternal and postnatal HF diet between 3 weeks to 15 weeks in (A) male and (B) female offspring (CC, light blue; CHF, dark blue; HFC, pink; HFHF, red) and body weight at 15 weeks in (C) male and (D) female offspring. **** $P < 0.0001$, †, $P < 0.1$. # $P < 0.05$ pre*postnatal interaction group affected. Data are displayed as predicted means ± SEM from maximal models. n = 6-13 per group (see Table 2.2 in Methods).

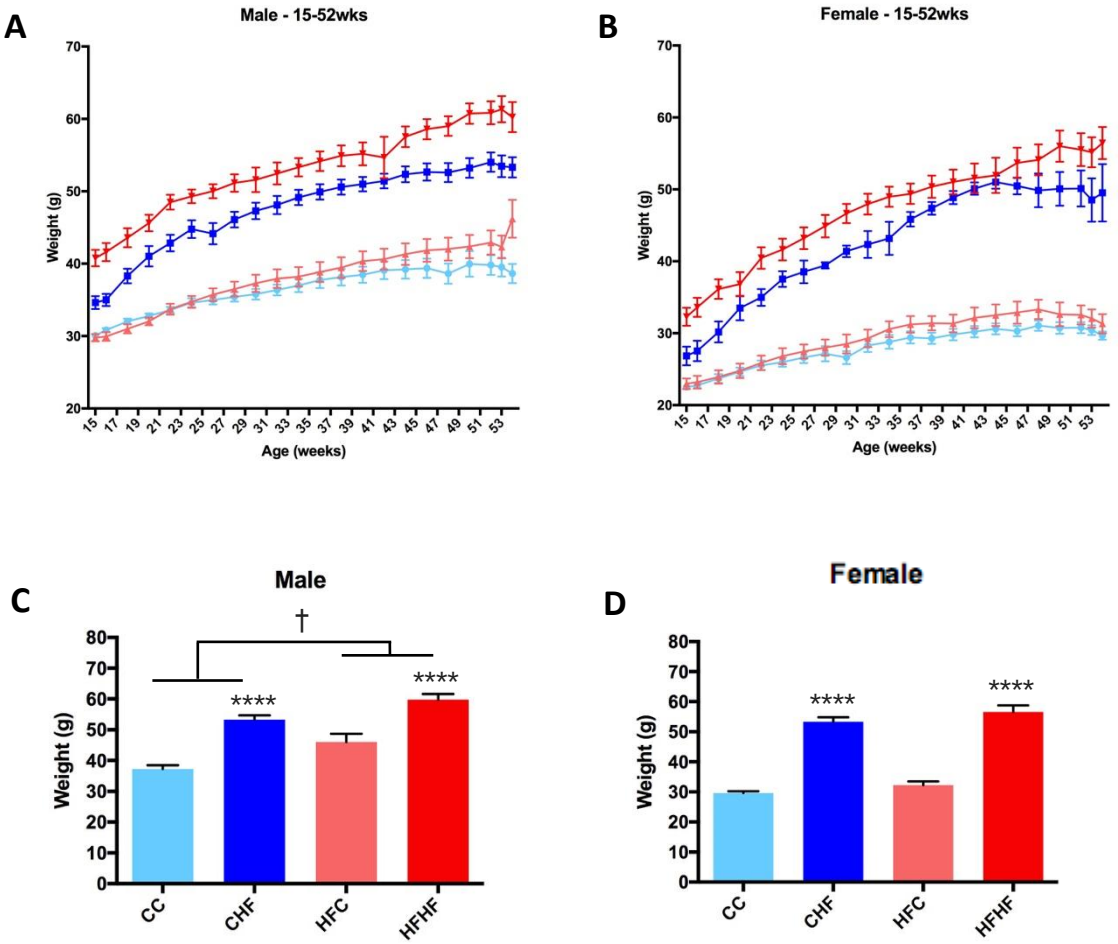

Figure 3.4 – **52 Week offspring body weights**. The effect of maternal and postnatal HF diet between 3 weeks to 52 weeks in (A) male and (B) female offspring (CC, light blue; CHF, dark blue; HFC, pink; HFHF, red) and body weight at 52 weeks in (C) male and (D) female offspring. ****, $P < 0.0001$; †, $P < 0.1$. Data are displayed as predicted means ± SEM from maximal models. n= 5-13 per group (see Table 2.2 in Methods).

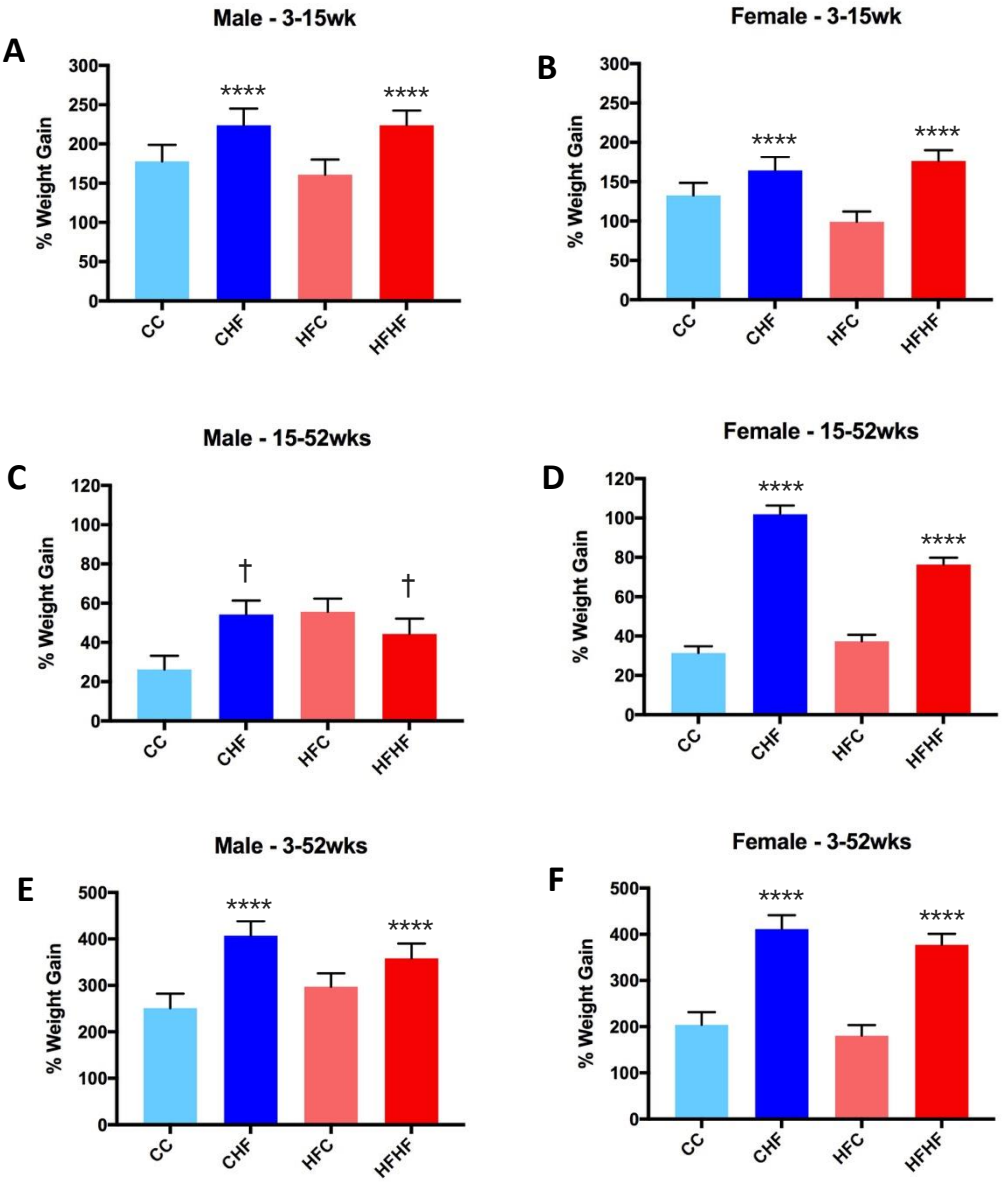

Figure 3.5 – **Weight gain between weeks 3-15, 15-52 and 3-52**. The effect of maternal and postnatal HF diet on percentage weight gain between 3 to 15 weeks in (A) male and (B) female offspring, 15 to 52 weeks in (C) male and (D) female offspring and 3 to 52 weeks in (E) male and (F) female offspring. ****, $P < 0.0001$, †, $P < 0.1$. Data are displayed as predicted means ± SEM from maximal models. n= 5-13 per group (see Table 2.2 in Methods).

3.4.4 Pup Retrieval and Pup USVs

There was no significant difference in total pup retrieval time between HF-fed vs C-fed mothers (data not shown), however there was a strong trend for HF-fed mothers to spend more time during the interaction period ($P = 0.051$, Figure 3.6A). HF-fed mothers also had a higher incidence of failing to retrieve pups to the nest (4 incidences of failure from HF-fed mothers in comparison to no incidences from C-fed mothers).

USV cluster calls at P7 tended to be increased in male but not female pups from HF-fed mothers ($P = 0.053$, Figure 3.6B). Cluster calls were correlated to Individual calls in both male and female offspring (data not shown, $P < 0.0001$ for both sexes, $R^2 > 0.8$ in both sexes). Males emitted more USVs than females ($P < 0.048$).

USV individual and cluster calls were not correlated to any pup retrieval total time, investigation latency, interaction time or nest latency scores (data not shown).

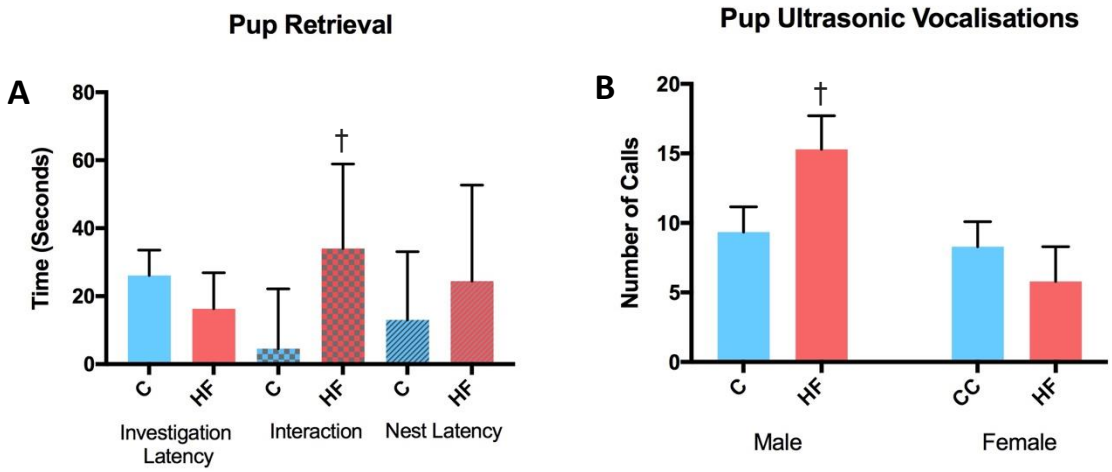

Figure 3.6 – **Pup Retrieval and USV**. The effect of maternal HF diet on (A) pup retrieval time and (B) pup USVs and the correlation between dam weight at conception and pup calls. †, $P < 0.1$. Data are displayed as predicted means ± SEM and were statistically analysed by independent *t*-test (A) or mixed model (B). n = 2 offspring per mother for pup retrieval test and n = 2 per sex per mother for pup USV.

3.4.5 Open Field Test

3.4.5.1 Exploratory Behaviour

At 15 weeks of age, there was no effect of maternal or postnatal obesity in both sexes on ambulatory time or distance travelled. However, there was an interaction between maternal and postnatal HF diet ($P < 0.05$) for ambulatory time in the HFC group which tended to be increased from CC ($P = 0.083$, Figure 3.7A).

At 52 weeks of age, postnatal HF diet significantly reduced both ambulatory time and distance travelled ($P < 0.0001$, Figure 3.7B and D), however there were no effects of maternal HF diet.

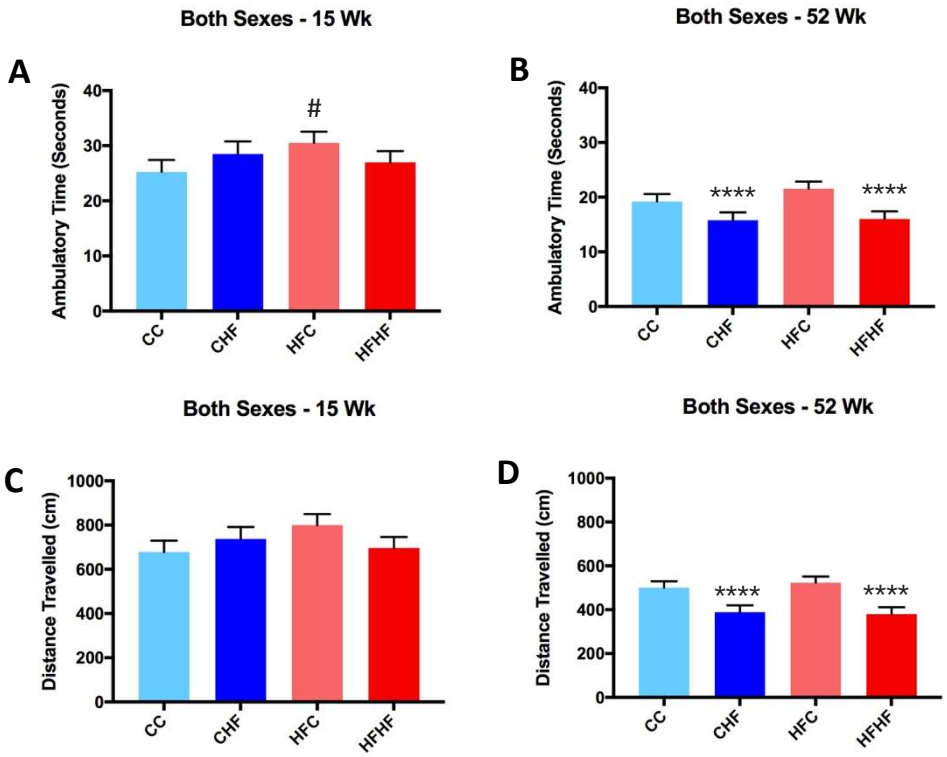

Figure 3.7 – **Exploratory Behaviour at 15 and 52 weeks of age.** Effect of maternal and postnatal HF diet in both sexes (A-D) on ambulatory time (15 weeks; A, 52 weeks; B) and distance travelled (15 weeks; C, 52 weeks; D). **** $P < 0.0001$. # $P < 0.05$ pre*postnatal interaction group affected. Data are displayed as predicted means ± SEM from maximal models. n = 5-13 per sex per group (see Table 2.2 in Methods).

3.4.5.2 Vertical Movement

At 15 and 52 weeks of age, there was a significant effect of postnatal HF diet to decrease jump counts in both males and females (15 weeks; $P < 0.01$ Figure 3.8A and C, 52 weeks; $P < 0.0001$ Figure 3.8B and D) but at 15 weeks only the HFHF group was significantly reduced from CC when analysed separately in both males ($P < 0.002$) and females ($P < 0.008$). In 52 week-old female offspring there was a significant effect of maternal HF diet to reduce jump counts ($P < 0.001$, Figure 3.8D) but this effect was not seen in males. Postnatal HF tended to decrease vertical counts at 15 weeks ($P = 0.099$, Figure 3.8E), and significantly reduce vertical counts at 52 weeks ($P < 0.0001$, Figure 3.8F) in both sexes. There was also a trend for maternal HF to reduce vertical counts at 52 weeks ($P = 0.092$). Female offspring jumped significantly more than males at 15 ($P < 0.01$) and 52 weeks ($P < 0.05$), and there was no effect of sex on vertical counts at either age.

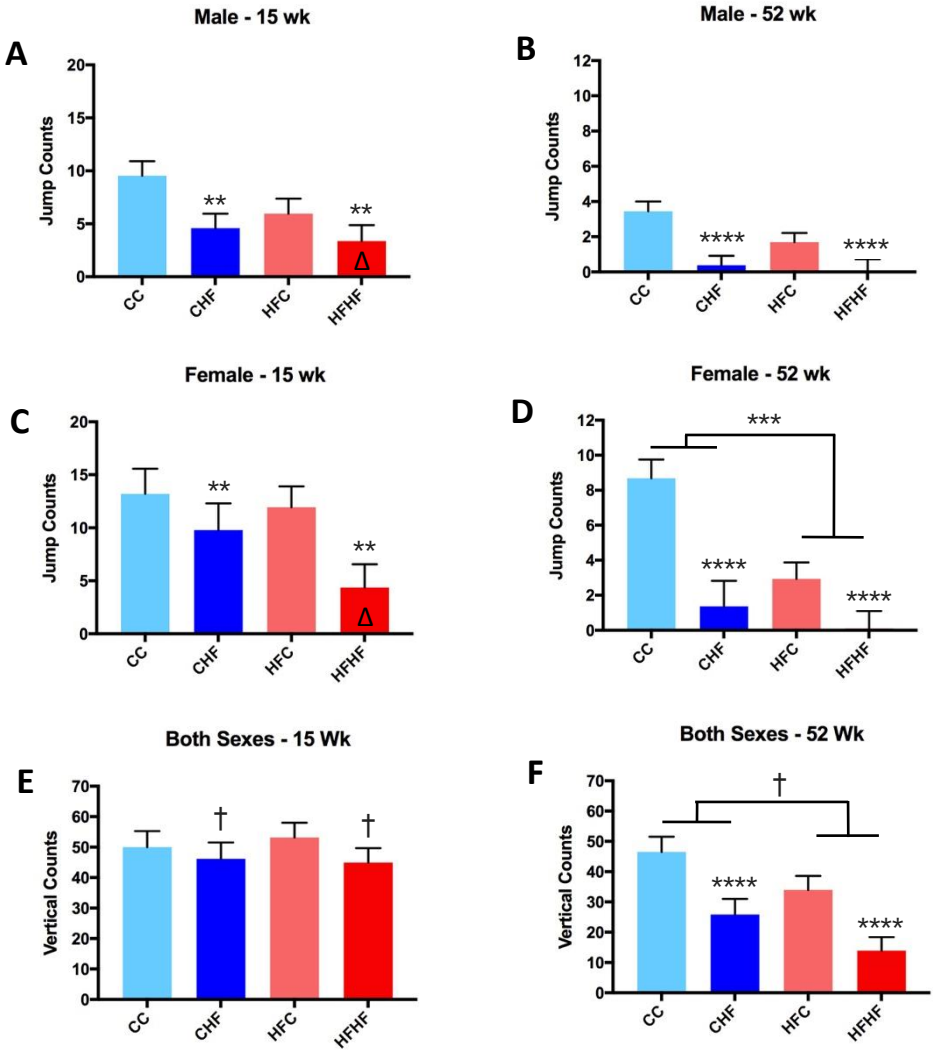

Figure 3.8 – **Vertical Behaviour at 15 and 52 weeks of age.** Effect of maternal and postnatal HF diet in males (A, B) and females (C, D) and both sexes combined (E, F) on jump counts (15 weeks; A-B, 52 weeks; C-D) and vertical counts (15 weeks; E, 52 weeks; F). **** $P < 0.0001$, ** $P < 0.01$, † $P < 0.1$. Δ, only HFHF significantly different from CC. Data are displayed as predicted means ± SEM from maximal models. n = 5-13 per sex per group (see Table 2.2 in Methods).

3.4.5.3 Velocity and Resting Time

At 15 weeks of age there was a significant reduction in velocity in offspring fed a HF diet postnatally in both sexes of offspring ($P < 0.05$; Figure 3.9A). At 52 weeks of age, in both sexes, there was a significant effect of postnatal HF diet to reduce velocity ($P < 0.0001$), and there was an additional significant effect of maternal HF diet to reduce velocity ($P < 0.05$; Figure 3.9B).

In 15 week-old offspring, there was a significant interaction between maternal and postnatal diet to significantly increase resting time in HFHF ($P = 0.036$; Figure 3.9C). But at 52 weeks of age postnatal HF diet alone increased resting time ($P < 0.0001$; Figure 3.9D).

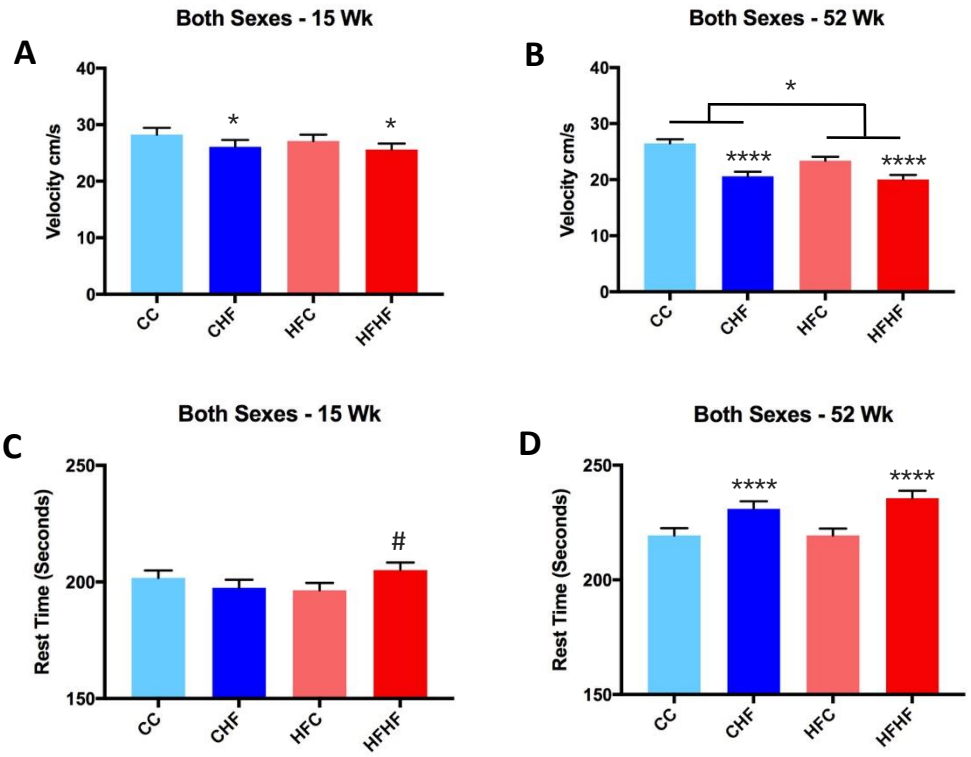

Figure 3.9 – **Velocity and Resting Time at 15 and 52 weeks of age.** Effect of maternal and postnatal HF diet in both sexes combined (A-D) on velocity (15 weeks; A, 52 weeks; B) and resting time (15 weeks; C, 52 weeks; D). **** $P < 0.0001$, * $P < 0.05$. # $P < 0.05$ pre*postnatal interaction group affected. Data are displayed as predicted means ± SEM from maximal models n = 5-13 per sex per group (see Table 2.2 in Methods).

3.4.5.4 Stereotypic Behaviour

At 15 weeks of age, there was no effect of maternal or postnatal obesity in both sexes on stereotypic time. However, there was an interaction ($P < 0.05$) between maternal and postnatal diet in the HFC group which tended to be increased from CC ($P = 0.053$) and HFHF ($P = 0.060$, Figure 3.10A). At 52 weeks of age, postnatal HF diet decreased stereotypic time ($P < 0.0001$; Figure 3.10B) in both sexes. However, only the HFHF group was significantly decreased from CC ($P < 0.001$) when analysed separately.

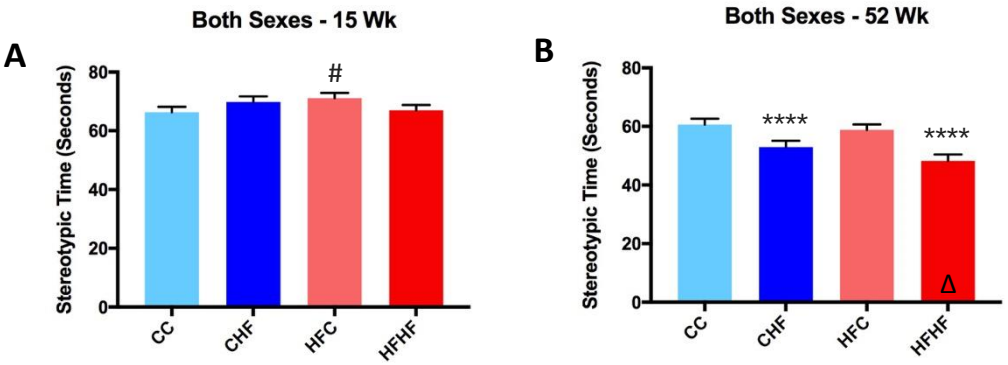

Figure 3.10 – **Stereotypic Behaviour at 15 and 52 weeks of age.** Effect of maternal and postnatal HF diet in both sexes combined on stereotypic time in 15 (A) and 52 week-old (B) offspring. **** $P < 0.0001$. # $P < 0.05$ pre*postnatal interaction group affected. Δ, only HFHF significantly different from CC. Data are displayed as predicted means ± SEM from maximal models n = 5-13 per sex per group (see Table 2.2 in Methods).

3.4.5.5 Zone Data

Diet did not affect the percentage of distance travelled (Figure 3.11A), time spent resting (Figure 3.11C) or number of entries (Figure 3.11E) in the centre area in 15 week-old mice. In 52 week-old offspring there was an effect of postnatal HF diet to decrease percentage of distance travelled ($P < 0.01$; Figure 3.10B), time spent resting ($P < 0.05$; Figure 3.11D) or number of entries ($P < 0.0001$; Figure 3.11F) in the centre area. In addition, maternal HF diet decreased percentage of distance travelled in the centre area ($P < 0.05$; Figure 3.11B). The HFHF group was the most significantly decreased group from CC across all 3 parameters, and the only significantly different group from CC in the percentage time spent resting in the centre ($P < 0.005$). In 52 week-old offspring, body weight correlated negatively with; zone entries ($P < 0.001$, $R^2 > 0.158$) and % distance travelled in centre ($P < 0.01$, $R^2 > 0.132$), and total percentage weight gain between 3 to 52 weeks also correlated negatively with zone entries ($P < 0.001$, $R^2 > 0.149$), % distance travelled in centre ($P < 0.05$, $R^2 > 0.086$), and % time resting in the centre ($P < 0.05$, $R^2 > 0.057$) but not percentage weight gain between 15-52 weeks of age which when separated by sex was only true in female offspring. Dam weight at conception was not correlated with OF anxiety at either age.

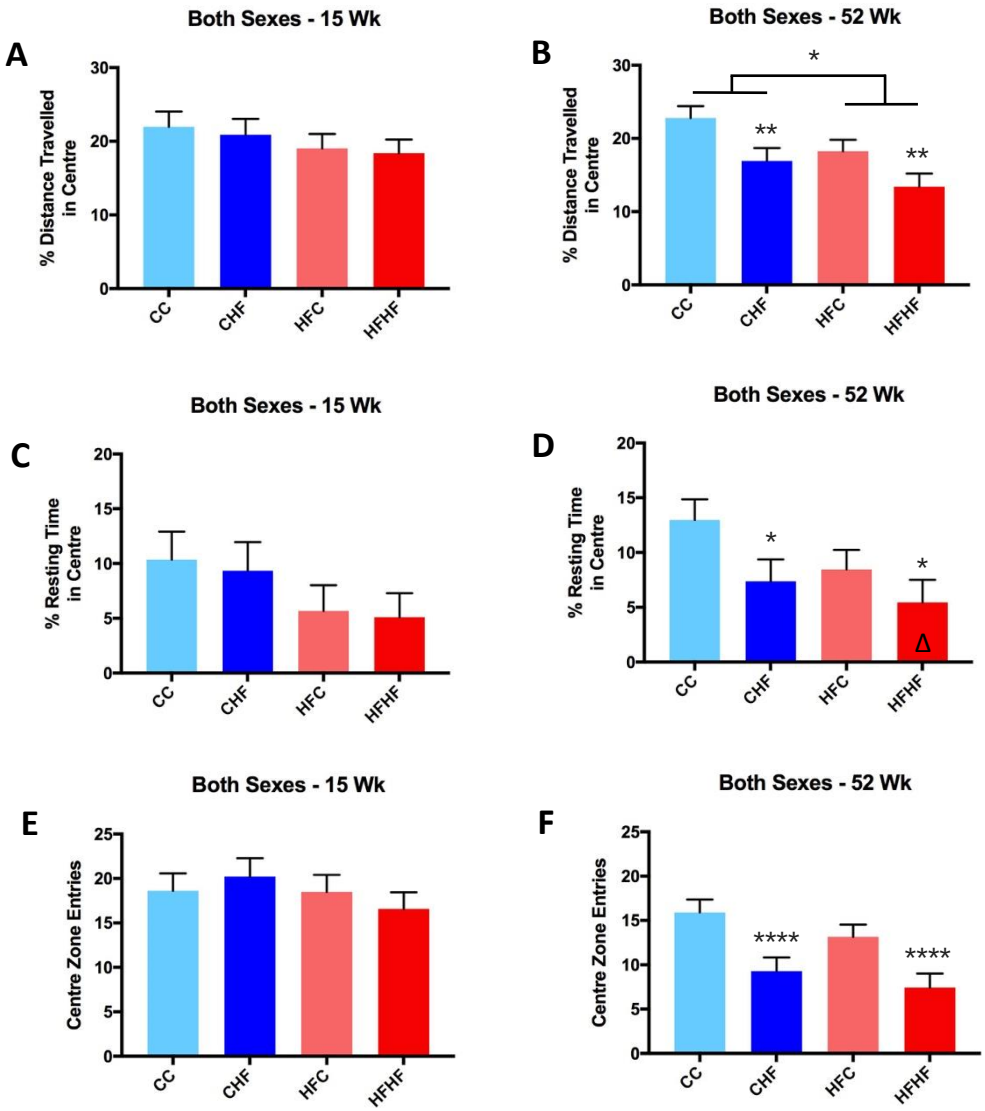

Figure 3.11 – **Centre Zone Behaviour at 15 and 52 weeks of age.** Effect of maternal and postnatal HF diet in both sexes combined on % centre distance travelled (15 weeks; A, 52 weeks; B), % centre resting time (15 weeks; C, 52 weeks; D) and centre zone entries (15 weeks; E, 52 weeks; F). **** $P < 0.0001$, ** $P < 0.01$, * $P < 0.05$. Δ, only HFHF significantly different from CC. Data are displayed as predicted means ± SEM from maximal models. n = 5-13 per sex per group (see Table 2.2 in Methods).

3.4.6 Elevated Plus Maze

3.4.6.1 Entry into the Open Area

At 15 weeks of age there was a trend ($P = 0.096$, Figure 3.12A) for male offspring fed a postnatal HF diet to spend more time on the open arms, but only the HFHF group was significantly different from CC ($P < 0.05$) when analysed separately. At 52 weeks of age, there was no effect of maternal or postnatal diet on time spent on the open arms in male offspring (Figure 3.12B). Additionally, no effect was observed on open arm entries at 15 weeks of age (Figure 3.12E), however, at 52 weeks of age, maternal obesity caused an increase in open arm entries in male offspring ($P < 0.05$, Figure 3.12F) and this was largely due to the HFC group which, when analysed separately, was significantly different ($P < 0.05$) to the maternal C-fed dietary groups (CC and CHF).

No effects of diet were seen in female offspring (Figure 3.12C and G) at 15 weeks. At 52 weeks of age, postnatal HF diet caused a significant reduction in time ($P < 0.05$, Figure 3.12D) and a trend in reduction of time in entries ($P = 0.064$, Figure 3.12H) to the open area of the EPM in females. Female offspring at both ages had greater time and entries in the open arm area than males overall ($P < 0.05$, Figure 3.12A-H).

When analysing the percentage of time spent in the open arm, the effects observed were statistically the same as the raw data of time on the open arm (and were strongly correlated; $P < 0.0001$, $R^2 > 0.989$), except in 52 week-old male offspring where there was a trend ($P = 0.068$) for maternal HF diet to increase percentage time spent on the open arms (CC, 0.48 ±1.39%; CHF, 2.43 ±1.36%; HFC, 2.99 ±1.34%; HFHF, 3.2 ±1.75%).

At 52 but not 15 weeks of age in female offspring there was a significant negative correlation between open arm time and weight ($P < 0.05$, $R^2 > 0.163$) and total 3-52 week weight gain ($P < 0.01$, $R^2 > 0.262$), as well as a negative trend between 15-52 week percentage weight gain and open arm time ($P = 0.055$, $R^2 > 0.121$). There was no correlations between weights or weight gain to open arm time in males at either age. No correlations were found between EPM results in time/entries on the open arm and OF thigmotaxis results at either age or sex. Maternal obesity was not correlated with EPM anxiety at either age or sex.

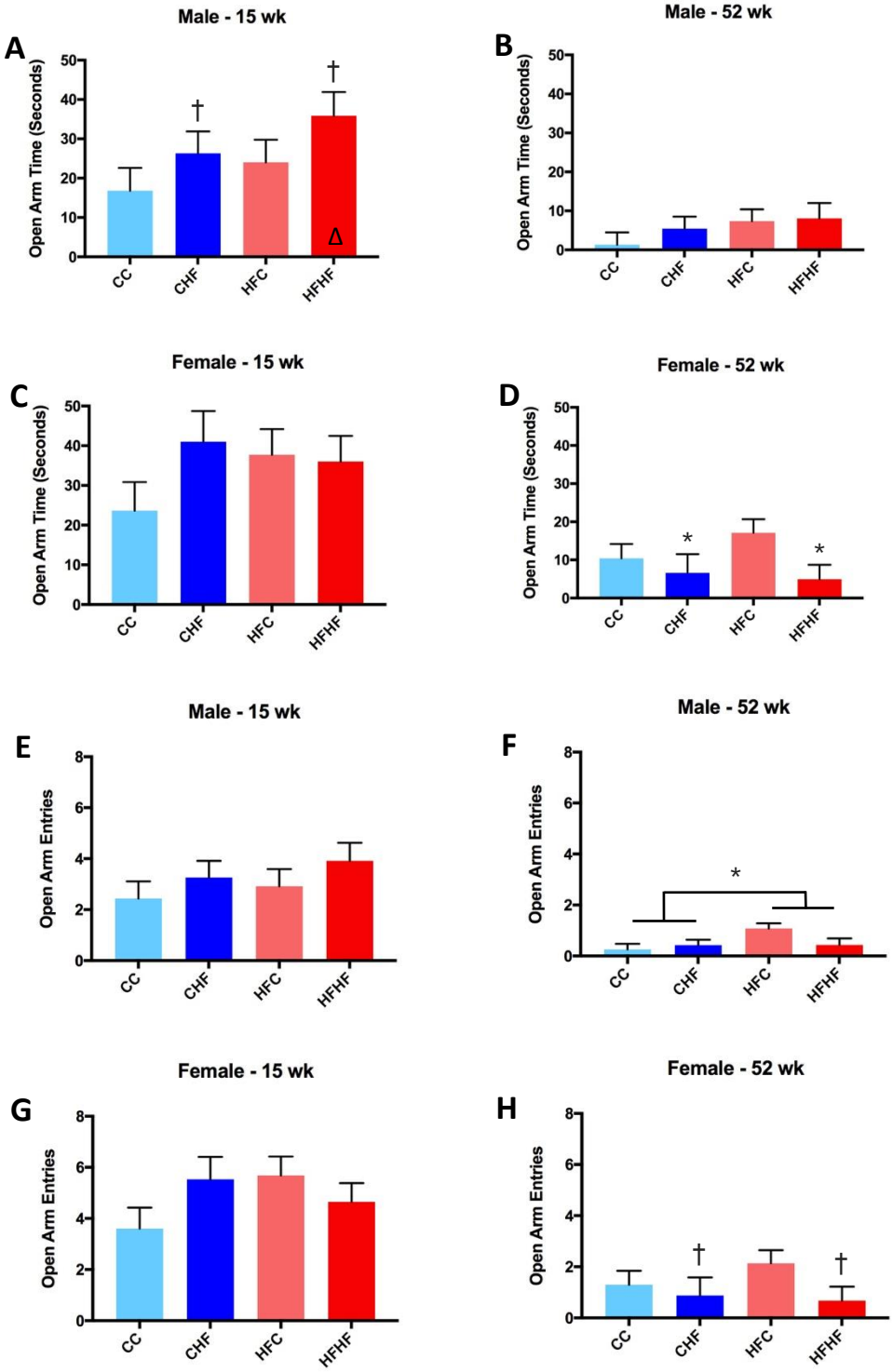

Figure 3.12 – **Elevated Plus Maze: Open Area.** Effect of maternal and postnatal HF diet in male (A, B, E, F) and female (C, D, G, H) offspring on open arm time (15 weeks; A, C, 52 weeks; B, D) and open arm entries (15 weeks; E, G, 52 weeks; F, H). * $P < 0.05$, † $P < 0.1$. Δ, only HFHF significantly different from CC. Data are displayed as predicted means ± SEM from maximal models. n = 5-13 per sex per group (see Table 2.2 in Methods).

3.4.6.2 Entry into the Closed Area

At 15 weeks of age, both sexes of offspring fed a postnatal HF diet spent less time in the closed arms of the EPM ($P < 0.01$; Figure 3.13A) but this was not reflected in closed arm entries (Figure 3.13C and E). At 52 weeks of age however, maternal HF tended to cause less time to be spent in the closed arm of the EPM ($P = 0.092$, Figure 3.13B). There was an interaction between maternal and postnatal diet ($P = 0.004$) which when analysed pairwise showed the male HFC group closed arm entries was elevated and tended to be increased from the CC group ($P = 0.098$) and significantly increased from the HFHF group ($P < 0.01$, Figure 3.13D). Overall, female offspring entered the closed area more than males at 52 weeks ($P < 0.05$; Figure 3.13F).

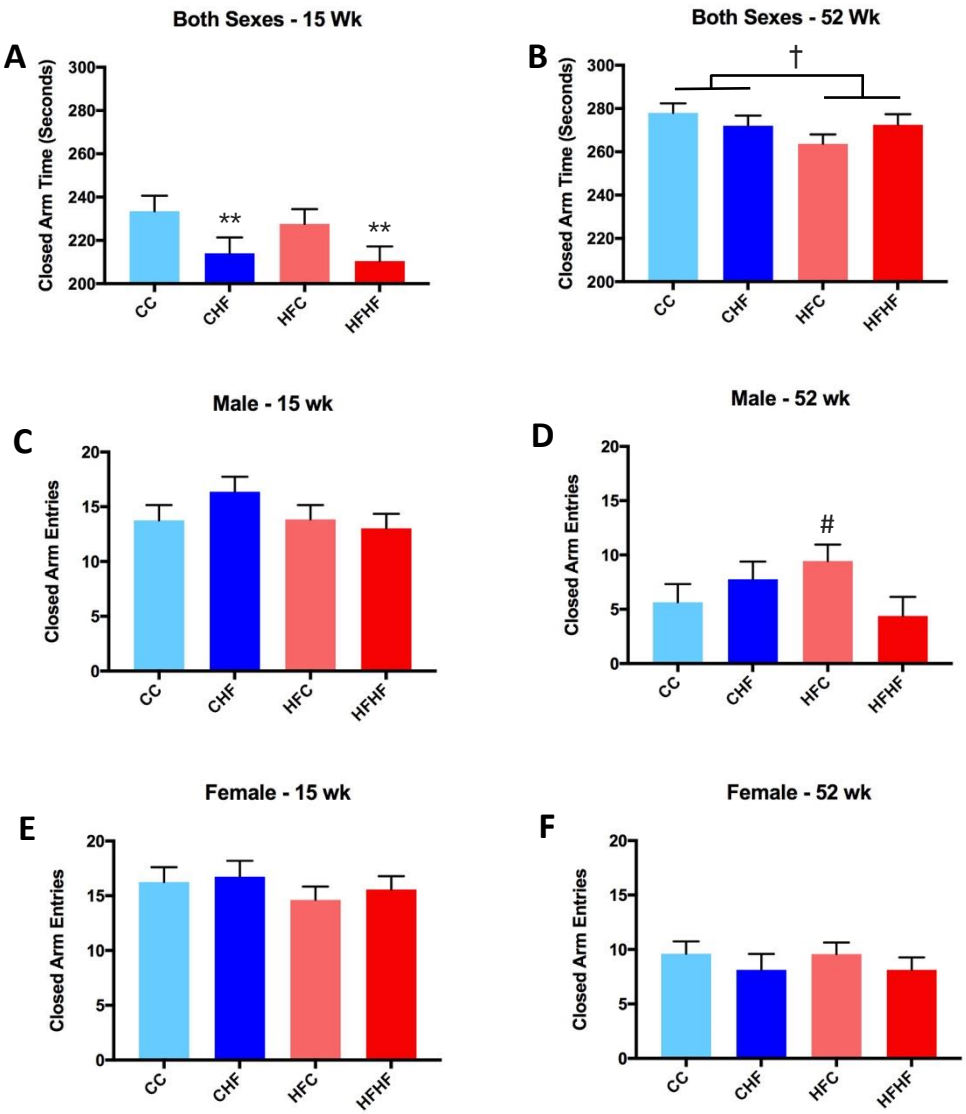

Figure 3.13 – **Elevated Plus Maze: Closed Area.** Effect of maternal and postnatal HF diet in both sexes combined (A, B), male (C, D) and female (E, F) offspring on closed arm time (15 weeks; A, 52 weeks; B) and closed arm entries (15 weeks; C, E, 52 weeks; D, F). ** $P < 0.01$, * $P < 0.05$, † $P < 0.1$. # $P < 0.05$ pre*postnatal interaction group affected. Data are displayed as predicted means ± SEM from maximal models. n = 5-13 per sex per group (see Table 2.2 in Methods).

3.4.6.3 Time in the Centre Area

Time spent in the centre area was increased by postnatal HF diet at 15 weeks of age in both sexes ($P < 0.01$, Figure 3.14A), but centre entries remained unaffected by diet in both male and female offspring (Figure 3.14C and E). At 52 weeks, there was a significant ($P < 0.047$) interaction between maternal and postnatal HF diet such that pairwise comparisons showed that there was a trend ($P = 0.08$, Figure 3.14B) for the HFC group to be increased from CC. Postnatal HF diet tended to decrease female offspring centre entries ($P = 0.069$, Figure 3.14F), but no effect of maternal or postnatal HF diet was seen in male offspring at 52 weeks of age. There was a significant ($P < 0.003$) interaction effect between maternal and postnatal HF diet and subsequent analysis showed that the HFC diet group was significantly increased from HFHF ($P < 0.01$) and tended to be increased from the CC group ($P = 0.056$, Figure 3.14D) in males at 52 weeks. This effect of increased entries in the HFC group was also observed in open and closed arms in males at the same age. Females entered the centre square area more often at both ages (both ages; $P < 0.05$, Figure 3.14C-F).

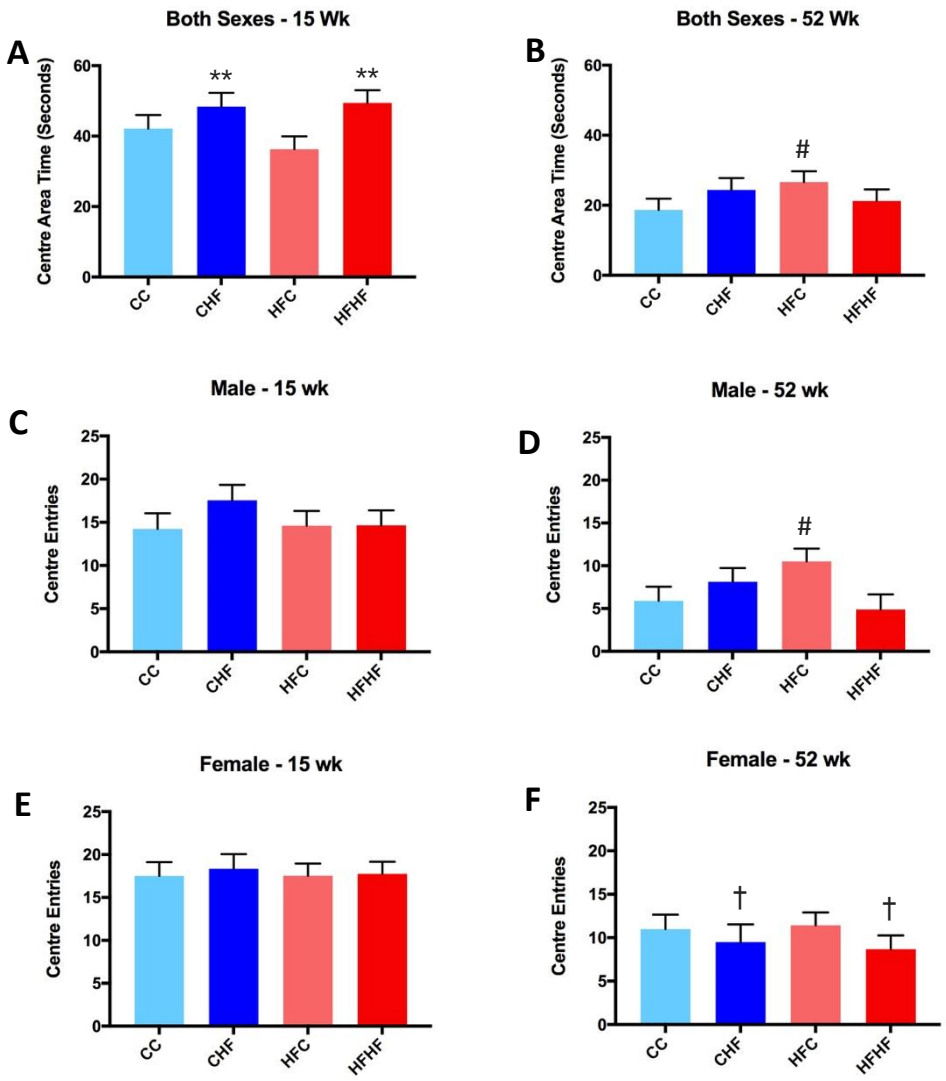

Figure 3.14 – **Elevated Plus Maze: Centre Area.** Effect of maternal and postnatal HF diet in both sexes combined (A, B), male (C, D) and female (E, F) offspring on centre area time (15 weeks; A, 52 weeks; B) and centre area entries (15 weeks; C, E, 52 weeks; D, F). ** $P < 0.01$, † $P < 0.1$. # $P < 0.05$ pre*postnatal interaction group affected. Data are displayed as predicted means ± SEM from maximal models. n = 5-13 per sex per group (see Table 2.2 in Methods).

3.4.6.4 Entry into the Far Reaches of the Open Area

Maternal HF diet increased far arm entries in 15 week-old offspring ($P < 0.05$, Figure 3.15A), which may be due to the HFC group which when analysed separately was the only group significantly increased from CC ($P < 0.05$). No effect of diet was seen at 52 weeks of age. Far arm entries at 15 weeks were positively correlated to open arm behaviour (when both sexes were combined) from section 3.4.6.1 in both time ($P < 0.001$, $R^2 = 0.67$) and entries ($P < 0.001$, $R^2 = 0.5$).

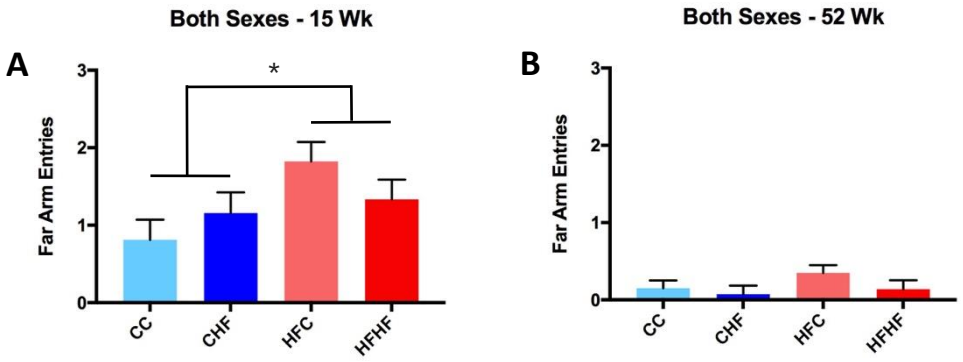

Figure 3.15 – **Elevated Plus Maze Far Open Area.** Effect of maternal and postnatal HF diet on time spent in far open area in both sexes at 15 (A) and 52 weeks (B). * $P < 0.05$. Data are displayed as predicted means ± SEM from maximal models. n = 5-13 per sex per group (see Table 2.2 in Methods).

3.4.7 Novel Object Recognition Test

3.4.7.1 Acquisition Trial

Acquisition trial results showed that at each age, male and female mice spent equal time with left or right identical objects (Figure 3.16A-D). This lack of preference validates this experiment and demonstrates that mice in a novel environment will explore two identical objects approximately equally, regardless of sex and age.

During acquisition trials, postnatal HF-fed 15 week-old male mice spent more time exploring both of the identical objects together in general ($P < 0.01$; Figure 3.16E). However, at 52 weeks of age there was a trend for maternal HF-fed males to spend more time with the objects in the acquisition trial ($P = 0.061$, Figure 3.16F). These effects were not seen in females at either age (Figure 3.16G, H). 52 week-old females overall spent longer exploring objects in the acquisition test than males ($P < 0.05$).

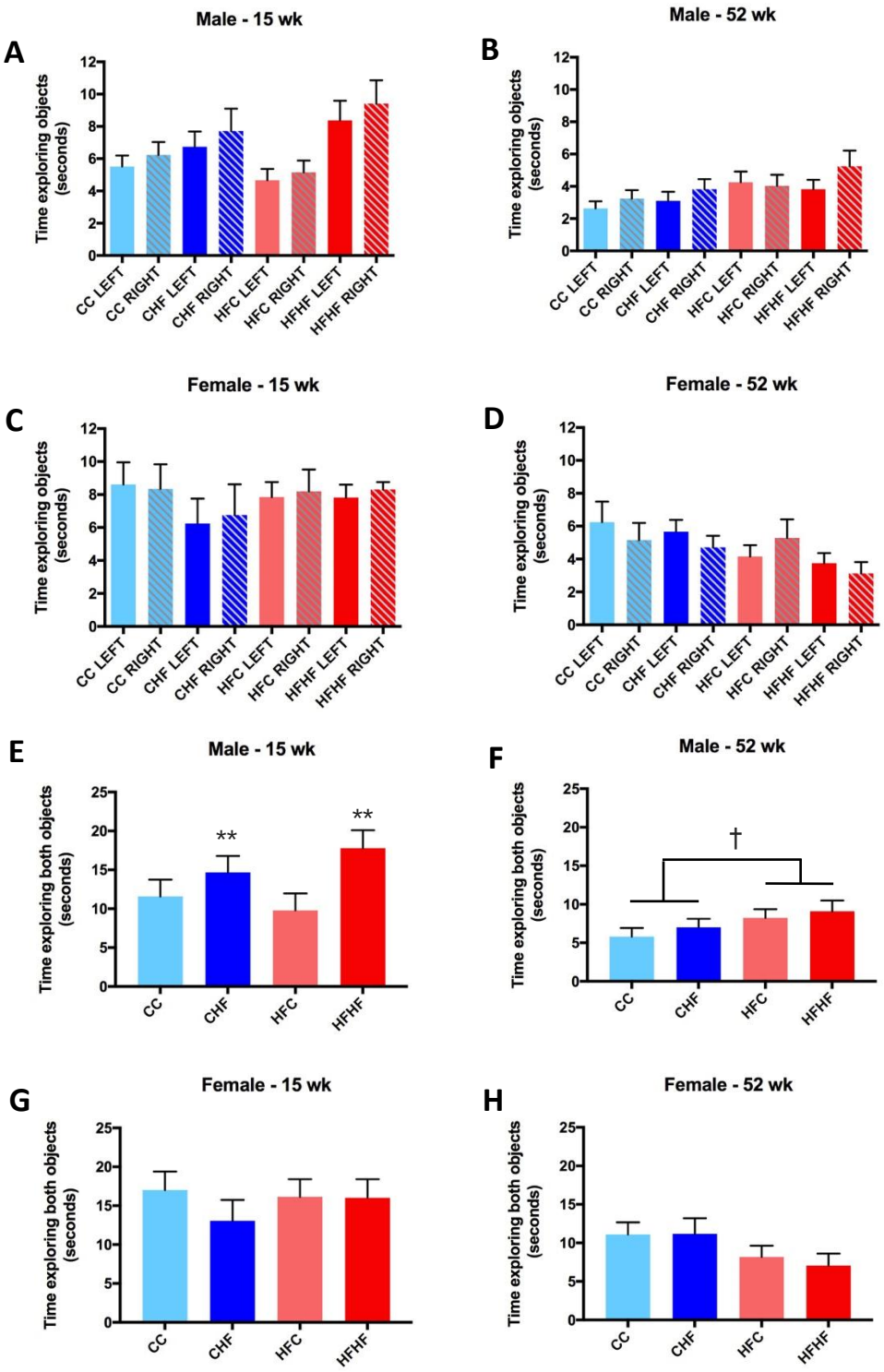

Figure 3.16 – **Acquisition Trials.** Effect of maternal and postnatal HF diet in male (A, B, E, F) and female (C, D, G, H) offspring on acquisition time with separate objects (15 weeks; A, C, 52 weeks; B, D) and acquisition time with both objects together (15 weeks; E, G, 52 weeks; F, H). ** $P < 0.01$, † $P < 0.1$. Data are displayed as predicted means ± SEM from maximal models (E, F) or statistically analysed by paired t-test for each diet group (A-D). n = 5-13 per sex per group (see Table 2.2 in Methods).

3.4.7.2 Retention Trial

When comparing the time spent with familiar (F) *vs.* novel (N) object in both males and females at 15 weeks, time spent with the novel object tended to ($P < 0.1$) or was significantly greater than ($P < 0.05$) time with the familiar object in all groups (Figure 3.17A and C), except the male HFHF group (Figure 3.17A). At 52 weeks of age however, a lack of discrimination between familiar and novel objects was only observed in HFC male, and CC and CHF female offspring (Figure 3.17B and D). There was no effect of dietary groups on the total time mice spent with both of the two objects during the retention trial (Figure 3.17E and F).

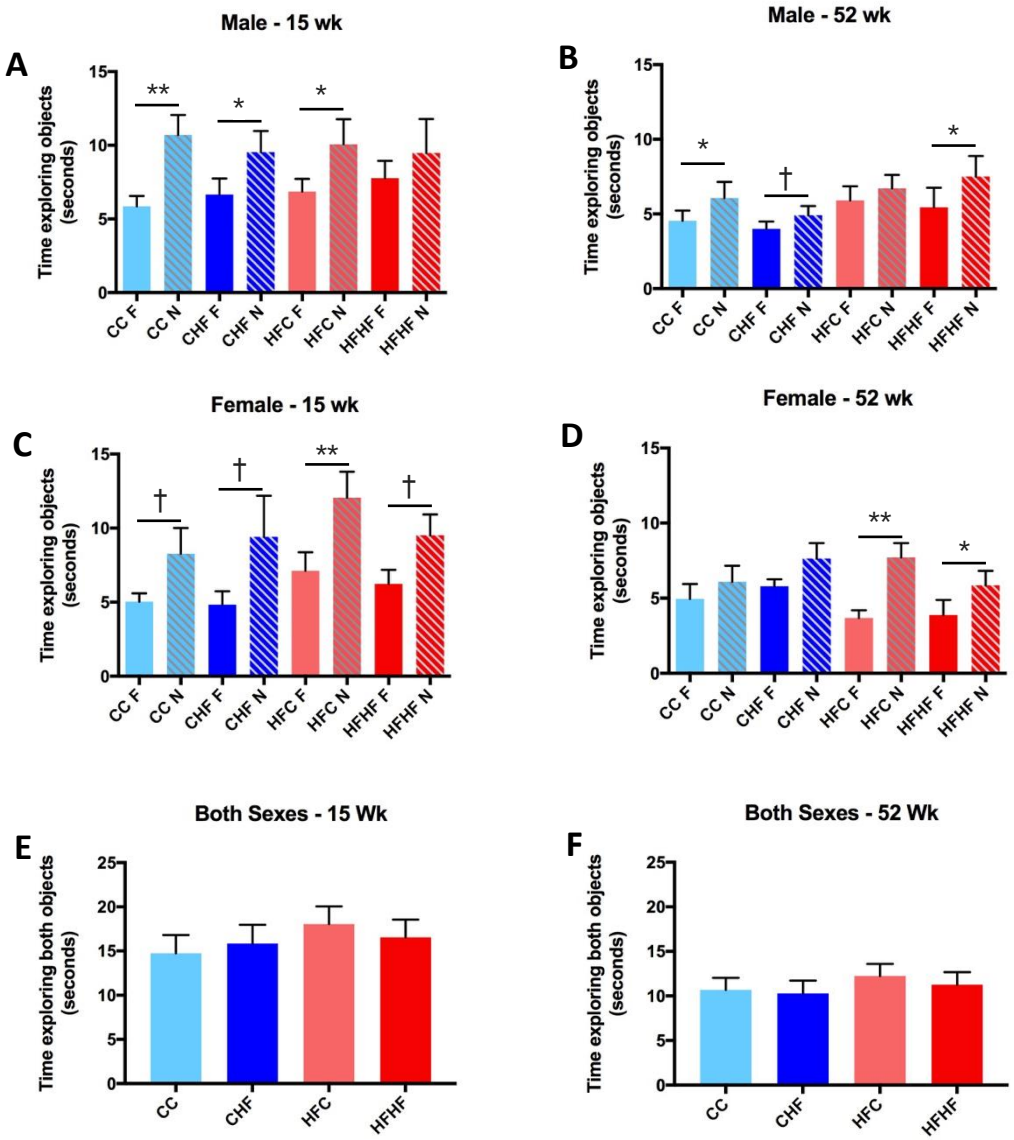

Figure 3.17 – **Retention Trials.** Effect of maternal and postnatal HF diet in both sexes (E, F), male (A, B) and female (C, D) offspring on acquisition time with separate objects (15 weeks; A, C, 52 weeks; B, D) and retention time with both objects (15 weeks; E, 52 weeks; F). ** $P < 0.01$, * $P < 0.05$, † $P < 0.1$. Data are displayed as predicted means ± SEM from maximal models (E, F) or statistically analysed by paired t-test for each diet group (A-D). n = 5-13 per sex per group (see Table 2.2 in Methods). F = familiar, N = novel.

3.4.7.3 Discrimination Index

At 15 weeks of age the discrimination index tended to be reduced in maternal HF-fed male offspring ($P = 0.095$, Figure 3.18A) and when analysing separately only the HFHF group was significantly decreased from CC ($P < 0.05$). No effects were seen at 52 weeks in males (Figure 3.18B). In females, no effects of diet were observed at 15 weeks (Figure 3.18C), but there was a significant increase in the discrimination index in maternal HF-fed mice at 52 weeks ($P < 0.05$, Figure 3.18D). Neither dam weight at conception nor offspring weights were correlated with DI at either age or sex. DI was not correlated to EPM or OF anxiety measures.

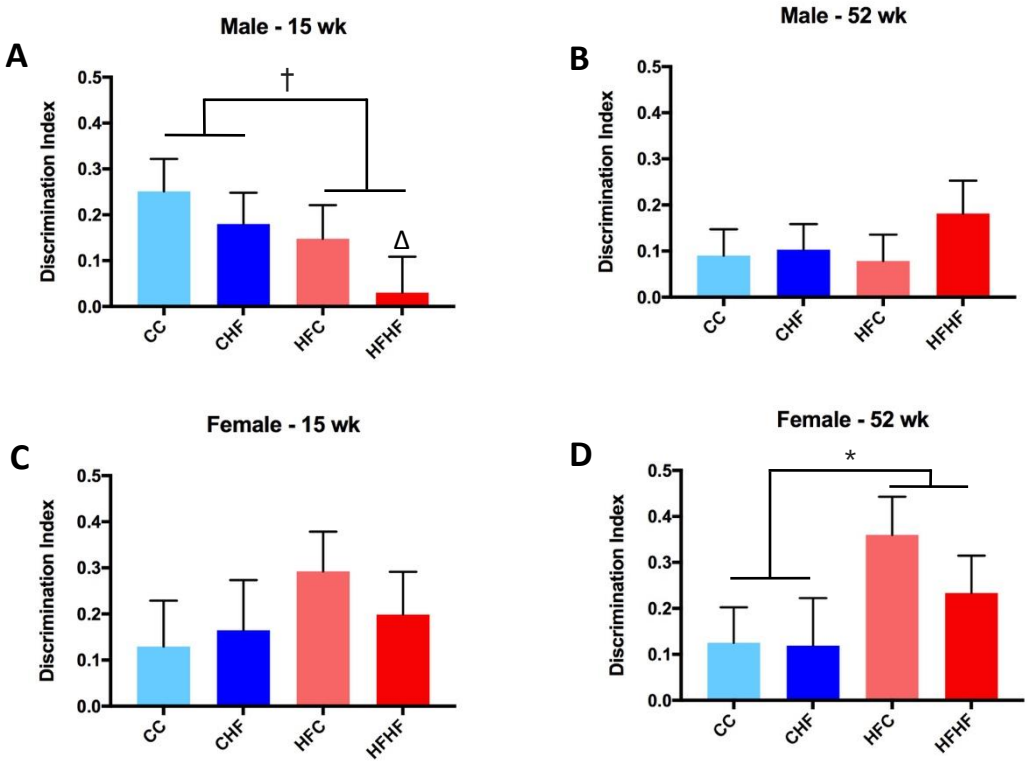

Figure 3.18 – **Discrimination Index.** Effect of maternal and postnatal HF diet in male (A, B) and female (C, D) offspring on discrimination index (15 weeks; A, C, 52 weeks; B, D). * $P < 0.05$, † $P < 0.1$. Δ, only HFHF significantly different from CC. Data are displayed as predicted means ± SEM from maximal models. n = 5-13 per sex per group (see Table 2.2 in Methods).

3.5 Discussion

In order to assess potential neurological alterations resulting from a change in maternal and postnatal diet, behavioural tests were performed in HF and C-fed dams and their offspring. Maternal care was assessed via pup retrieval tests and P7 pup anxiety was assessed via USV tests. Effects of obesogenic HF diet were seen on maternal care and in pup anxiety. In 15 week-old adult offspring, OF testing was used to assess general locomotor activity and anxiety, EPM to assess anxiety phenotypes and NOR to assess non-spatial recognition memory. Effects of postnatal diet were observed on activity and anxiety in both sexes. Effects of maternal diet were seen on memory. Changes in activity, anxiety and memory were largely sex-specific with exacerbation of maternal and postnatal HF diet combined effects seen more predominantly in male offspring.

3.5.1 Effect of HF diets on maternal and offspring weight

At the start of the trial, 6 weeks prior to conception, there was no significant difference between dam weights, this ensured that all mice had an equal start to the trial and no chance that initial weight differences would affect long-term outcome. Female mice that had at least a 15% increase in body weight over a period of at least 6 weeks on the HF diet were included in the study and were mated. At conception, during pregnancy and post-weaning, HF-fed dams remained heavier than their C-fed counterparts, as expected based on previous use of this model (82). Differences in pregnancy weight may have occurred due to difficulties in plug checking in HF mice, as timing of plug checking is used to assign offspring gestational age. Weight gain during the first 2 weeks of pregnancy appeared similar between diet groups despite the reduced average littersize in HF-fed dams. This could imply greater birth weights in the HF-fed offspring; however, birth weight was not measured since we have previously noted incidences of rejection from HF-fed mothers following human interaction. Cannibalistic tendencies of obese dams is noted in other studies also (262). There was no effect of maternal diet on offspring weight at P7 but there was an effect of sex, with males being significantly heavier than females which persisted into adulthood. Despite the lack of significant effect of maternal HF on pup weight, dam weight at conception was negatively correlated to pup weight at P7. The fewer pups born to HF mothers would suggest that these pups would be able to access more milk and grow faster. However, this potential reduction in weight could be due to other factors such as poor maternal care or poor lactation, as previously reported in obese dams (289). The same study also reported lower weights of pups from obese dams until P11, which then became significantly increased at P19 (289). Lower weight in pups from obese mothers compared to control has been noted in other studies: Li *et al.* showed that 58% HF-feeding in rats from conception resulted in reduced male pup weight at postnatal day 2 (400) and another study measuring weight throughout the lactation period reported that rat

offspring from 52% HF-fed mothers were consistently lower in weight than offspring from C-fed mothers (401). Milk composition from HF-fed rodent dams has previously been shown to only have an increase in lipids from approximately P10 until weaning at P21 (402) which could explain why pups from HF mothers have a delayed increase in body weight due to increased adiposity. At 3 weeks of age it appeared that weight of pups from HF mothers were elevated, however only females showed a trend for elevated weight. Previous studies generally report that at weaning (at P21) rodent pups from HF mothers have increased body weight compared to control (403, 404).

The effect of postnatal obesogenic HF diet was evident as offspring aged, and there was a significant increase in body weight in these offspring in both sexes at 15 and 52 weeks. Effects of maternal obesity were subtle with trends for increased weight at both ages in males but only at 15 weeks in females suggesting they may be relatively protected from the effects of maternal HF during aging. Confirming our previous findings in this model (83), at 15 weeks of age only, an interaction effect between maternal and postnatal HF-feeding (HFHF) was observed so that the maternal obesogenic HF diet during pregnancy and lactation exacerbated the effects of postnatal HF diet in both sexes to increase body weight. This interaction between maternal and postnatal HF-feeding to induce increased offspring body weight compared to offspring exposed to postnatal HF alone has been observed in other similar rodent studies (405-407) and suggest that maternal HF primes a greater response to postnatal HF diet in young adult offspring.

Interestingly, weight gain between 15 and 52 weeks of age in males was only weakly affected by postnatal HF diet via a trend, and the HFC group had similar weight gain to the CHF and HFHF group. This further suggests that maternal HF diet may prime male offspring to have increased weight gain, even when fed a C diet postnatally, but only during this period of development. No other effects of maternal obesity were observed to influence offspring weight gain. In general, the effect of weight gain post-weaning in both sexes showed strong significant effects of postnatal HF diet to increase weight gain between testing ages (3-15 weeks and 15-52 weeks) and total adult life weight gain (3-52 weeks).

3.5.2 Effect of HF diets on maternal care and pup anxiety

Despite the increased incidence of HF-fed mothers failing to retrieve pups to their nest, no significance was found in the pup retrieval test between HF and control fed mothers in any of the three phases of the test. However, a trend of increased time spent during the interaction phase of the pup retrieval test was found in HF-fed obese mothers, which may indicate that these mothers have difficulty in recognising their own pups. Others have also shown an increase in pup recognition time in HF-fed obese mothers (408). These results in the current study may have been

limited by small group sizes. There is also anecdotal evidence to suggest that HF diet affects mice behaviour, as those with excessive weight gain lead to cannibalisation of offspring, as mentioned above. One particular HF-fed obese mother with rapid weight gain of over 57% over 6 weeks was excluded from the study due to cannibalisation of young in two separate pregnancies. These effects were not observed in C-fed mothers. This cannibalism has also been noted in other studies (408).

To my knowledge, the effect of maternal obesity on offspring USVs has not been previously investigated. Pup anxiety at P7 was assessed by measuring USVs and these were analysed as individual calls, and cluster calls (multiple individual calls expressed within 1 second of each other) which represent a single attempt to call the mother. Pup USV cluster calls tended to be increased by maternal HF in males but not females. A similar effect was observed in USV individual calls but no significance was observed. Pup USVs function as 'distress calls' to invoke maternal retrieval (409) as pups cannot live for long outside the safety of the nest due to the risk of low temperature (pups lack fur and subcutaneous fat) and predators. Some authors suggest USVs indicate behaviour related to thermoregulation rather than distress (410), however in my experiments the separated pup is placed on a heat mat of approximate temperature to its home nest conditions to reduce any effect of lower temperature. Mouse pups bred for high anxiety phenotypes emit more frequent USVs (369), suggesting a link between stress and USVs. In the current study, male offspring exposed to maternal HF may be attempting to call to mothers, which could reflect the poorer maternal retrieval during the interaction phase in HF mothers. The sex effect could indicate that male offspring are potentially more susceptible to early life environmental changes than their female siblings.

It was not possible from the current study to determine whether maternal care influenced pup USV or, conversely, the level of pup USVs influences maternal care. Although the same pups were used for USV measurements and pup retrieval, no correlations were observed between pup USVs (cluster or individual) and pup retrieval at all phases or total retrieval time. It remains possible that maternal HF diet has differential effects on anxiety in male and female offspring. Further studies are required to fully investigate the effect of HF diet on maternal care and pup anxiety.

3.5.3 Effect of HF diets on locomotor activity and anxiety in young and mature adulthood

Two gold standard tests were used to assess anxiety. In the OF test, anxiety was assessed by thigmotaxis (wall hugging) behaviour (and locomotor activity was also measured in addition as a proxy of exploratory behaviour). Since rodents are neophobic they will find the centre of the OF

test aversive and will generally choose to stay close to the walls, therefore the more time the mice spend in the centre, the less anxious they may be during testing. This test is used as a gold standard test of anxiety in mice. Another gold standard of anxiety phenotype measurement is the EPM in which a mouse is allowed to explore closed vs. open arms. Typically, the less anxious mice will choose to explore the more 'dangerous' open area. Although age appeared to have an effect to OF reduce locomotor activity and increase EPM anxiety, this is a well-documented consequence of aging in C57BL/6 mice (411-413) and was not statistically analysed in this study.

3.5.3.1 Maternal Obesity

I hypothesised that maternal obesity would increase anxiety in adult mice (increased thigmotaxis and decreased locomotor/exploratory behaviour in the OF, and decreased time/entries in open arms of the EPM). However, using well-validated measures of anxiety (OF and EPM), despite some indications that maternal obesity may influence some aspects of anxiety phenotypes, particularly in males, there were no consistent effects of maternal obesity on anxiety.

3.5.3.1.1 Locomotor Activity

Maternal obesity affected some aspects of offspring locomotor activity. In exploratory locomotor behaviour assessment at 15 weeks of age, there were no significant effects of maternal obesity on either ambulatory time and distance travelled, although there was an interaction in the HFC group which had a tendency to be elevated from CC animals for ambulatory time, an effect which has been previously observed in male but not female offspring (324). This same effect was also observed in stereotypic time. This could be suggestive of hyperactivity in these mice and is supported by previous studies (75, 414) who also show increased locomotor activity in mouse offspring from obese mothers in an OF test regardless of sex. In humans there is an association between pregravid maternal obesity and ADHD/hyperactive children (73, 415), and there is a gender bias in ADHD with diagnosis twice as likely in males than females (416), however it is largely unknown what mechanisms are able to induce this behavioural change.

Rearing/vertical behaviour in the OF test was impaired in 52 week-old offspring from obese mothers. Vertical counts were reduced in both sexes and jump counts reduced in female offspring and this trend to rear less may be associated with anxiety as rearing/vertical behaviours are associated with exploration (417, 418) and are viewed as anxiolytic behaviour (419, 420). The reduction in velocity in males and females from obese mothers could also relate to more cautious behaviour in these animals and potentially indicate increased anxiety. There was no difference in weight or weight gain between CC and HFC groups in either sex, therefore these changes in

locomotor behaviours are not likely to be linked to body weight, but instead potential developmental changes due to maternal HF diet.

3.5.3.1.2 Anxiety Behaviour

In terms of OF thigmotaxis analyses, results from previous studies of maternal obesity are inconsistent, with one study showing that 60% HF maternal diet in rats increased centre zone entries and centre ratio of time in mid-adolescent (P35-45) offspring (27), but another showed the same maternal HF diet decreased centre ratio of time in female mouse offspring but not male at the same age (324). Other studies suggest maternal obesogenic 60% HF diet in rats does not influence thigmotaxis in young adult offspring at 2.5 months (279) or 40% HF diet (non obesogenic; just during gestation and lactation) at 4 months (421). Overall, these studies suggest that effects of maternal obesity on thigmotaxis may be time/age or species dependent. In my study maternal obesity decreased percentage distance travelled in the centre in both sexes at 52 weeks, indicating increased anxiety. As mentioned previously, the effect of maternal obesity on offspring thigmotaxis has been shown to vary with age and species which may be why no effects were seen at 15 weeks of age. There are few studies examining older age so the current study adds value to the data of the effects of maternal obesity in mature adulthood.

Anxiety was further assessed in the EPM. Previous research suggests that maternal obesity (via 58%-60% HF-feeding) can cause an increase in time on open arms in both male and female young adult mice aged between 1.5 to 3 months (27, 75); and the study by Balsevich *et al.* also showed hyperactivity in these 3 month-old offspring in OF testing, similar to some of the locomotor activity findings of my study. Conversely, there are similar studies that show decreased time in open arms of both male and female 3 month-old rodent offspring fed a maternal 60% HF diet (77, 277). These differences in research outcomes, despite similar experimental design, suggest further research on the effects of maternal diet on offspring anxiety was warranted. Although there were no effects of maternal obesity on anxiety behaviour at 15 weeks of age in my study, further investigation at 52 weeks of age revealed changes in anxiety in these same mice. In male offspring at 52 weeks of age there was an increase in open arm entries due to maternal obesity and this was reflected as a trend in the percentage of time spent on the open arms (but not open arm time on its own). This effect of maternal obesity on male open arm entries appears to be largely due to the HFC group which, when separately analysed, was significantly increased from both maternal C-fed offspring groups (CC and CHF), whereas the HFHF group was not. This effect of increased/highest entries in the HFC group was consistent in all areas of the EPM in male 52 week-old offspring, which could be indicative of hyperactivity (or indecisiveness (422)) in this group. However, markers of hyperactivity were not observed in the OF test at this age, although

there were some trends for increased ambulatory and stereotypic time in this group at 15 weeks of age. I speculated that the influence of maternal obesity on decreased anxiety on the EPM in 52 week-old male offspring may be influenced by the weight gain in the HFC group between 15 and 52 weeks of age but there was no correlation between these factors.

Although a test used to assess memory, findings from the NOR test also corroborate with EPM results of reduced anxiety in 52 week-old male offspring exposed to maternal obesity. During the NOR acquisition trial there was an increase in exploration of both identical objects, suggesting reduced anxiety in a novel environment, as found in the EPM.

Maternal obesity also had an effect on the number of entries into the far reaches of the open arms. As entry onto the open arms could be brief and the mice could stick to the 'safer' end closest to the centre square, a halfway point of each open arm was marked off to denote a 'danger' zone at the furthest reaches. I posited that a completely open area far from safety like this would show that a mouse that reaches this far would be the least anxious. The results show that maternal obesity increased entries into this far open area at 15 weeks when both sexes combined were analysed. It is difficult to make conclusions about this measure at 52 weeks as there were so few entries to the far end. This measure of movement in the EPM extremities has been used previously (423), and has been shown to be decreased across 3 strains of stressed rats and correlate with general open arm exploration (including open arm entries, time in open arms and head dipping behaviour) (424), and also decreased in transgenic mice bred for anxiolytic properties (via chronically activated GR) which also corresponds to open arm behaviour in general (425). This method however has currently not been well-validated as a measure of anxiety, and therefore is less widely used an output of the EPM. A study of prenatal stress in rats showed increased far end open arm exploration in late adolescent 45 day-old male offspring and decreased far open arm exploration in in 60 day-old females indicating differential effects of maternal environment on sex and age (426). In that study only the effects observed in the females corresponded to open arm behaviour in general but the far arm behaviour in males was not correlated with open arm behaviour (426). In my study, this reduced far arm anxiety behaviour in 15 week-old offspring from obese mothers was significantly correlated to open arm behaviour despite no significant effects of maternal obesity in these parameters. This may suggest that larger animal numbers may elucidate effects of maternal obesity on general open arm behaviour. However, this potential effect of maternal obesity to decrease anxiety was not associated with OF thigmotaxis behaviour at this age, so the effects of maternal obesity on anxiety at 15 weeks are unclear. Although there is an overall effect of maternal obesity, when analysed separately only the HFC group is significantly elevated from CC. This increase in the HFC group in particular has

been noted in the locomotor parameters of ambulatory and stereotypic time which may link to the idea that the mice in this group may be prone to hyperactivity.

3.5.3.2 Postnatal Obesity

My hypothesis that postnatal obesity would increase anxiety behaviours was supported, with notable effects dependent on age/time on the HF diet. Postnatal obesity affected locomotor/exploratory behaviour, thigmotaxis and time spent/entries in an open area. Some of these effects were initially observed at 15 weeks but became more pronounced at 52 weeks, indicating that length of time being obese may be a factor influencing anxiety.

3.5.3.2.1 Locomotor Activity

Although there were no changes to exploratory behaviour at 15 weeks of age, at 52 weeks, postnatal obesity reduced both ambulatory time and distance travelled in both sexes. This, coupled with reductions in velocity, jump and vertical counts at both ages and increased resting time at 52 weeks of age may be indicative of anxiety, however it could also be due to the increased weights in these animals which may make movement more difficult. Postnatal HF-fed obese 12 month-old mice have generally been reported to decrease locomotor activity compared to control (427), which may conflate any reductions in activity due to anxiety.

Stereotypic behaviour entails repetitive functionless motor behaviour which in the OF test break the same beams repetitively; most rodents exhibit stereotypic behaviours such as grooming, sniffing, face washing behaviours and head bobbing. Conditions inducing chronic mild stress have been shown to reduce grooming (428-430), however grooming is also noted in animal models of stress and anxiety (431, 432) which indicates this is a complex behaviour that cannot be simplified and may be related to degree of stressor. While stereotypic time displayed an interaction effect in the HFC group at 15 weeks of age, there was a significant effect of postnatal HF to reduce these behaviours at 52 weeks of age in both male and female offspring. In my study the reduction of stereotypic activity observed in both sexes of offspring fed a postnatal HF diet could be linked to the overall reduction in locomotor activity observed in these same mice. Indeed, a previous study of 45% postnatal HF-feeding in rats has shown to be linked to reduced grooming behaviours in multiple behavioural tests after 16 weeks on the diet (but not 12 weeks) (433), and another study using 60% HF diet to induce obesity in mice noted no difference in grooming behaviour after 18 weeks despite reduced locomotor activity (434), which indicates this activity shift may be fat composition, species and time dependant. In my study, although the reduction in stereotypic behaviour is likely linked to increased weight and overall reduction in locomotor activity, due to the strong effects of anxiety behaviour observed in the OF in both sexes and EPM in the females

due to postnatal HF diet, I posit that the reduction in stereotypic behaviour may also be associated with anxiety and that the HF diet may be acting similarly to chronic mild stress.

Despite differences in weight between postnatal HF and C-fed groups at 15 weeks, there were few changes in locomotor activity in offspring. The higher weight in postnatal HF-fed obese animals at 15 weeks reduced jump behaviour and velocity, but had no effect on other measures of activity behaviour such as distance travelled or stereotypic time. The postnatal effects that did occur at 15 weeks were exacerbated at 52 weeks, and in general locomotor activity was decreased in both male and female 52 week-old mice. While large weight gain in these animals will contribute to this, it could also be associated with anxiety, as these results also correlate with OF zone data. However, it is difficult to separate effects of obesity and effects of anxiety at this age.

3.5.3.2.2 Anxiety Behaviour

In male and female 15 week-old mice, there were no effects of postnatal obesity on anxiety behaviour in the OF test. In general, thigmotaxis anxiety behaviours due to postnatal obesity are reported at various ages in the mice with some mouse studies showing significant effects from 12 weeks in C67BL/6 male mice fed a 58% HF diet (51) to 16 weeks in C57BL/6 male mice fed a 45% fat diet (50) (which could stress the importance of percentage fat diet). No behavioural effects have been shown earlier than 12 weeks with 8 weeks of 60% fat feeding in C57BL/6 male mice showing no behavioural changes in the OF test (435) and one study also in C57BL/6 male mice showed no differential effect of 22 weeks of 60% HF-feeding on OF test thigmotaxis behaviour (264), indicating there may be crucial time windows to anxiety behaviour and that variations between methodology of behavioural testing may also influence anxiety phenotypes. Regardless of the postnatal obesogenic HF diets ability to significantly increase weight gain at 15 weeks of age, 12 weeks of postnatal HF-feeding may not be a considerable enough time to influence anxiety-related changes in the OF test in these animals. Despite this, 15 week males showed a tendency for reduced anxiety on the EPM (increased open arm time) due to postnatal obesity. This result is contrary to current literature on the effect of postnatal obesity, which suggests anxiety (via decreased EPM open arm time and entries) is increased in obese young adult (male) C57BL/6 mice fed a 58%-60% HF diet for 12 to 22 weeks (54, 264) or 45% HF diet in rats for 22 weeks (436), although EPM anxiety in the latter study was not accompanied by changes to OF thigmotaxis (264).

The finding of reduced EPM anxiety in males was supported by results from the NOR test acquisition trial. When faced with two different objects during the acquisition trial, 15 week-old male mice fed a postnatal HF diet spent more time overall exploring both objects, suggesting

inquisitive and less anxious behaviour in a novel environment. As the OF test is a habituated test, this may explain the difference in anxiety outcome. Taken together, these findings suggest obesity induced by a postnatal HF diet may reduce anxiety behaviour in young adult male offspring at 15 weeks of age.

Although closed and centre arm behaviour are not generally used for analysis of anxiety, a significant postnatal obesity effect of reducing time spent in the closed arm in both sexes further indicated reduced anxiety in 15 week-old mice. There was also an increase in time spent in the centre area due to postnatal HF. The centre area provides equal access to open and closed areas and provides no opaque cover over the mouse. How to score the central area is contested, with some researchers implying that as the mouse is started in this area, they are habituated to it (374) and others include time spent in the centre to open arms (437). Time spent in the centre could also indicate indecisiveness as the centre square provides access to all 4 arms, a concept that has been suggested previously (438, 439). Anecdotally, from my observations, mice that spend longer in the centre turn around within the square and smell the entrances; increased time in this area in postnatally HF-fed offspring could suggest that diet has a role in decision-making. In fact, HF diet in rats has been shown to affect decision-making skills (422), which could also be indicative of poor memory with rodents not remembering which arms they have previously visited. Spatial memory was not measured in this study, and further work assessing the potential association with centre time is warranted to make a conclusion.

The effect of age on anxiety became apparent in the 52 week-old mice. At this age there was a significant effect of postnatal obesity to increase all thigmotaxis related behaviour; the percentage of distance travelled and percentage of time resting in the centre zone, as well as total centre zone entries. These data indicate postnatal obesity induces anxiety in both male and female aged adult mice and is in agreement with previously reported data in an obesogenic 60% HF mature adulthood (11 months) rodent model (440). In my study, there was also a decrease in EPM open arm time and a trend for decreased open arm entries at 52 weeks of age due to postnatal HF that is supported in the literature, but this effect was only in the female offspring. In males at 52 weeks of age, the effect of postnatal obesity on increased open arm time (decreased anxiety) observed at 15 weeks was lost. As mentioned above, there is a considerable body of literature on the effects of postnatal HF diet/obesity reducing time on open arms of the EPM (52, 54, 264) due to anxiety and depressive-like behaviours. In some studies (54) these effects only become apparent at a certain age, suggesting mice may need to reach a certain weight or be exposed to a particular diet for a certain period of time before anxiety or depressive-like behaviour manifests. Unfortunately, the literature generally assesses the effect of HF diet on EPM

in young adult, but not mature, age, therefore there are limited long-term studies of the effects of HF for comparison.

From my data, there is a significant effect of sex on anxiety behaviour in the EPM at 52 weeks of age. Although the majority of previous studies are performed on male offspring only, the effect of maternal obesity on EPM open arm time has also been shown to display contradictory results in sex differences. *Bilbo et al* showed that in rats 60% maternal HF diet only affected male offspring anxiety on the EPM at 3 months, as shown by a decrease of open arm time whereas there was no effect in female offspring (277). However, a similar study using the same 60% maternal HF diet and behavioural testing age in mice showed no evidence for sex differences (77). These differences between studies could be attributed to variations in behavioural testing conditions, female oestrus cycles, circadian rhythm, species and so further research in this area is warranted. There was also an overall effect of sex on EPM behaviour where females had significantly increased open arm entries and time than males at both ages. This behaviour could indicate a susceptibility to anxiety in males in general, regardless of diet, and a general differential effect of sex on the EPM as there were no effects of sex on OF thigmotaxis. The only sex effects observed in the OF was in vertical behaviour which may be related to the elevated weights seen in males.

At 52 weeks of age there was also a trend for postnatal obesity to cause female offspring to have a reduction in centre entries, and this alongside the tendency for reduced open arm entries displayed hypoactivity in these mice concurrent with reduced locomotor activity. As mice are placed facing an open arm at the start of the EPM, they make a conscious choice to turn around and enter the closed arms, indicating time and entries into the closed arms are chosen, at least at the start of the test. Overall, the effect of postnatal obesity on reduced activity and also reduced time on the open arm indicate a strong main effect of postnatal obesity on anxiety in the 52 week-old female offspring, but not at 15 weeks of age.

3.5.3.3 Interaction between effects of maternal and postnatal obesity

I hypothesised that maternal obesity would increase susceptibility to the effects of postnatal obesity however few interactions between maternal and postnatal obesity were found in my study such that the HFHF group was adversely affected. There are extremely limited previous studies investigating these potential interactions, particularly into old age, therefore my study provides novel insights into the effects of maternal and postnatal HF on aging and its consequences on anxiety.

3.5.3.3.1 Locomotor Activity

During exploratory analysis, in both sexes at 15 weeks, the HFHF group spent increased time resting. This is likely to be linked to the increased weights of these mice to reduce activity. As expected at 52 weeks of age both male and female offspring displayed increased resting time in the postnatal HF groups (CHF and HFHF) which is likely to be due to their significantly elevated weights. No other significant interactions were found at 15 weeks, however there was a subtle change in vertical behaviour such that the HFHF group was the only group significantly decreased from CC in jump counts and this may be related to the interaction effect observed in weight at this age such that HFHF was the most increased. There was no interaction effect or subtle change on locomotor activity at 52 weeks indicating no cumulative effect of both maternal and postnatal obesity at this age, although large weight gains in these animals due to postnatal HF may be masking any effects, including subtler non-interaction effects also.

3.5.3.3.2 Anxiety Behaviour

In terms of anxiety behaviour, there were no significant interactions between maternal and postnatal HF at either age. Although no interactions were found, there were some subtle changes in anxiety that were exacerbated in the HFHF group. In the 15 week-old males, there was an increased trend in open arm time (and % open arm time) due to postnatal HF that when analysed separately revealed the HFHF group was the only group significantly increased from the CC group indicating this group was the least anxious. However, at 52 weeks of age in both sexes the HFHF group was the most significantly decreased from CC in OF thigmotaxis outcomes (the only significant group decreased from CC in % time resting in the centre), indicating subtle effects of both a maternal and postnatal HF diet that may be brought out with more animals.

The interactions I proposed may not have an effect at 15 weeks of age (12 weeks of postnatal HF-feeding) due to not enough time consuming HF diet which has been shown to generally have an effect after 12 weeks of HF-feeding as mentioned above. However, mice at 52 weeks of age (49 weeks of postnatal HF-feeding) might be too heavy or lethargic to show the effects that maternal diet may cause, as no significant interactions between maternal and postnatal HF were found. There was also a reduction of locomotor activity in older mice, which corresponds with literature (411, 412), which may also mask effects of maternal obesity. As there were some subtle effects of maternal and postnatal diet influencing anxiety separately at 52 weeks, these may be better elucidated with more animals and at an earlier age to minimise the strong effects of obesity and age on anxiety and locomotor activity. If studied further, I would use an age in between the current study, such as between 25-30 weeks based on previous studies ability for both maternal and postnatal diet to affect stress behaviour.

3.5.4 Effect of obesogenic HF diets on memory in young and mature adulthood

The NOR test was performed to assess the effect of maternal and postnatal HF diets on non-spatial recognition memory either directly or due to the link between anxiety and memory (441). The discrimination between familiar and novel objects was dependent on age and sex and was affected by diet.

At 15 weeks of age there was a trend for a reduced discrimination index (DI) in offspring exposed to maternal obesity, suggesting that these mice could not differentiate new from familiar objects and implying poorer recognition memory. Maternal obesity via 60% HF-feeding has been shown to have no effect on NOR testing in 9 week old rats (279), but at 12 weeks of age in C57BL/6 mice a reduction in NOR performance has been reported in males but not females, similar to the outcome of my study (395). Although mice at 12 months of age have been shown to worsen NOR memory in a model of Alzheimer's disease (210), this 15 week memory impairment was lost at 52 weeks of age in male offspring. This lack of effect at older age may potentially be due to previously reported general reduction in the ability to discriminate between familiar and novel objects as mice age (442) and/or reduced locomotor activity observed in aged mice, which could be masking effects of the diet.

In the females, maternal obesity appeared to have a significant long-term effect to enhance non-spatial recognition memory in female offspring at this age. This improvement in recognition memory in 52 week-old females was unexpected, although another study by Bilbo *et al.* has also previously observed improved memory after multiple memory tests in 3 month-old female rats exposed to a 60% maternal HF diet (277). In my study, this effect of maternal obesity on memory was not correlated with an effect on anxiety, since anxiety was largely affected by postnatal obesity. There is a scarcity of studies examining the effects of maternal obesity on non-spatial recognition memory assessed by NOR in female offspring and in aged offspring, thus it is difficult to compare my data to previous studies.

In my study no effects of postnatal obesity on memory were observed, however, the effect of postnatal obesity itself (induced by sucrose, HF or both) has been shown to vary between different rodent studies. While it has been shown to impair memory in the NOR test in high sucrose fed mice (443), and mice fed 40% HF diet after 21 weeks of age (444), obesity has also been shown to have no effect on memory in the NOR. For example, memory was not affected in 12 week-old mice fed a 60% HF diet (445) or in 6 month-old mice exposed to a diet containing 32% fat (446). In fact postnatal obesity may be able to worsen non-spatial memory in an age dependant manner, since a study of male mice fed a 60% HF diet only showed a poor performance during the NOR test at 11-12 months of age and older but not at 3-4 or 7-8 months

of age (447). This aged effect of postnatal obesity could explain why memory impairments were not seen in the NOR test due to postnatal obesogenic HF-feeding in my experiments as my mice may not have been old enough for impairments to take place. The percentage fat diet used in different studies to induce obesity may also be a factor in any effects on memory impairments.

My results show that in male offspring, a HF diet fed throughout young adult (15 week-old) mice's life alone is not sufficient to influence recognition memory, as measured by the NOR. The dietary group most affected is when a 'second hit' of HF diet is fed both postnatally following maternal HF-feeding (HFHF) and is the only diet group significantly different to the CC group, suggesting this deficit could be primed prenatally and then revealed when fed a postnatal HF diet. This 'second hit' of maternal and postnatal obesogenic (60-65%) HF diet has previously been shown to negatively impact spatial memory during tests using a Morris Water Maze wherein HFHF mice (fed postnatal HF for 12-14 weeks) were unable to retain memory of previous test days compared to C-fed mice, with HFC only performing marginally better (278, 448). Another similar study using maternal and postnatal HF diet exposure noted a significant maternal effect to impair memory in the same test, with (45% HF-fed for 13 weeks postnatally) HFHF group again performing the worst (211). I observed no effect of diet in the 15 week-old female offspring, but unfortunately, the tests above only assessed male offspring, therefore there were limited studies for comparison to these differential sex effects, although, a LepRdb/+ mouse model of maternal obesity has noted impaired spatial memory in male, but not female, HFHF groups (449). Although the data were quite variable, it appears that there is a reduction in discrimination index with age in males but not females at 52 weeks, however this was not statistically analysed.

3.5.5 Conclusion

In this chapter I have observed an age and sex- dependent main effect of postnatal obesity on anxiety and maternal obesity on memory, but no major effects of both maternal and postnatal obesity combined. Generally, the data do not consistently support the hypothesis that maternal obesity increases anxiety and impairs memory, and that interaction with further postnatal obesity worsens these effects. This is surprising considering the majority of evidence currently available suggests that both maternal obesity and postnatal obesity separately induce anxiety and impair memory. However, I only studied the effects of diet on young and mature adulthood, the latter of which may be masking the effects of anxiety and memory due to age effects, and the former of which may not be enough time to detect changes in behaviours. There were also fewer numbers of animals at 52 weeks of age for testing, although patterns of maternal obesity influencing anxiety are emerging, such as in OF zone data. Regardless, there may be subtle exacerbation effects in the HFHF group to alter anxiety and memory as can be seen in Figure 3.19.

My results also show there may also be subtle differences in behaviour between male and female offspring, particularly anxiety behaviour on the EPM and memory in the NOR test. This could indicate increased sensitivity of male offspring to HF diet as they appear to be more susceptible to the effects of maternal obesity and that this starts from pup age and persists into old adulthood. There is currently limited animal research on the effects of maternal obesity on both male and female offspring behaviours however, so further research needs to be done to confirm any sex-specific behavioural defects.

These behavioural differences due to maternal and/or postnatal obesogenic diet consumption may have potential implications for mental health problems and cognition in adult life, some of which may be due to alterations of the HPA axis. The HPA axis has a role in regulating anxiety and memory, and HF diet, both maternal and postnatal, have been shown to be able to alter HPA function basally and in response to stress. The HPA axis is also known to have sex-specific effects which can influence multiple disease outcomes (450) and may explain the discrepancies between male and female data. Therefore, next steps in this thesis involved determining if behavioural alterations are connected to disruption of the HPA-axis.

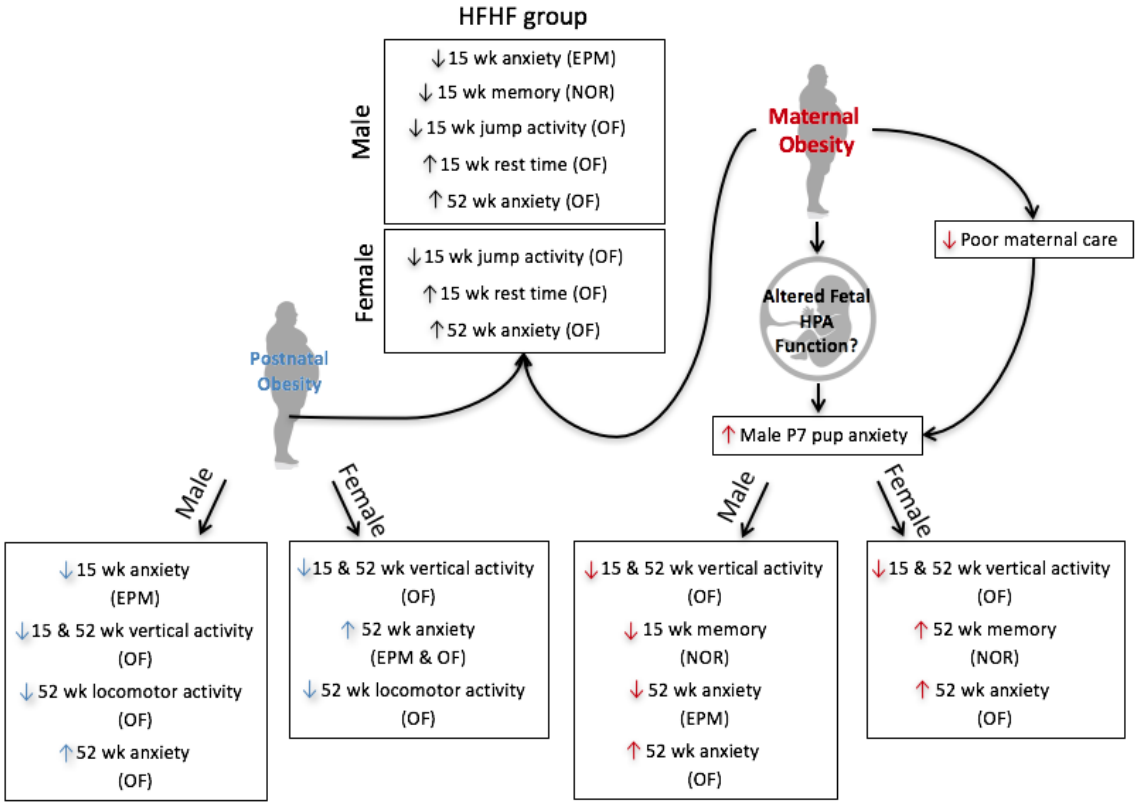

Figure 3.19 – **Schematic diagram of overarching results from Chapter 3.** Overall independent effects of maternal (red) and postnatal (blue) obesity on behaviour and memory in male and female offspring at 15 and 52 weeks of age. Combined box shows effects that were exacerbated in the HFHF group (significantly different from CC group). OF, open field; EPM, elevated plus maze; NOR, novel object recognition.

Chapter 4 The effect of maternal and postnatal obesity on offspring HPA axis

4.1 Introduction

In Chapter 3, the effect of maternal and postnatal obesity was investigated in young and mature offspring behaviour and the effect of HF obesogenic diet on maternal care was also explored. I observed that mothers fed a HF diet showed a trend toward impaired maternal care and that changes in offspring anxiety occurred in males from P7. There were some subtle changes in anxiety and memory at 15 weeks of age and these effects were more prevalent in males. At 52 weeks of age however, anxiety was significantly associated with postnatal HF-feeding in females and improved memory with maternal HF diet, whereas in males, results assessing anxiety were mixed. Overall these results show that obesogenic HF-feeding at different stages of the life course is able to alter behaviour in mice and that these changes may be age and sex-dependent. Neuropsychiatric disorders and changes in behaviours and cognition are strongly linked to disturbances in HPA axis function (451). Therefore, the next step in this thesis was to investigate the effect of maternal and postnatal obesity on aspects of HPA axis function in these offspring at 15 and 52 weeks of age.

As outlined in Chapter 1, the HPA axis is a homeostatic system that acts in response to stressors to increase the output of glucocorticoids. This axis mediates the responses to stressful environments and situations such as inflammation and emotional distress. Dysregulation of this axis can increase risk of health problems such as metabolic syndrome, weight gain and inability to regulate stress responses which can lead to chronically elevated stress (452, 453). The hypothalamus is the main centre of HPA function in the brain, with other regions such as hippocampus also playing a role in HPA control (144). Both maternal and postnatal obesity have independently been shown to affect HPA axis activity. HPA axis function also has well-established differences between the sexes (140).

Maternal obesity has been reported to influence several components of the offspring HPA axis. In a rat study of maternal 60% HF diet before and during pregnancy and lactation, 3 month-old offspring were reported to have reduced levels of basal corticosterone in both sexes, and reduced response to stress in females only (76). Levels of MR and GR in brain amygdala were also elevated in offspring from HF-fed mothers and this was coupled with changes in behaviour; anxiety was

shown to be elevated in both OF and EPM testing (76). In another study of maternal 58% HF diet, male offspring were shown to have unaltered basal corticosterone and unaltered response to stress at both 3 and 12 months of age (75). At 12 months of age however, GR and FKBP51 levels were increased in the PVN of the hypothalamus suggesting subtle changes to the HPA axis took place and these changes were coupled with increased anxiety at 12 months but not at 3 months of age (75).

Postnatal obesity itself is also known to alter HPA axis function. A study of chronic (12 weeks) 45% HF-feeding in male mice resulted in decreased basal corticosterone plasma levels, decreased morning GR and CRH mRNA levels in the PVN and increased evening CRH mRNA levels. These results suggest that HPA activity is reduced in these animals and that there may be complex changes in diurnal regulation (454). Other similar studies of chronic HF-feeding in rodents also suggest the HPA response to stress is altered, with an increase in output levels of corticosterone (259) and ACTH (260). However, Auvinen *et al.* systematically reviewed that effects of obesogenic HF-feeding on HPA axis activity varied widely between studies due to inconsistencies between methodologies such as the time that HPA activity was assessed, diet composition, duration of diet, rodent strain/species and housing situation, amongst many other variables (256). This shows that the HPA axis is vulnerable to even the smallest of changes and necessitates further research in this area to clarify the potentially contradictory effects of obesity on offspring HPA axis function. There is also a paucity of studies that take into account the potential interaction between maternal and postnatal HF diet together. Therefore, this chapter focuses on the effect of maternal and postnatal obesogenic HF diet, sex differences and age on HPA axis regulation.

4.2 Hypothesis and Aims

Hypothesis: Maternal HF diet will alter regulation of HPA axis activity in offspring, affecting plasma corticosterone and ACTH output. This effect will be exacerbated by a postnatal HF diet.

Aims: To investigate the effects of maternal and postnatal HF diet on:

1) Corticosterone concentrations basally and during restraint stress test at 15 and 52 weeks of age
2) ACTH concentrations basally and during restraint stress test at 52 weeks of age
3) Hypothalamic and hippocampal markers that regulate HPA axis activity at 15 and 52 weeks of age

4.3 Methods

4.3.1 Mouse cohorts

4.3.1.1 Cohort B: High-fat mouse model - 15 week molecular data

In a previous study performed by Dr Felino Cagampang, non-virgin female C57BL/6 mice were fed either a control (C, 7% kcal fat) or high-fat (HF, 45% kcal fat) diet for 6 weeks before mating and during pregnancy and lactation to induce obesity (C, *n* = 9; HF, *n* = 7). Offspring were then fed a C or HF diet post-weaning from 3 weeks of age onwards, creating 4 dietary groups: CC, CHF, HFC and HFHF, as detailed in section 2.1.1 and in in appendix A. Animals were sacrificed at 15 weeks and brains were collected between 13.00-16.00 hours and snap frozen in liquid nitrogen and stored at -80˚C until PVN and CA3 regions were micropunched (details in section 2.1.2).

4.3.1.2 Cohort C: High-fat mouse model – restraint stress and 52 week molecular data

Non-virgin female C57BL/6 mice were fed either a control (C, 7% kcal fat) or high-fat (HF, 45% kcal fat) diet for 6-11 weeks (depending on length of time to reach minimum compulsory 15% weight gain) before mating and during pregnancy and lactation (C, *n* = 5; HF, *n* = 8). Offspring were then fed a C or HF diet post-weaning from 3 weeks of age onwards, creating 4 dietary groups: CC, CHF, HFC and HFHF, as detailed in section 2.1.1 and in in appendix A. Animals were sacrificed at 52 weeks and brains were collected between 13.00-16.00 hours and snap frozen in liquid nitrogen and stored at -80˚C until PVN and CA3 regions were micropunched (details in section 2.1.2).

4.3.1.3 Lipopolysaccharide (LPS)-treated mice

LPS-treated mice were used as a control measure to assess a widely used mouse model of inflammation that I could compare my data to. C57BL/6 female mice between 4-5 months of age (n = 2) were injected with 200 µl of LPS at 10 µg/ml concentration into the intraperitoneal cavity. Animals showed sickness behaviour suggesting evidence of brain inflammation. Mice were sacrificed 4 to 6 hours after treatment and perfused with saline. Whole brain, pituitary and adrenals were collected and snap frozen in liquid nitrogen and stored at -80˚C until PVN and CA3 regions were micropunched (details in section 2.1.2).

4.3.2 Restraint stress test

Experiments were performed as previously described in sections 2.2.2.5. All restraint testing occurred at 16 and 52-54 weeks of age. Mice were placed in the testing room for 30 minutes prior to testing to acclimatise before each experiment took place.

Briefly, each mouse was placed into a restraint tube for 30 minutes and a tail snip of < 1 mm was performed. Blood was taken (~50 µl) via tail 'milking' immediately (0 minutes), 15 minutes and 30 minutes after being placed in the restraint tube, and then again after 30 minutes of recovery time (60 minutes from start). Blood was centrifuged at 2000 RPM for 5 minutes and plasma pipetted into a separate tube and stored at -80°C until using to measure corticosterone and ACTH concentrations.

4.3.3 Corticosterone and ACTH ELISAs

Plasma corticosterone concentrations were assessed by ELISA (Enzo Life Sciences, USA), as outlined in section 2.5.1. Briefly, ELISA-prepared plasma samples, standards and controls were pipetted into appropriate wells of a 96-well ELISA plate, and then conjugate and antibody added to appropriate wells. After incubation and washing, Substrate Solution was added to every well. Post-incubation, Stop Solution was added to every well and the plate read immediately at optical density 405 nm. Results were corrected for blank wells. Calculation of results can be found in section 2.5.1.1.

Plasma ACTH concentrations were assessed by ELISA (Enzo Life Sciences, USA), as outlined in section 2.5.1. ELISA-prepared sample, standards and controls were pipetted into designated wells of a Streptavidin coated 96-well plate. Biotinylated Antibody and Enzyme Labelled Antibody was added to the same wells. After incubation and wash steps, TMB Substrate solution was added to each well and incubated again. Stop Solution was added to each well and the plate read immediately at optical densities: 450 nm and 405 nm. Samples and controls were read using the 450nm results for ACTH concentrations up to 150 pg/ml. ACTH concentrations above 150 pg/ml was interpolated using the 405 nm calibrator results. Calculation of results can be found in section 2.5.2.1.

Using Graphpad Prism, corticosterone AUC (above baseline) was calculated for 30 minutes of restraint time plus 30 minutes of recovery time in each sample at 15 and 52 weeks of age. ACTH AUC (above baseline) was calculated for 30 minutes of restraint time plus 30 minutes of recovery time in 52 week-old offspring only.

4.3.4 Analysis of gene levels

Total RNA was extracted by methods outlined in section 2.4.1. Briefly, PVN and CA3 regions were micropunched from cryostat-cut sections of whole brains from 15 and 52 week-old mice. Tissue was homogenised in Tri-reagent, isolated using chloroform and isopropanol, and washed in

ethanol to prevent contamination. Nanodrop Spectrophotometer (Nanodrop 1000, Thermo Scientific, USA) was used to calculate concentration and purity of RNA for each sample.

cDNA synthesis was performed using TaqMan® Reverse Transcription Reagents (ThermoFisher Scientific, Basingstoke, UK) using Multiscribe Reverse Transcriptase and random hexamers. 400 ng RNA was used for each reaction. Real-time qPCR was performed using pre-validated primers of GOI (Sigma-Aldrich, UK) and SYBR Green (Bio-Rad, UK) on Roche LC480 PCR system (Roche, UK). The geometric mean of housekeeping genes PGK1, ATP5B and CYC1 were used to normalise GOI results. GOI data were analysed by plotting log concentration of standards against CT value and calculating mRNA levels using the CT values of the samples of interest. All samples were split for gene analysis by brain region; all ages, sexes, diet groups in PVN or CA3 samples underwent cDNA synthesis and real-time qPCR on the sample plate.

Table 4.1 - **Details of genes used for relative mRNA expression analysis**

GENE	Accession No.	Company	Primer	Sequence 5'-3'
GR (NR3C1)	NM_008173.3	Sigma Aldrich	Forward Reverse	5'-TGGAAGGACAGCACAATTACCTT-3' 5'-CTTCGTTTTTCGAGCTTCCAGG-3'
MR (NR3C2)	NM_001083906.1	Sigma Aldrich	Forward Reverse	5'-CTGGCCAAGGCAGCTATGGA -3' 5'-GTTCGGAGTAGCACCGGAAA -3'
CRH	NM_205769.5	Sigma Aldrich	Forward Reverse	5'-TCTGGATCTCACCTTCCACCT-3' 5'-CCATCAGTTTCCTGTTGCTGT-3'
FKBP51	NM_010220.4	Sigma Aldrich	Forward Reverse	5'-AGCAACGGTAAAAGTCCACCT -3' 5'-TTCCCCAACAACGAACACCA -3'

4.3.5 Statistical Analysis

A mixed effects model was used to analyse corticosterone, ACTH and 52 week mRNA levels offspring data (see section 2.6 for details). This statistical model was used as it factored in the effects of littersize and which dam each individual originally came from. Therefore, all results are independent of dam and littersize.

Results used in graphs are predicted means ± SEM derived from maximal models (including diet group and littersize) applied to observed data. Depending on normal distribution, data were either not transformed or z-transformed before mixed model statistical analysis. This made sure dependant variables were standardised allowing the effect estimates to be compared directly.

15 week mRNA level data were statistically analysed by 2-way ANOVA. In Cohort B litters were standardized and only one offspring was taken from each mother, so each offspring was independent. Therefore, mixed model analysis was not required.

Overall statistical differences were assessed in both sexes combined and in each sex separately. Results are presented separately due to the well-known differential sex effects in HPA axis function and indeed in this study there were general effects of sex on HPA activity across ages and brain regions. LPS-treated mouse data difference from CC groups per sex and age were analysed by *t*-test per brain region. SPSS version 24 and GraphPad Prism were used for all analysis.

4.4 Results

4.4.1 Baseline and stress-induced corticosterone concentrations

The time courses of corticosterone (CORT) concentrations during the restraint stress test are shown in Figure 4.1A-D. At 15 weeks of age, there was a trend for maternal HF to increase baseline CORT in males ($P = 0.075$, Figure 4.2A) and only the HFHF group tended to be increased from both CC ($P = 0.062$) and CHF ($P = 0.061$), but there was no effect of diet in female offspring (Figure 4.2C). However, at 52 weeks, there was a significant effect of postnatal HF to increase baseline CORT in both male ($P < 0.0001$, Figure 4.2B) and female offspring ($P < 0.01$, Figure 4.2D). There was no effect of maternal HF at this age.

At 15 weeks of age, in response to stress, there was a trend of HFHF diet group to be decreased from CC in male offspring ($P = 0.056$, Figure 4.2E) and no effect of diet in female offspring (Figure 4.2G). However, at 52 weeks there was a significant effect of postnatal HF diet to decrease CORT AUC in both male ($P < 0.0001$, Figure 4.2F) and female offspring ($P < 0.05$, Figure 4.2H). There was also an overall significant effect of maternal HF diet to increase CORT AUC in male offspring ($P < 0.05$, Figure 4.2F).

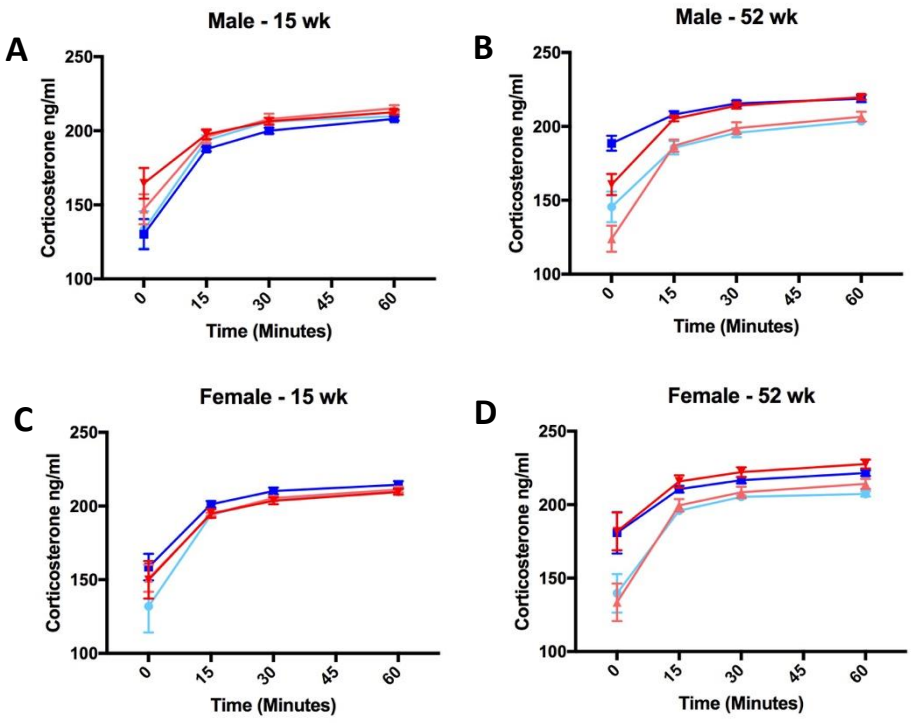

Figure 4.1 – **Corticosterone concentrations during restraint stress test.** Effect of maternal and postnatal HF diet in male (A, B) and female (C, D) offspring (CC, light blue; CHF, dark blue; HFC, pink; HFHF, red) on CORT time course during restraint stress test (15 weeks; A, C, 52 weeks; B, D). Data are displayed as predicted means ± SEM from maximal models. n = 5-13 per sex per group (see Table 2.2 in Methods).

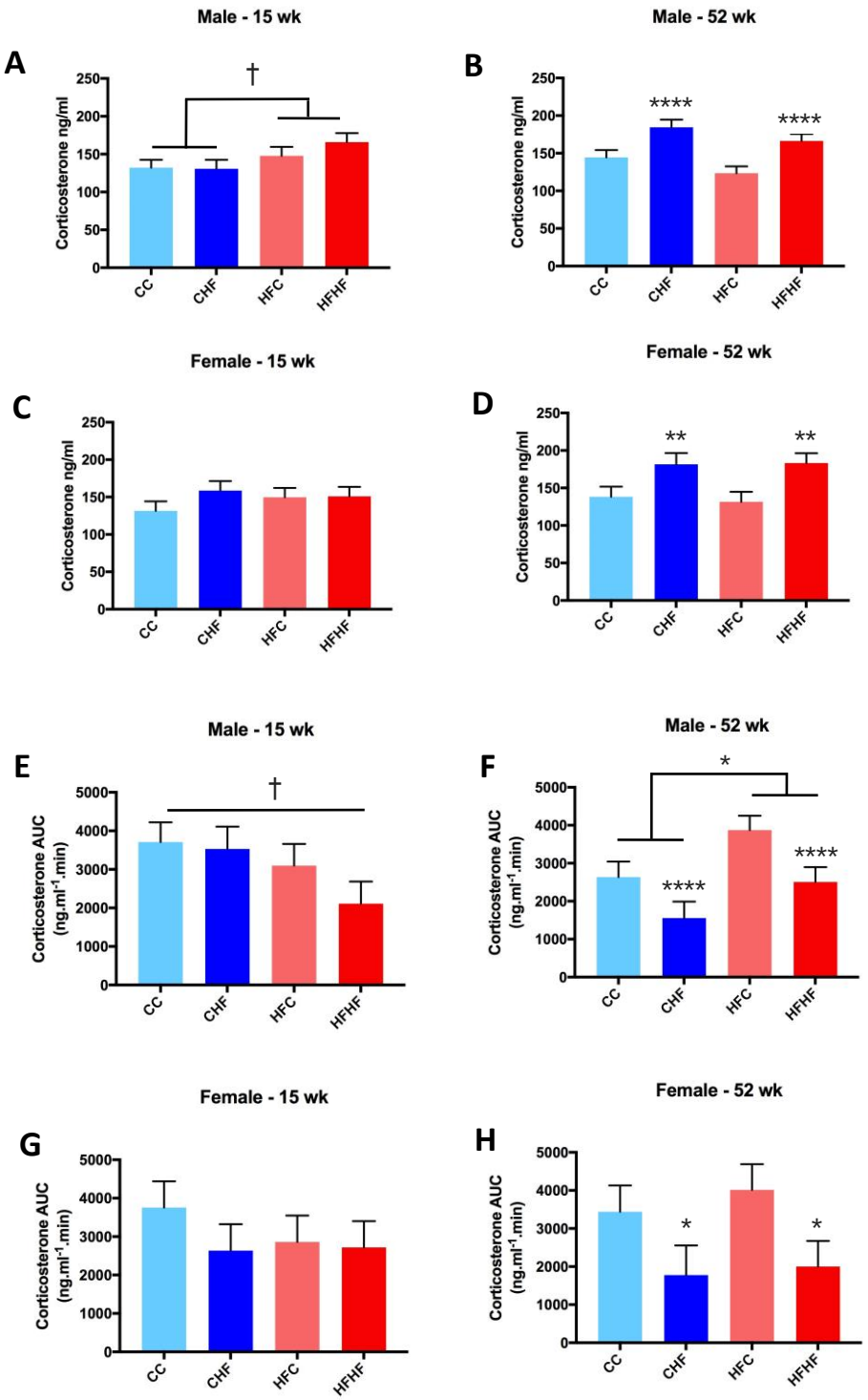

Figure 4.2 – **Corticosterone concentration at baseline and AUC during restraint stress test.** Effect of maternal and postnatal HF diet in male (A, B, E, F) and female (C, D, G, H) on baseline CORT (15 weeks; A, C, 52 weeks; B, D) and CORT AUC (15 weeks; E, G, 52 weeks; F, H). **** $P < 0.0001$, ** $P < 0.0001$, * $P < 0.05$, † $P < 0.1$. Data are displayed as predicted means ± SEM from maximal models. n = 5-13 per sex per group (see Table 2.2 in Methods).

4.4.2 Baseline and stress-induced ACTH concentrations

The time course of ACTH concentrations during the restraint stress test in 52 week-old males and females are shown in Figure 4.3A and B. In male offspring there was a trend of postnatal HF diet to decrease basal ACTH ($P = 0.064$, Figure 4.4A), but in female offspring postnatal HF diet significantly increased basal ACTH ($P < 0.0001$, Figure 4.4B). In females, there was also a significant interaction ($P = 0.03$) between maternal and postnatal HF diet which revealed ACTH concentrations in the HFHF diet group were significantly higher than maternal or postnatal HF alone. This HFHF group was the only group significantly increased ($P < 0.01$) from CC. In females but not males, basal ACTH levels significantly positively correlated with basal CORT levels at 52 weeks ($P < 0.001$, $R^2 = 0.436$). There was an effect of sex on postnatal HF diet in females such that this group was significantly greater than males ($P < 0.0001$).

In male offspring no effect of diet was seen on ACTH AUC (Figure 4.4C). In female offspring there was a significant effect of postnatal HF diet to reduce ACTH AUC ($P < 0.05$, Figure 4.4D). In females but not males, ACTH AUC significantly positively correlated with CORT AUC at 52 weeks ($P < 0.01$, $R^2 = 0.314$). Male offspring had increased overall ACTH AUC response overall compared to females ($P < 0.05$).

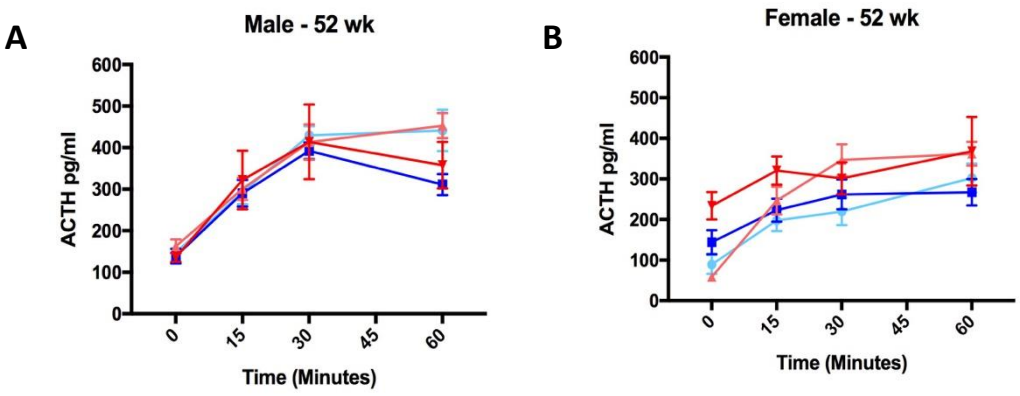

Figure 4.3 – **ACTH concentrations during restraint stress test.** Effect of maternal and postnatal HF diet in male (A) and female (B) offspring (CC, light blue; CHF, dark blue; HFC, pink; HFHF, red) on ACTH time course during restraint stress test. Data are displayed as predicted means ± SEM from maximal models. n = 5-13 per sex per group (see Table 2.2 in Methods).

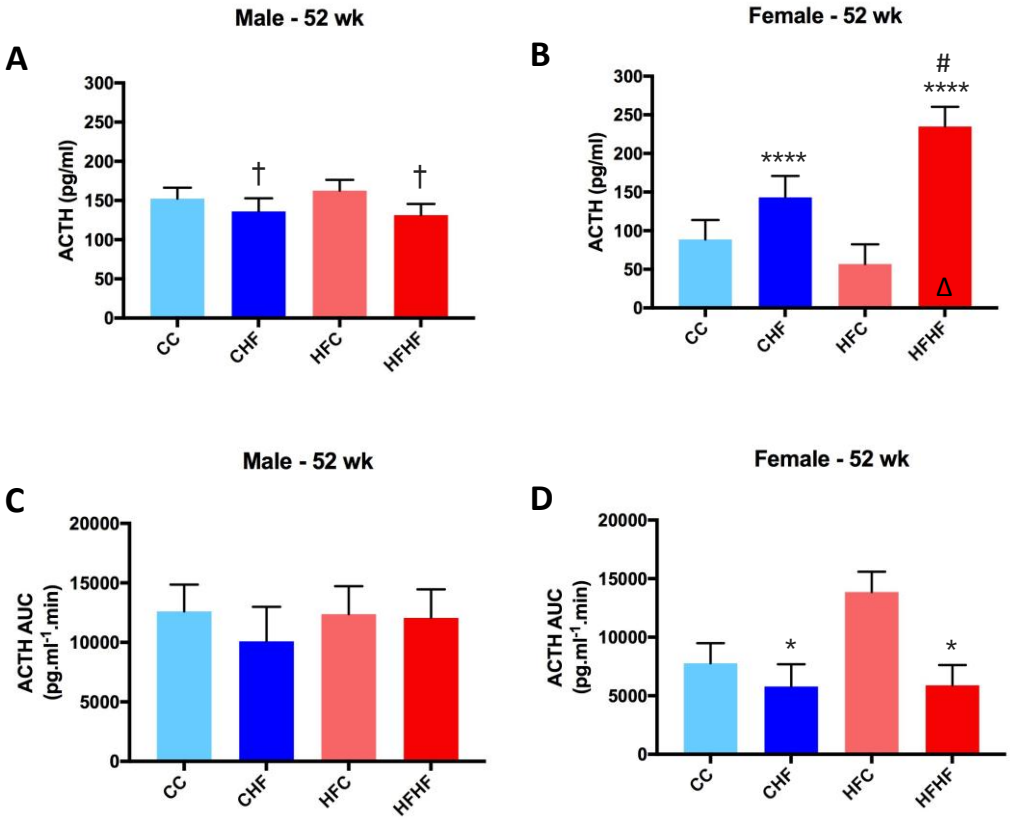

Figure 4.4 – **ACTH concentration at baseline and AUC during restraint stress test.** Effect of maternal and postnatal HF diet in male (A, C) and female (B, D) on ACTH baseline (A, B) and AUC (C, D) at 52 weeks of age. **** $P < 0.0001$, * $P < 0.05$, † $P < 0.1$, # $P < 0.05$ pre*postnatal interaction group affected. Δ, only HFHF significantly different from CC. Data are displayed as predicted means ± SEM from maximal models. n = 5-13 per sex per group (see Table 2.2 in Methods).

4.4.3 Molecular analysis of key regulators of HPA axis activity (basal measurements)

4.4.3.1 Glucocorticoid and mineralocorticoid receptor mRNA levels in the PVN and CA3

There was no effect of maternal or postnatal HF diet on GR in the PVN or CA3 compared to control In both 15 week-old offspring (Figure 4.5A, C, E, G) and 52 week-old offspring (Figure 4.5B, D, F, H). However there was an interaction ($P < 0.05$) between maternal and postnatal HF in the female PVN at 15 weeks, which when analysed between groups showed that HFC was significantly increased from the CC group ($P < 0.05$, Figure 4.5C). Male offspring GR levels were increased overall from females in the PVN at 52 weeks ($P < 0.0001$, Figure 4.5B and D)) but decreased from females in the CA3 at 15 weeks of age ($P < 0.0001$, Figure 4.5E and G).

MR levels were increased by a maternal HF diet in 15 week-old male offspring PVN ($P < 0.05$, Figure 4.6A) and CA3 ($P < 0.0001$, Figure 4.6E) but only the HFHF group was significantly elevated from CC in PVN at this age ($P < 0.05$). MR levels in CA3 tissue were also decreased by postnatal HF diet in males at 15 weeks ($P < 0.05$, Figure 4.6E). The only effect in 15 week-old females was a trend ($P = 0.064$) for maternal HF diet to reduce MR levels (Figure 4.6G).

At 52 weeks of age there was no effect of diet on PVN MR levels in male offspring (Figure 4.6B) but MR levels were reduced by maternal HF diet in the CA3 ($P < 0.0001$, Figure 4.6F) but only the HFHF group was significantly reduced from CC ($P < 0.01$). In the females at this age there was a significant effect of postnatal HF diet to increase MR levels in PVN ($P < 0.05$, Figure 4.6D) but reduce MR levels in CA3 ($P < 0.01$, Figure 4.6H).

There was a significant effect for MR levels to be greater in the females than males in both PVN and CA3 at 15 weeks of age (both $P < 0.01$, Figure 4.6A, C, E, G), and for the males to be greather than females in the PVN and CA3 at 52 weeks of age (both $P < 0.01$, Figure 4.6B, D, F, H).

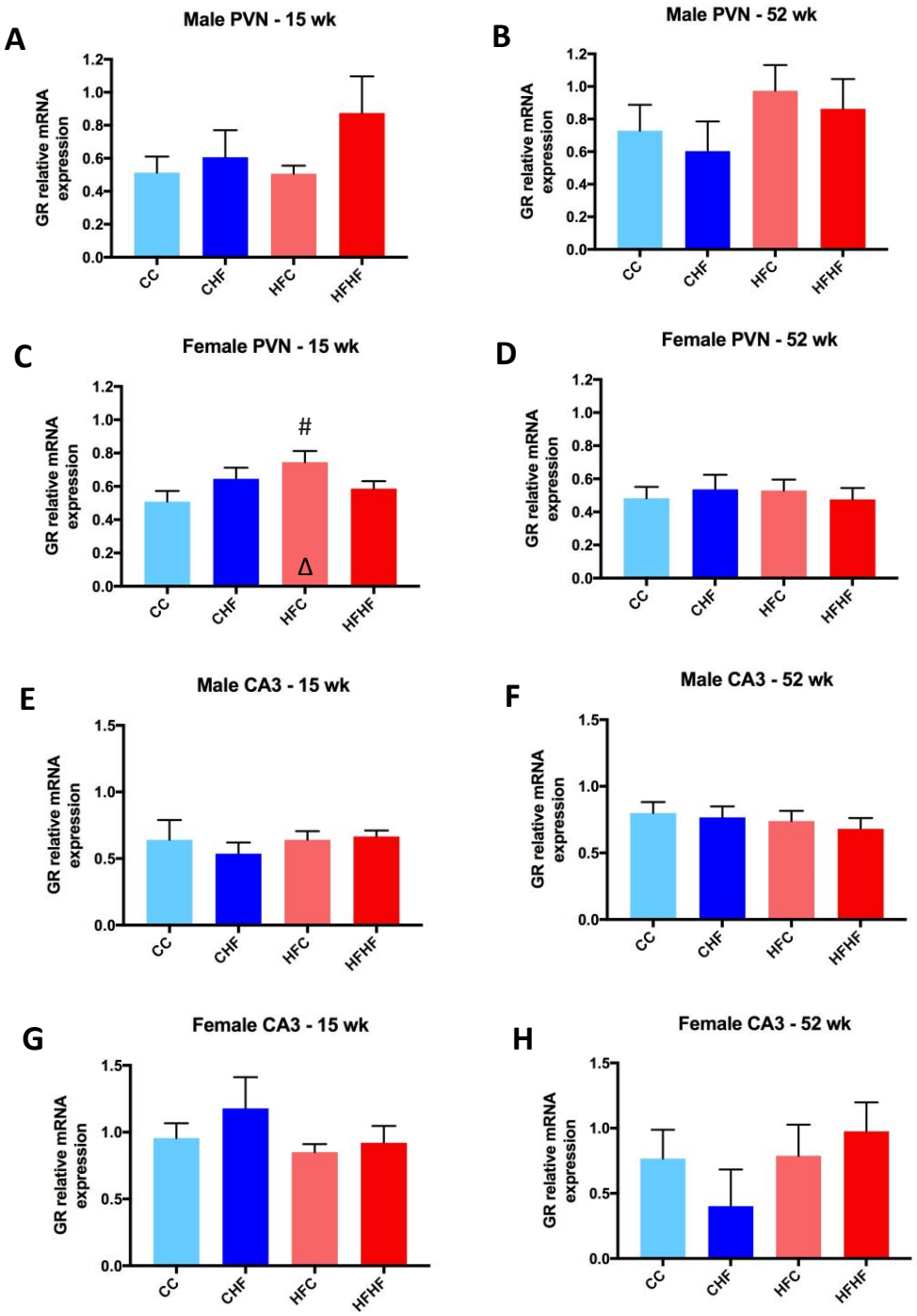

Figure 4.5 – **GR mRNA levels in PVN and CA3.** Effect of maternal and postnatal HF diet in male (A, B, E, F) and female (C, D, G, H) on GR levels in the PVN and CA3 (15 weeks; [PVN: A, C] [CA3: E, G] and 52 weeks; [PVN: B, D] [CA3: F, H]). # $P < 0.05$ pre*postnatal interaction group affected. Δ, only HFC significantly different from CC. Data are displayed as predicted means ± SEM from maximal models. n = 3-9 per sex per group (see Table 2.2 in Methods).

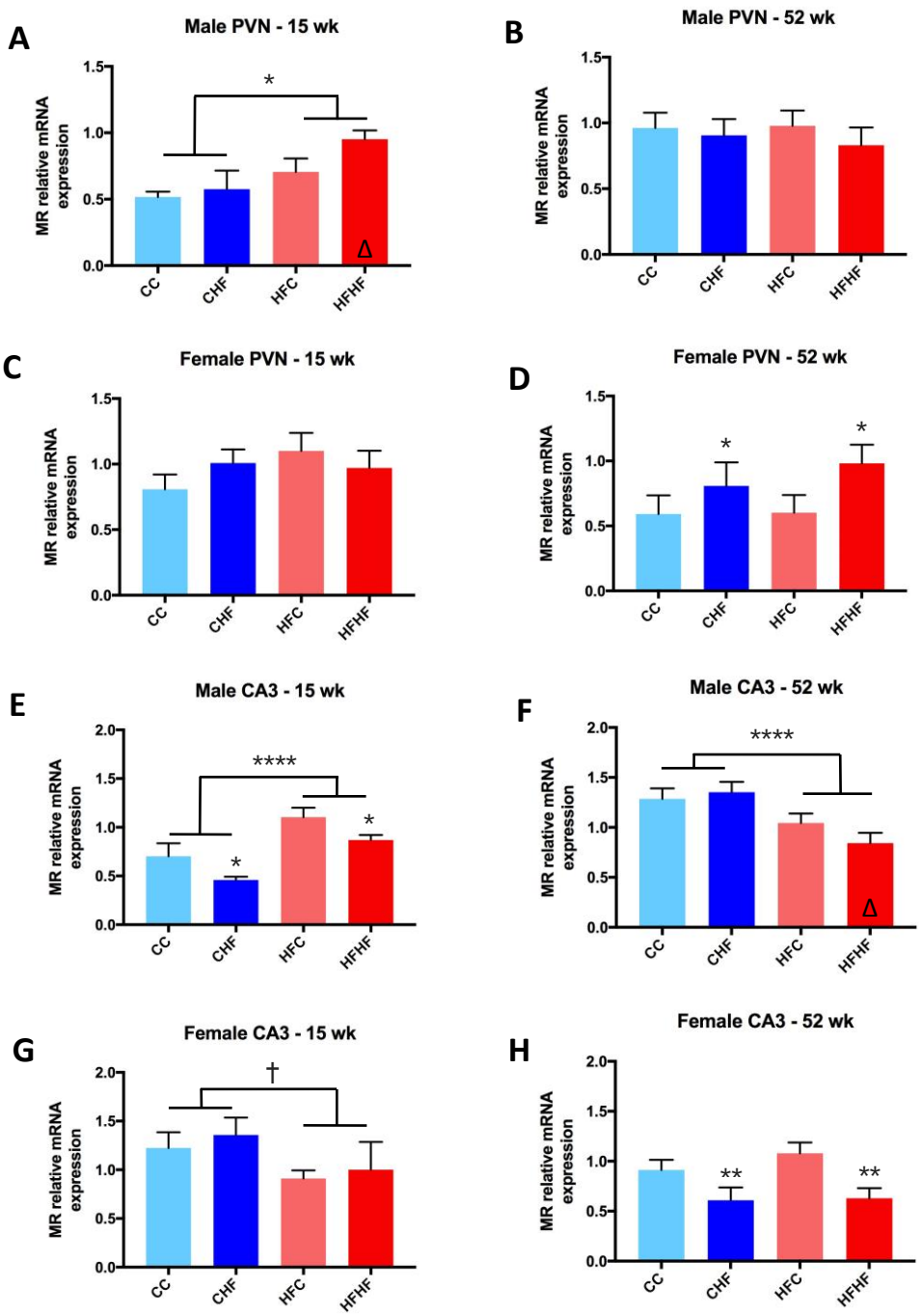

Figure 4.6 – **MR mRNA levels in PVN and CA3.** Effect of maternal and postnatal HF diet in male (A, B, E, F) and female (C, D, G, H) on MR levels in PVN and CA3 brain tissue (15 weeks; [PVN: A, C] [CA3: E, G] and 52 weeks; [PVN: B, D] [CA3: F, H]). **** $P < 0.0001$, ** $P < 0.01$, * $P < 0.05$, † $P < 0.1$. Δ, only HFHF significantly different from CC. Data are displayed as predicted means ± SEM from maximal models. n = 3-9 per sex per group (see Table 2.2 in Methods).

4.4.3.2 CRH mRNA levels in the PVN and CA3

CRH mRNA levels were increased by maternal HF diet in male offspring at 15 weeks in the PVN ($P < 0.001$, Figure 4.7A) and when analysed separately HFHF was the only group significantly elevated from CC ($P < 0.01$). Maternal HF diet also increased CRH in the CA3 ($P < 0.05$, Figure 4.7E), and there was an additional effect of postnatal HF to increase CRH levels in the CA3 only ($P < 0.05$) such that the HFHF group was significantly elevated from all other groups (CC, $P < 0.001$; CHF and HFC, $P < 0.009$). At 52 weeks of age, maternal HF reduced CRH levels in the PVN ($P < 0.0001$, Figure 4.7B) but there was no effect of diet on CRH levels in the CA3 (Figure 4.7F).

In female offspring there was a trend ($P = 0.063$) for increased CRH levels in the postnatal HF groups at 15 weeks (Figure 4.7C) in the PVN (again the HFHF group was the only group significantly elevated from CC [$P < 0.05$]) and no effect of diet in CA3. There was no effect of diet on CRH levels in 52 week PVN (Figure 4.7D) but maternal HF tended to increase CRH levels ($P = 0.078$, Figure 4.7H) in the CA3. There was overall increased CRH levels in the PVN in males compared to females at 15 ($P = 0.059$) and 52 weeks of age ($P < 0.0001$). In the CA3, there was increased CRH levels in females compared to males at 15 weeks of age ($P < 0.01$) but no sex effect at 52 weeks of age.

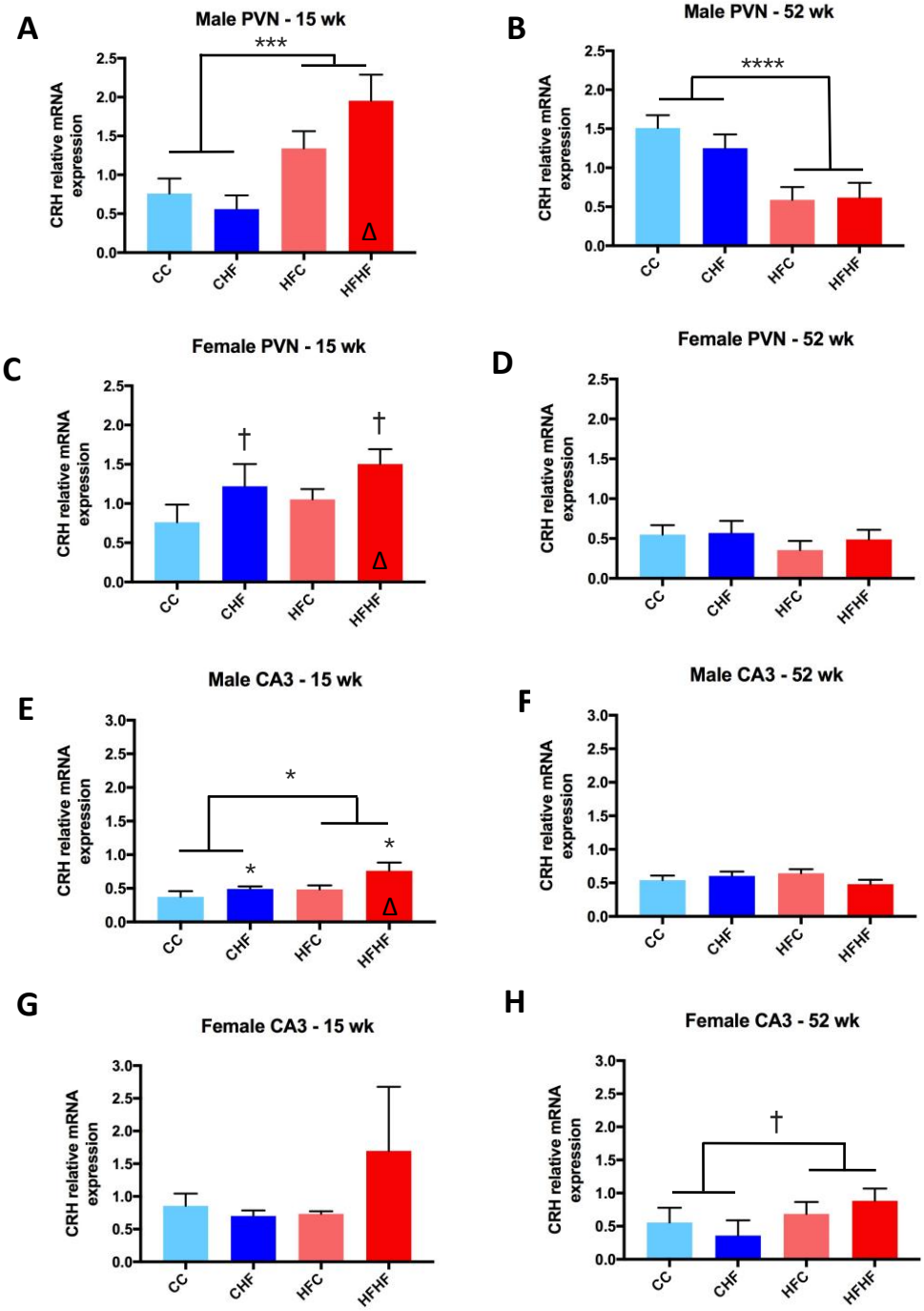

Figure 4.7 – **CRH mRNA levels in PVN and CA3.** Effect of maternal and postnatal HF diet in male (A, B, E, F) and female (C, D, G, H) on CRH levels in the PVN and CA3 (15 weeks; [PVN: A, C] [CA3: E, G] and 52 weeks; [PVN: B, D] [CA3: F, H]). **** $P < 0.0001$, *** $P < 0.001$, * $P < 0.05$, † $P < 0.1$. Δ, only HFHF significantly different from CC. Data are displayed as predicted means ± SEM from maximal models. n = 3-9 per sex per group (see Table 2.2 in Methods).

4.4.3.3 FKBP51 mRNA levels in PVN and CA3 brain tissue

The only effect of diet on FKBP51 mRNA levels in PVN tissue occurred in 52 week-old female offspring where there was a significant effect of postnatal HF diet to increase FKBP51 levels ($P < 0.0001$, Figure 4.8D). However, in CA3 tissue, effects were observed from 15 weeks of age. In male offspring there was an effect of postnatal HF diet to increase FKBP51 levels ($P < 0.0001$) and there was an interaction between maternal and postnatal HF diet ($P < 0.05$) such that the HFHF group was significantly increased from all other diet groups (CC and HFC: $P < 0.0001$, CHF: $P < 0.01$, Figure 4.8E). In 15 week-old females there was an effect of maternal HF diet to reduce FKBP51 levels in the CA3 ($P < 0.01$, Figure 4.8G), however this effect was lost at 52 weeks (Figure 4.8H). In 52 week-old males there was an effect of both maternal and postnatal HF diet to increase FKBP51 levels in the CA3 (both $P < 0.01$) such that the HFHF group was the most significantly increased from all other groups (CC, $P < 0.0001$; CHF and HFC, $P < 0.05$, Figure 4.8F)

There was a significant effect for FKBP51 levels to be greater in the males than females in both PVN and CA3 at 15 weeks of age (both $P < 0.01$, Figure 4.8A, C, E and G) and in the CA3 at 52 weeks of age ($P < 0.01$, Figure 4.8F and H). There was a significant effect for FKBP51 levels to be greater in the females than the males in the PVN at 52 weeks of age ($P < 0.001$, Figure 4.8B and D).

Data from LPS-treated mice showed an increase in FKBP51 compared to CC mice for all PVN [15 week male; CC: 0.79 ±0.15 ($P < 0.05$), 15 week female: 0.43 ±0.06 ($P < 0.0001$), 52 week male; CC: 0.97 ±0.17 ($P < 0.01$), female: 0.76 ±0.28 ($P < 0.01$), LPS: 1.73 ±0.03], but not CA3 [15 week male; CC: 0.85 ±0.12, 15 week female: 0.93 ±0.23, 52 week male; CC: 0.91 ±0.11, female: 0.98 ±0.22, LPS: 0.73 ±0.11].

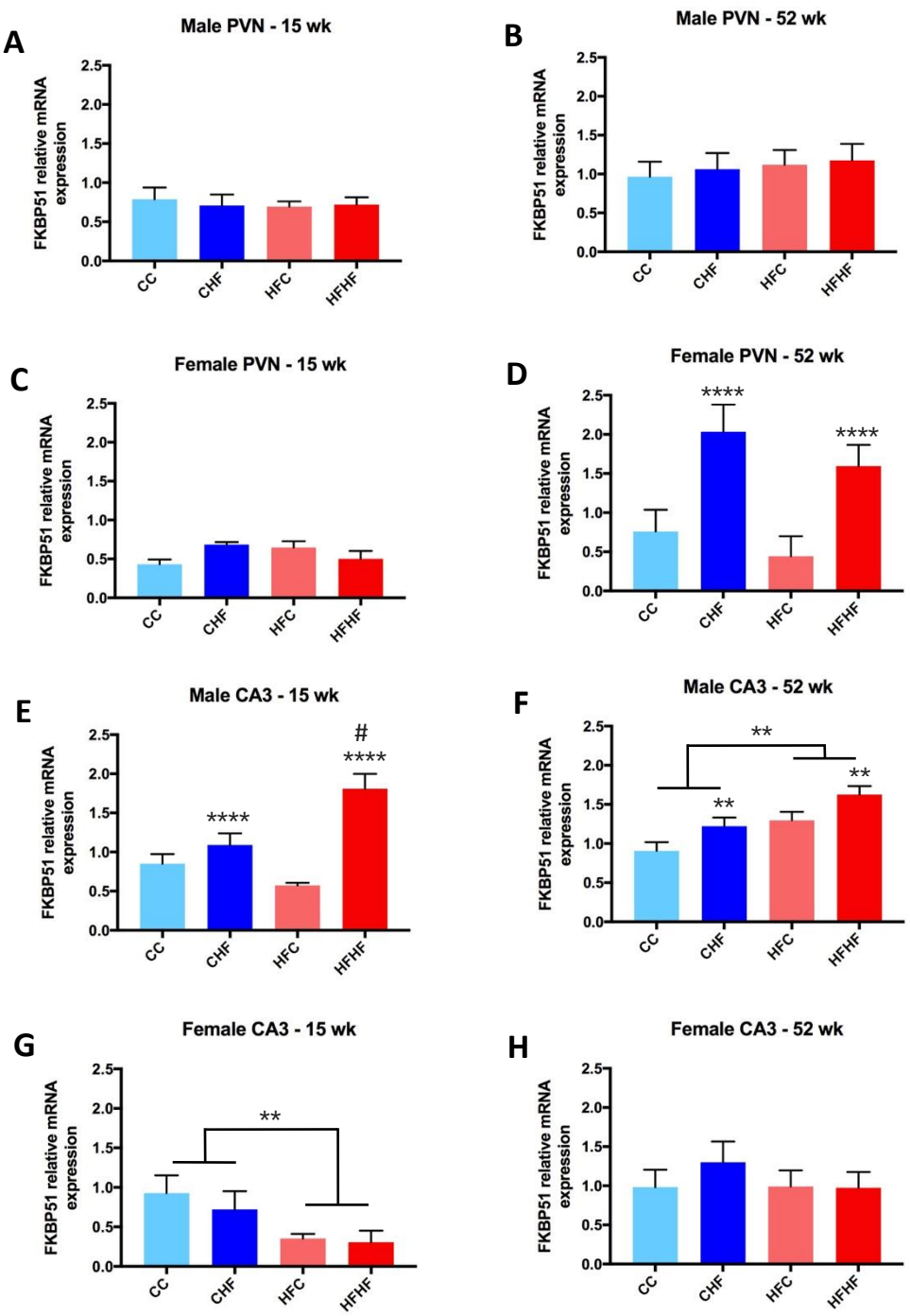

Figure 4.8 – **FKBP51 mRNA levels in PVN and CA3.** Effect of maternal and postnatal HF diet in male (A, B, E, F) and female (C, D, G, H) on FKBP51 levels in the PVN and CA3 (15 weeks; [PVN: A, C] [CA3: E, G] and 52 weeks; [PVN: B, D] [CA3: F, H]). **** $P < 0.0001$, ** $P < 0.01$, * $P < 0.05$, † $P < 0.1$. # P < 0.05 pre*postnatal interaction group affected. Data are displayed as predicted means ± SEM from maximal models. n = 3-9 per sex per group (see Table 2.2 in Methods).

4.5 Discussion

In Chapter 3, I reported changes in anxiety and memory due to maternal and postnatal obesity. As anxiety and memory are in part mediated by changes to the HPA axis I investigated whether they were linked. In order to assess changes in HPA function resulting from a change in maternal and postnatal diet, stress tests and mRNA level analysis of brain tissue were performed in young (15 weeks) and mature (52 weeks) adult offspring from HF and C-fed dams. Restraint stress tests performed on the same offspring at 15 and 52 weeks of age showed main effects of postnatal HF diet in both male and female offspring but maternal HF affected males only, inferring sex-specific effects of maternal obesity. mRNA levels of genes relating to HPA function were assessed in banked 15 week tissue, and in 52 week tissue from my own cohort of mice (same used for restraint testing). Again, effects of postnatal diet were observed in both male and female offspring, whereas significant effects of maternal diet were largely observed in male offspring.

4.5.1 Effect of maternal and postnatal obesogenic HF diets on HPA function

There were overall effects of maternal and postnatal obesity to alter different parts of the HPA axis and these effects were sex and age-specific. The effects will be discussed separately below.

4.5.1.1 Postnatal Obesity

There were significant effects of postnatal obesity to increase baseline and reduce AUC of CORT at 52 weeks of age in both male and female offspring. The same effects were also observed in ACTH data in female offspring, but in males there was an opposite effect of postnatal HF diet to have a tendency to instead reduce baseline ACTH and have no effect on AUC.

4.5.1.1.1 Effects on corticosterone in both sexes.

The effect of postnatal HF diet to increase basal CORT confers with previous literature. In humans, obesity is generally considered to be associated with elevated glucocorticoid levels as measured by daily output and diurnal slope measurement (238, 455), but due to confounding methodology and other variable factors in human research, results are often inconsistent between studies. Animal studies have been useful due to highly controlled environments and therefore allow us to directly assess the effects of obesity for example through precise HF-feeding. The effect of HF diet in postnatal life to increase baseline CORT has been previously reported in a number of animal models. For example, a study of 45% HF-feeding for 12 weeks in male rats and a study of 60% HF diet for 18 weeks in C57BL/6 mice showed significantly elevated basal CORT levels measured in the morning (265, 456). Another study of 45% HF diet on male rats

fed from weaning until 24 weeks of age showed that HF-feeding caused an increase in basal CORT levels measured in the morning (the same time as my mice were measured) (436). This study also showed that there was no significant difference between morning and evening levels of baseline CORT in the HF group (as was seen in the control group) indicating that the normal diurnal variation in these mice is disrupted and that CORT is chronically upregulated throughout the day at this age (but a similar study did not find diurnal corticosterone consistency at 12 weeks 45% HF-feeding (256) . A long-term study of 45% HF-feeding in rats from weaning to 9-12 months of age also showed a trend for increased basal CORT (measured in the morning) (427). These data all consistently showed increased CORT from rodents fed a HF diet in both young and old adulthood. Overall, the 52 week data from my study matches previously reported outcomes linking obesity to increased basal CORT.

The chronically elevated CORT observed in my study could be indicative of a hyperactive HPA axis. However, the effects observed in my study due to postnatal HF diet only become apparent with age; there are no effects at 15 weeks of age in either sex. Despite McNeilly *et al.* describing increased CORT levels after 12 weeks of 45% HF-feeding in rats (265), there is no effect after 12 weeks of feeding in my study. This could be due to differences between rats and mice, and also this study also did not state from what age the diet was taken. Despite the early weight gain in the postnatal HF-fed groups, the effect of postnatal obesity on the HPA axis of these mice may only become apparent as they age, having spent longer on the HF diet. It would be interesting to assess the effects of postnatal HF diet at a mid-point age in my mouse model to pinpoint the length of time on the diet that it takes to alter CORT output. Few studies have examined the effects of HF-feeding in conjunction with aging, and many studies only assessed males, therefore my study adds value to this particular area of research.

CORT AUC was significantly decreased due to postnatal HF diet in response to an acute restraint stress test in both male and female 52 week-old offspring. This may suggest that chronically elevated basal CORT has reset the negative feedback for when there is an acute stressor. It has been proposed that chronic stress is able to attenuate glucocorticoid response to an acute stressor (457), and in male rats chronic restraint stress for 14 days has been shown to reduce ACTH response to stress so that it is comparable to non-stressed mice, but diminish CORT response to stress, indicating differential changes in HPA function similar to that of my study (458). In my study postnatal obesity may be acting as a 'stressor'. In humans, obesity is generally considered to be associated with elevated glucocorticoid levels and responsiveness (238, 455), with reactivity being highly associated with abdominal obesity in particular. Limited research exists assessing the effect of obesity on stress responses in animals. However, one particular study similar to my own, of 45% HF-feeding in mice for 12 weeks, showed that at 18 weeks of age

male offspring fed a HF diet had a decreased CORT response to a 30 minute restraint stress test plus 30 minute rest period (459). This study corresponds to the results observed at 52 weeks of age in my study. This could indicate that similarly to basal CORT results, the effect of diet on stress response may be age/time on HF dependent.

Altered adrenal corticosteroid output could be due to changes in the adrenal itself (e.g. altered sensitivity or steroidogenic enzyme capacity) or altered stimulation by circulating ACTH from the pituitary. To assess the possible causes, I measured ACTH concentrations at 52 weeks, as well as hypothalamic and hippocampal factors that regulate the HPA axis. Sex effects were observed in both basal and stimulated ACTH.

4.5.1.1.2 Females

For females, the increase in basal CORT at 52 weeks of age due to postnatal obesity could be due to increased basal ACTH, the results of which were significantly correlated in postnatal HF groups. Interestingly, preliminary research performed using my 52 week adrenal glands (from the same animals) suggests reduced ACTH receptor (melanocortin 2 receptor; MC2R) due to postnatal HF diet (data not published), which may be an attempt to reduce HPA activity. The driving force behind this elevated circulating ACTH did not appear to be CRH which was unaffected by postnatal obesity at 52 weeks of age. There was however, a trend for increased CRH in 15 week-old females, and this may be a precursor to HPA hyperactivity in the mature offspring, however due to using different cohorts the mice cannot be directly compared. The lack of effect of postnatal HF on CRH at 52 weeks may indicate changes to CRH receptors levels in the pituitary, receptor sensitivity or CRH level itself. CRH receptors (corticotropin-releasing factor receptor 1 or 2; CRF-R1 or CRF-R2) are expressed in the CA3 and hippocampus also (460). Future work measuring these receptors would be beneficial to understand the connection between CRH and ACTH at this age.

Alterations in the feedback of the HPA axis may be contributing to this HPA hyperactivity, therefore steroid receptors GR and MR were evaluated in the PVN and CA3 regions of the hypothalamus and hippocampus respectively. FKBP51, a modulator of steroid receptor binding affinity, and therefore sensitivity, was also measured. Despite elevated basal CORT levels, there was no change in relative GR levels in either brain region. Few studies have assessed the long-term effect of obesity on GR expression in the hypothalamus, however previous research suggests a diurnal change of GR in the PVN after 12 weeks of HF-feeding, wherein 45% HF-feeding caused a reduction of GR levels in the PVN at 9.00 but not 18.00 hours in male C57BL/6 mice (454). These effects were not elucidated in my study as samples were only assessed at a single afternoon PM time point.

Due to the lack of changes in response to obesity in the GR, relative MR levels was also investigated as this receptor has ten times higher binding affinity for CORT (461). Changes were found in MR levels due to postnatal HF diet that were specific to the brain region. In the PVN there was an increase in 52 week-old females, alongside the increase in basal CORT observed in these same mice. This suggests MR levels may be upregulated in response to elevated basal CORT in order to enhance the negative feedback of the HPA axis. Alongside GR, MR has been shown to be important for the long-term adaptation of the brain to stress and altered HPA function (462) and studies using selective antagonists of MR show that MR and not GR are crucial to the habituation response to repeated stress (in my study obesity is the "stressor") on the release of CORT through negative feedback (463). Indeed, enhanced negative feedback may explain the response to an acute stress test. ACTH AUC during restraint stress testing was decreased due to postnatal HF diet in 52 week-old female offspring and significantly positively correlated with CORT AUC. The correlation indicates potential overall reductions in HPA output in response to acute stress. It would also be interesting to measure CRH mRNA levels in the brain of a stressed mouse in order to get a clearer picture of total HPA output, however this was not performed in my study.

Studies of postnatal HF diet/obesity in rats has been shown to be associated with reduced MR levels in the hippocampus (51, 464). In addition, prolonged stress has previously been shown to decrease MR levels in the rat hippocampus (465). In my study there was an effect of postnatal HF to reduce MR levels in CA3 tissue 52 week-old female offspring, which may be related to the increased circulating CORT. No changes in MR were seen at 15 weeks of age which agrees with the lack of changes in HPA output at this age. It is unclear why there are differential effects of MR levels in the two brain regions. However, elevations in FKBP51 levels in the PVN, and not the CA3, may partially explain this.

As mentioned previously, FKBP51 is a negative modulator of GR and MR activity, and hormone regulated GR directly increases FKBP51 levels as an auto-regulatory process that decreases glucocorticoid sensitivity. FKBP51 (measured in amygdala) has also been strongly associated with anxiety-related psychiatric disorders (359). In FKBP51 knockout mice (females) GR mRNA levels in both the PVN and CA3 brain regions are unaffected (361). In my study postnatal obesity increased FKBP51 at 52 weeks but this was not associated with changes in GR levels. This increased FKBP51 levels could be associated with the anxiety phenotypes observed in these same mice and potentially also indicate that while GR mRNA levels may not be altered in these same mice, their sensitivity to their increased CORT may be decreased. Such glucocorticoid 'resistance' in the postnatal HF groups could lead to compensatory high levels of basal circulating CORT. This would also suggest that the reduced interaction between CORT and the GR could be blocking the

negative feedback mechanism, causing persistently elevated basal (but not stress induced) circulating CORT levels. Glucocorticoid resistance due to elevated FKBP51 has been previously noted in squirrel monkeys (466) and in humans (467) and diagnostic assays are being developed using FKBP51 as a marker of glucocorticoid sensitivity (468). In addition, aged FKBP51 knockout mice show decreased CORT levels after response to stress (10 minutes restraint) and elevated anxiety (469), although these authors did not state in which sex this was performed. It is unclear what role FKBP51 plays in the decreased CORT response to stress in the female 52 week-old mice in my study. As FKBP51 decreases steroid receptor sensitivity, an increased response to stress would be expected, however the increase in PVN MR may be mediating this effect.

Overall, in the females, effects of postnatal obesity were largely at 52 weeks and not 15 weeks, suggesting the length of time exposed to obesity and/or age is an important factor in regulation of HPA axis function.

4.5.1.1.3 Males

In the male offspring, basal ACTH at 52 weeks tended to be decreased by postnatal obesity, indicating a differential effect on the HPA axis compared to the females. In humans, obesity has been previously shown to exhibit overall elevated basal plasma ACTH in adult males (263). However, animal models assessing the effect of obesity on basal ACTH levels are limited and the effects of diet-induced obesity on adrenal function/morphology have not been studied in detail, particularly in older age (256). Despite this, the studies that do exist suggest that obesity does not affect ACTH baseline levels at young adult age; a study of 60% HF-feeding for 18 weeks in C57BL/6 mice revealed no changes in basal plasma ACTH levels compared to C-fed mice (456), and another study of 40% HF-feeding in male rats showed no changes in basal ACTH either (470) (both studies measured ACTH in the morning). To my knowledge this assessment of ACTH at 52 weeks of age in maternal and postnatal HF-fed mice is novel.

To explain the trend for decreased basal ACTH but increased CORT we see in the same mice at 52 weeks of age, it could be suggested that there may be changes in ACTH MC2R levels in the adrenal glands – increase receptor numbers or enhance receptor signalling. However, preliminary results from further research conducted on the adrenal glands from my study have not revealed any effects of either maternal or postnatal HF diet on MC2R levels (data not published). Therefore, the changes we observe may be due to changes in ACTH signalling in the adrenal. Due to the lack of studies assessing obesity on both MC2R and CRFR it is unclear whether they are involved in the dysregulation of the HPA axis we see due to obesogenic HF-feeding in postnatal life. Research has recently shown a melanocortin 2 receptor accessory protein (MRAP) is necessary for the interaction of ACTH with MC2R in order to traffic MC2R from the endoplasmic reticulum to the

cell surface and facilitate signalling (471, 472). MRAP has since been found to be a critical component of the HPA axis, involved in adrenal responsiveness to ACTH, however to date there are no studies (to my knowledge) assessing the effect of obesity on MRAP levels (473), therefore it is unknown whether this gene may play a part in potentially increasing adrenal sensitivity to ACTH that could explain the increase in CORT. Another possible explanation is that there may be increased local CORT synthesis that is associated with excess adipose tissue in the adrenal. Males are significantly heavier than females at 52 weeks of age so this could contribute to elevated CORT observed, and there may be increased fat storage in the adrenal itself (research using mouse adrenal glands from this study is currently assessing this). Indeed, relative levels and activity of 11β -HSD1 that converts inactive cortisone into active CORT has shown to be increased in male mice regardless of C or HF diet, and this was reduced by estrogen administration suggesting that females may be protected from some of the adverse effects of adiposity (474). There could also be a role for upregulated steroidogenic enzymes, so that despite low ACTH, basal CORT can still be increased by postnatal HF, and indeed current research using adrenal glands from the same mice in my study have found that postnatal obesity increases steroidogenic enzymes at 52 weeks in males (data unpublished).

The trend for reduced basal ACTH in male postnatal HF groups at 52 weeks was not associated with basal CRH levels in either PVN or CA3. Reductions in basal ACTH levels may be arising through an alternate pathway for example via changes in pituitary CRF receptor levels or sensitivity. Obesity has been linked to increased CRFR1 levels in PVN tissue in a model using obese Zucker rats (475) and also to increased CRFR2 levels in dorsal raphe nucleus found in the brainstem in Marmosets fed a 12.7% HF diet (476). To my knowledge there is no current literature on the effect of either maternal and postnatal HF diet on CRFR1 and 2 levels and sensitivity to CRH in the pituitary gland, and further research in this area may reveal the reason behind the changes to basal ACTH seen in my study.

The mechanism underpinning the lack of correlation between ACTH and CORT response to stress in 52 week-old males is unclear. As mentioned previously the HF diet may be having an effect on MC2R sensitivity (as measured by MRAP) or levels during the restraint stress test. It is interesting to note that ACTH response to stress is generally increased in the males compared to females which may be masking effects of diet. For the current study an additional in vivo cohort for assessment of adrenal and pituitary changes immediately following a restraint challenge would have been useful to assess HPA disturbances when faced with an acute stressor.

In order to assess whether the effect of postnatal obesity on CORT levels were associated with other components of the HPA axis, GR, MR and FKBP51 were also assessed in males. Although

there were no changes to CORT at 15 weeks of age due to postnatal HF, there was an increase in CRH in the CA3 but not PVN at this age. These changes in CRH levels did not correspond directly to changes in GR or indeed the CORT output in these animals at this age, and cannot therefore be explained in the context of this study. However, the increase could potentially be priming the offspring for altered hippocampal dependent learning and memory later in life, a concept previously put forward in the context of chronic stress (179, 182). The most affected group in these animals was the HFHF group which was significantly increased from all other groups and this was the group with the most impaired memory in the NOR test in Chapter 3. These changes in CRH in the CA3 linked to postnatal HF diet may correspond to other tests of long-term memory or cognitive function that it was not possible to measure in my study.

There was an effect of postnatal HF to reduce MR levels in CA3 tissue in 15 week-old males and this concurred with studies of both obese zucker rats and postnatal 60% HF diet (for 8 weeks) showing reduced MR levels in the male and female hippocampus respectively (51, 464). The reduction in MR at 15 weeks in my study were not associated with HPA hormone output. However, lower MR levels may be impairing negative feedback in this region, enhancing elevated CORT at later age, although no postnatal changes to MR levels were observed at 52 weeks of age. Despite no change to FKBP51 in the PVN in the male offspring, postnatal HF increased levels in the CA3 brain region at both 15 and 52 weeks. Not only does stress increase levels of FKBP51 in the PVN region, but increases have also been observed in the hippocampus of mice (352), which could indicate that changes to the HPA axis may be able to permanently affect the CA3 region which typically has high levels of FKBP51 (352). It is also likely that the increase in FKBP51 levels might be exacerbating the HPA axis hyperactivity observed in the male offspring in later age due to prevention of hippocampal contribution to negative CORT feedback via negative modulation of steroid receptors MR and GR.

While the elevation of FKBP51 levels in the females may partially explain the HPA axis hyperactivity we see in the 52 week-old offspring, it is interesting that we do not also observe a similar outcome in males of the same age. Unlike the females, the 52 week-old males do not consistently show anxiety during behavioural tests, and in addition, there is no significant effect of diet on basal circulating ACTH levels. This could indicate that the chronically elevated CORT levels in these male offspring may be permanently altered via an alternative mechanism to the females, indicating sex-specific effects of chronic obesogenic HF-feeding on HPA axis function.

4.5.1.2 Maternal Obesity

Effects of maternal obesity were more subtle compared to postnatal obesity, with main effects seen primarily in the male offspring at both ages.

4.5.1.2.1 Glucocorticoid receptor effects in both sexes

Maternal obesity via 58-60% HF-feeding has been shown to decrease hypothalamic GR levels (as measured via IHC) at 3 months (in the ARC and VMH) but increase at 12 months-old in male C57BL/6 mice (in the ARC, VMH and PVN) (75), and increase GR levels in the hippocampus at 1.5 months of age in rats (27). The study by Balsevich *et al.* also showed chronically elevated FKBP51 levels in the PVN of the same mice at both 3 and 12 months of age. However, we observed no effects of maternal obesity to alter GR or FKBP51 mRNA levels in the offspring, in either region, sex or age. This difference of outcomes may be due to the differing methodologies used such as the higher fat content in the diet or use of histochemistry to assess changes in gene levels.

4.5.1.2.2 Females

There were few effects of maternal obesity on HPA activity in females at either age. Female basal HPA hormone output from PVN and stress response remained unaffected by maternal obesity. The idea that females are more resilient to stress has been proposed and this may be the case in my study, regardless of maternal diet. Oestrogen produced in the brain has previously been reported to protect against the negative effects of chronic stress (during restraint) in female but not male mice (477). It was not possible to measure this in my study. Overall, the only effect to alter HPA output was a non-significant trend for increased CRH in the CA3 at 52 weeks and this was not associated with ACTH or CORT. In order to completely assess the effects of maternal obesity, steroid receptor levels and function were also investigated.

The trend for maternal HF diet to decrease MR levels in the 15 week-old female CA3 was not associated with changes to the HPA axis or behaviour/memory, however FKBP51 was also significantly reduced in the same animals due to maternal obesity and this may be influencing increased glucocorticoid binding affinity in the steroid receptors, which may compensate for the reduction observed in MR levels. These effects did not persist until 52 weeks of age, indicating the effect of maternal obesity is subtle and not long lasting. Although the studies on maternal obesity are limited, a study of prenatal stress during late pregnancy in rats has shown similar results of reduced MR levels in CA3 tissue of females between 16-17 weeks of age, but not males (478). This study also showed no effect of prenatal stress on GR levels in CA3 in either sex, similar to my study.

4.5.1.2.2.1 Females - Interactions between maternal and postnatal diet

For GR, there was an interaction between maternal and postnatal HF such that there was an in increase mRNA levels in the HFC but not HFHF group in the PVN of 15 week-old females only. This change to GR was not associated with any changes to anxiety or HPA axis activity (CORT or CRH

output) in the female mice at this age, nor at 52 weeks of age. There was another significant interaction observed as an increase in the ACTH baseline in the 52 week female HFHF group. This suggests there was potentially a priming effect of maternal HF diet to cause ACTH to further increase when exposed to postnatal HF over a long-term period of time. Due to the dearth of studies assessing long-term exposure to obesity both pre- and postnatally there is currently no literature to my knowledge for comparison. This elevation of ACTH in the HFHF group was not associated with a similar increase in CORT in the same mice. Current research from these same mice suggest that adrenal MC2R receptor levels is not lower in the HFHF groups (data unpublished), therefore this may be an issue of receptor insensitivity. Overall in the females there was only one statistically significant effect of maternal obesity on HPA function, and two interaction effects, indicating that females may be protected to some extent from the negative consequences of maternal obesity.

4.5.1.2.3 Males

There were more effects of maternal obesity on the male HPA axis than females, which were first observed at 15 weeks. There was a trend for increased basal CORT, although previous research suggests that maternal (60%) HF diet reduces basal CORT levels in both male and female rats (at approximately 13 weeks of age, although these animals had not undergone previous behavioural testing) (76), there was no such effect in my study. Further analysis revealed the HFHF group (and not the HFC group) tended to have higher basal CORT compared to the maternal C groups, which was also associated with decreased CORT AUC in HFHF males. This further strengthens the idea that persistently stressed mice may not be able to respond appropriately to stress, in a similar fashion to female mice exposed to postnatal obesity. Due to limited plasma volumes, ACTH was unable to be assessed at 15 weeks of age, however basal CRH mRNA levels (in both brain regions) were increased in the maternal HF groups, which may work to increase CORT levels, and supports the suggestion of offspring HPA hyperactivity following maternal obesity. MR mRNA levels were increased in the 15 week-old males (in both brain regions) exposed to maternal obesity which may be being upregulated to compensate for the increase in basal HPA activity via increased negative feedback. Previous research in rats has not found an effect of maternal obesity (via 60% HF-feeding) on hippocampal MR levels in 7 week old offspring (27), but at 13 weeks of age maternal 60% HF-feeding has been shown to either produce a significant reduction (279) or have no effect (76) on hippocampal MR mRNA levels in either sex. MR results from my study add to the current literature of the effects of maternal obesity on HPA and suggest that MR levels in the brain may be susceptible to the effects of maternal diet, but the effects may differ depending on environmental changes, methodologies or species.

At 52 weeks of age, in contrast to the weak effect at 15 weeks, maternal obesity significantly increased CORT AUC, suggesting long-term effects change with age, and an opposite effect to that of postnatal obesity. This outcome did not concur with a similar study using 58% maternal HF diet in 12 month-old male C57BL/6 mice by Balsevich *et al.* where CORT was measured during forced swim testing (75), however mice were also subjected to chronic social defeat stress for 3 weeks prior to testing which may explain the difference in results. In the same study, basal CORT and AUC in 3 month-old mice were not altered by maternal obesity (75), and a recent study in rats has also shown that a maternal HF diet does not affect levels of plasma CORT after a stressful test in 8 week-old male offspring (479), however these result did concur with my 15 week data. Another study assessing maternal obesity (via excess feeding) on offspring in sheep showed that elevated basal CORT and ACTH was not associated with changes in response to stress (273), however offspring were still only young adults. CORT measurements in my study show that the effect of diet may be dependent on age and/or length of time on the HF diet. Overall, few studies have assessed the effect of maternal HF diet on CORT in response to an acute stressor, and to my knowledge, the stress response outcomes in aged mice in my study are novel, and further work understanding the mechanisms are necessary.

CRH mRNA levels in the brain were reduced in 52 week-old male PVN, which was also opposite to the finding at 15 weeks of age, and may be associated with the weak trend for reduced basal CORT output in offspring from obese mothers. There was no association between CRH and basal ACTH at 52 weeks which might indicate altered pituitary CRF receptor levels or sensitivity in the maternal HF groups as a compensatory mechanism. Unfortunately, there are a paucity of studies addressing the effects of maternal obesity on offspring CRF receptor levels and further research in this area is warranted to fully elucidate whether this is a potential mechanism.

As mentioned previously, GR levels remained unaffected by diet, but MR mRNA levels decreased in the CA3 at 52 weeks of age (opposite to 15 week). This decrease, coupled with reduced CRH output in the PVN of the same animals, may be contributing to elevation of CORT AUC response to stress seen at this age due to less negative feedback, which may also be linked to the increase in CA3 FKBP51 in the same mice which may be causing reduced steroid receptor sensitivity in the MR and GR. To my knowledge there have been no comparable studies of the effect of maternal obesity on brain MR or FKBP51 levels in aged offspring.

Although FKBP51 was upregulated in the 15 week-old male CA3 by postnatal HF diet, there was an interaction so that the HFHF group were significantly increased than with either maternal or postnatal HF diet alone. This elevation of FKBP51 in the HFHF group could be associated with potentially increased GR and MR glucocorticoid resistance and reduced receptor sensitivity in this

group, which could be related to the increase in CORT at 15 weeks (particularly the HFHF group) due to maternal obesity, as negative feedback may be impaired in this diet group.

4.5.1.3 FKBP51 levels in LPS-treated mice

A small subset of LPS-treated mice were used to assess whether a mouse model of inflammation would alter levels in the brain compared to CC control mice. Interestingly, a consistent increase in expression of FKBP51 levels were found in the PVN of LPS-treated mice across age and sex, indicating FKBP51 may modulated by inflammation. This effect may be region specific however, as there was no change to FKBP51 in the CA3. This could suggest that the hippocampus may be more protected from inflammation than the hypothalamus, or that FKBP51 in the hypothalamus may be more sensitive to changes in inflammatory status. There is currently a paucity of data linking inflammation to FKBP51 in the brain, therefore this effect warrants further investigation.

4.5.2 Conclusion

Overall, the effects of maternal and/or postnatal obesity on the HPA axis are complex and there are age and sex-specific differences. In males there were effects of both maternal and postnatal obesity, and also differential effects at each age. At 15 weeks of age there appeared to be a main effect of maternal obesity to increase basal HPA axis activity (via CRH, CORT and MR levels). However, at 52 weeks of age both maternal and postnatal obesity affected the HPA axis, in different directions, and in a different way to the females at this age. In females, HPA function was generally unchanged at 15 weeks but there was a main overarching effect of postnatal obesity to increase basal HPA activity but reduce stress response at 52 weeks. These effects were accompanied by changes to MR and FKBP51, a gene related to psychiatric anxiety disorders. Few effects of maternal HF diet indicated female offspring are protected against the negative consequences of maternal obesity.

Generally, the data so far in the male offspring supports the hypothesis that maternal obesity will alter the functioning of the HPA axis, however subsequent postnatal obesity does not consistently worsen these effects, although some results in males show that the HFHF group is the worst affected compared to CC as can be seen in Figure 4.9. There is not a strong enough effect of maternal obesity throughout the HPA axis in females to apply this hypothesis to both sexes. This differential outcome due to sex difference is not surprising considering that the HPA axis has been widely reported to contrast between the two sexes. Despite few interaction effects, there may be some priming by maternal obesity to mediate the effects of further postnatal

obesity, for example in 52 week male stress response. All genes were measured basally, however changes to HPA activity during stress may elucidate hidden long-term changes in these animals. There is currently limited animal research on the effects of maternal obesity coupled with further postnatal obesity on both male and female offspring HPA axis however, particularly in aged offspring, so further research is required to confirm the observed effects.

These changes in HPA axis function due to maternal and/or postnatal obesogenic diet may potentially have long-term implications for increased risk of diseases linked to HPA dysfunction such as behavioural/anxiety and cognitive disorders. The next step in this thesis was to determine the potential causes of HPA axis dysfunction. Since obesity is linked with low-grade inflammation, including in the brain, next steps in this thesis involved determining whether neuroinflammation is involved in the effects on the HPA axis (Chapter 4) and behaviour (Chapter 3).

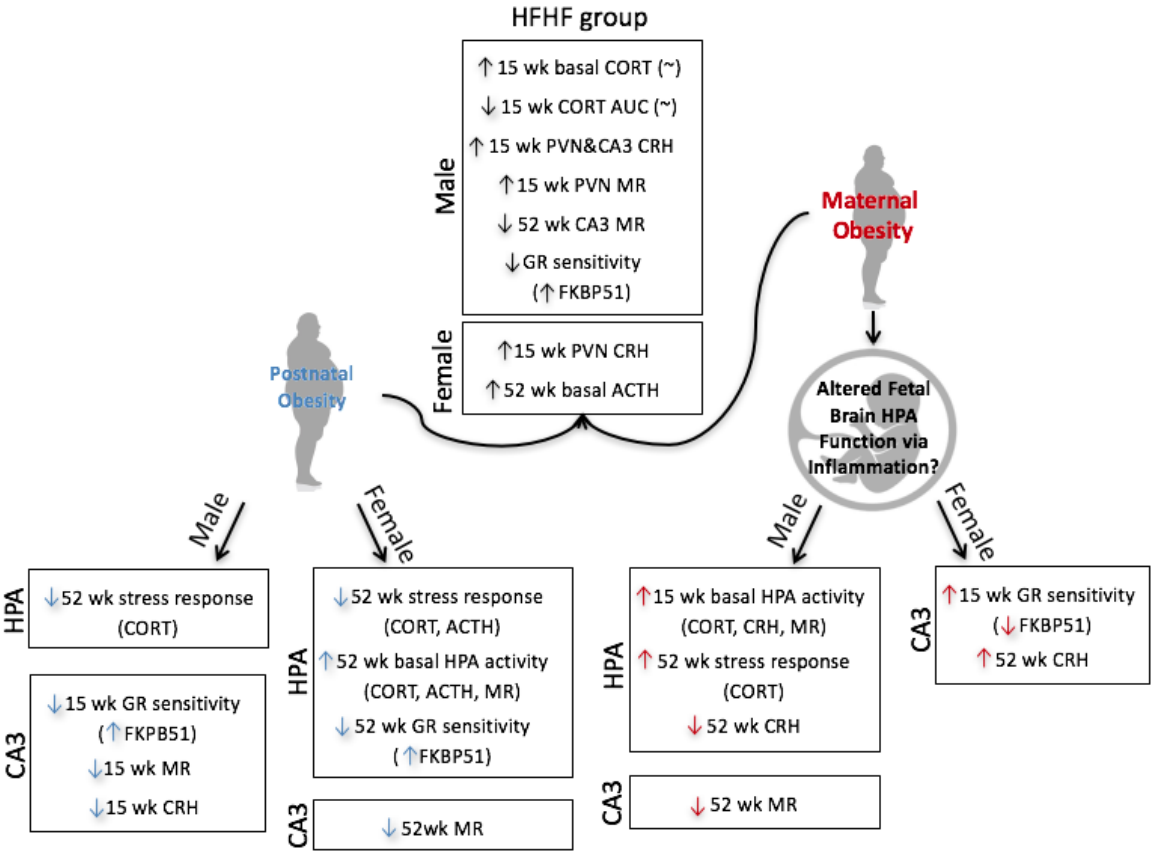

Figure 4.9 – **Schematic diagram of overarching results from Chapter 4.** Overall independent effects of maternal (red) and postnatal (blue) obesity on behaviour and memory in male and female offspring at 15 and 52 weeks of age. Combined box shows effects that were exacerbated in the HFHF group (significantly different from CC group). ~ = trend.

Chapter 5 The effect of maternal and postnatal high-fat diet on offspring inflammation

5.1 Introduction

Inflammation is the body's first line of defence against infection and injury and the resulting effect that has on cell and tissue damage. Activation of the immune system involves immune cells such as macrophages and cytokines in order to promote healing. This acute inflammatory response results in a beneficial outcome, whereas chronic inflammation often leads to detrimental outcomes such as tissue damage and long-term disease risk.

It is well documented that obesity is a form of chronic low-grade inflammation (480). This inflammation is linked to metabolic dysfunction (306), as well as a variety of neuropsychiatric symptoms displayed in animal models of obesity (481). In obesity, neuroinflammation has been demonstrated in areas of the brain such as the hypothalamus and hippocampus via microglial activation and elevated pro-inflammatory mediators (312, 482). Neuroinflammation, such as during viral infection, has consistently been shown to alter regulation of the HPA axis (483, 484), however there is limited and sometimes contradictory information in the existing literature about the effect of the chronic low-grade inflammation observed in obesity on HPA function.

During pregnancy it is hypothesised that increased inflammation from an obese mother may cross the placenta and affect the long-term development of the offspring via effects on the HPA axis. This in turn would affect behaviours associated with HPA function such as anxiety and memory. Current knowledge about maternal HF diet-induced obesity indicates that it alters inflammatory pathways in the offspring hippocampus. This has been shown in rat models as an increase of IL-6 and NfkB in 45 day old adolescent offspring from mothers fed a 58% HF diet (27). In a study using a 60% maternal HF diet, there were increases in CD11b and TLR4 in one day-old offspring and in IL-1B in 20 day-old and 3 month-old offspring (277). In the study by Bilbo *et al.*, this increase in inflammation coincided with a decrease in neurogenesis in the corresponding region (4). Offspring from mothers fed a 60% HF diet were also noted to have increased inflammation in the amygdala region of the brain which showed elevated IL-6 and NFkB mRNA expression (76). Less is known about the effects of maternal obesogenic HF diet on hypothalamic inflammation, however in non-

human primates 35% HF maternal diet has been shown to increase microglial activation in the hypothalamic and circulating cytokines (IL-1β) in the third trimester fetus (117). Increased inflammation during pregnancy, such as during infection, has been shown to negatively affect offspring during critical periods of neurodevelopment leading to behavioural deficits that have been linked to HPA dysfunction such as autism and schizophrenia in humans (319, 485) and non-human primates (486). Therefore, maternal obesogenic HF diet may have an influence on offspring neuroinflammation and this in turn may affect regulation of the HPA axis and behavioural outcomes.

In summary, further information on the effects of a maternal and postnatal obesogenic HF diet on offspring neuroinflammation is required. The current range of different experimental designs used in existing literature has yielded inconsistent results. Therefore, this chapter will focus on the effects of HF diet on inflammation in the offspring brain as a potential mechanism for the HPA and behavioural changes seen in Chapters 3 and 4.

5.2 Hypothesis and Aims

Hypothesis: Maternal HF diet will cause neuroinflammation in the offspring brain. This effect will be exacerbated by a postnatal HF diet.

Aims: To investigate the effects of maternal and postnatal HF diet on

4) Markers of neuroinflammation in hypothalamus and hippocampus by immunohistochemistry in 30 week-old offspring
5) Markers of neuroinflammation in PVN and CA3 brain regions in 15 and 52 week-old offspring
6) Circulating plasma IL-6 concentrations in 52 week-old offspring

5.3 Methods

5.3.1 Mouse cohorts

5.3.1.1 Cohort A: High-fat mouse model - 30 week immunohistochemical data

In a previous study by Dr Felino Cagampang, non-virgin female C57BL/6 mice were fed either a control (C, 7% kcal fat) or high-fat (HF, 45% kcal fat) diet for 6 weeks before mating and during pregnancy and lactation to induce obesity (mothers; C, *n* = 4; HF, *n* = 4). Offspring were then fed a C or HF diet post-weaning at 3 weeks of age creating 4 dietary groups: CC (n =3), CHF (n = 3), HFC (n = 2-3) and HFHF (n = 3), as detailed in section 2.1.1 and in appendix A. Animals were sacrificed and brains were collected between 13.00-16.00 hours and snap frozen in liquid nitrogen. Only male brains were used for preliminary IHC assessment of microglial markers.

5.3.1.2 Cohort B: High-fat mouse model - 15 week qPCR data

In a previous study by Dr Felino Cagampang, non-virgin female C57BL/6 mice were fed either a control (C, 7% kcal fat) or high-fat (HF, 45% kcal fat) diet for 6 weeks before mating and during pregnancy and lactation to induce obesity (C, *n* = 9; HF, *n* = 7). Offspring were then fed a C or HF diet post-weaning at 3 weeks of age creating 4 dietary groups: CC, CHF, HFC and HFHF, as detailed in section 2.1.1 and in in appendix A. Animals were sacrificed and brains were collected between 13.00-16.00 hours and snap frozen in liquid nitrogen.

5.3.1.3 Cohort C: High-fat mouse model - 52 week qPCR data

Non-virgin female C57BL/6 mice were fed either a control (C, 7% kcal fat) or high-fat (HF, 45% kcal fat) diet between 6-11 weeks (depending on length of time to reach minimum compulsory 15% weight gain) before mating and during pregnancy and lactation (C, *n* = 5; HF, *n* = 8). Offspring were then fed a C or HF diet post-weaning at 3 weeks of age creating 4 dietary groups: CC, CHF, HFC and HFHF, as detailed in section 2.1.1 and in appendix A. Animals were sacrificed and brains were collected between 13.00-16.00 hours and snap frozen in liquid nitrogen.

5.2.1.4 Lipopolysaccharide (LPS)-treated mice

LPS-treated mice were used as a control measure to assess a widely used mouse model of inflammation that I could compare my data to. C57BL/6 female mice between 4-5 months of age (n = 2) were injected with 200 µl of LPS at 10 µg/ml concentration into the intraperitoneal cavity. Animals showed sickness behaviour suggesting evidence of brain inflammation. Mice were sacrificed 4 to 6 hours after treatment and perfused with saline. Whole brain, pituitary and adrenals were collected and snap frozen in liquid nitrogen and stored at -80˚C.

5.3.2 Immunohistochemistry

Briefly, brain sections were taken, post-fixed in alcohol and quenched for endogenous peroxidase activity. Sections were then incubated with blocker, then incubated with primary antibody overnight. The following day sections were incubated with secondary antibody. For DAB staining the sections were then incubated in Avidin Biotin Complex. Staining was detected by using DAB as the chromogen. Haematoxylin was used as counterstain before dehydration and coverslipping.

For immunofluorescence, sections were taken and post-fixed in alcohol, incubated with blocker and then incubated with primary antibody overnight. The following day sections were incubated with secondary antibody. Sections were finally incubated for ten minutes in Hoescht to stain nuclei and then coverslipped immediately. Details of primary and secondary antibodies are given in section 2.3.2. Relative staining from the images were analysed using LAS AF Lite and ImageJ software as outlined in section 2.3.3.

5.3.3 Analysis of inflammatory marker gene expression

Total RNA was extracted by methods outlined in section 2.4.1. Briefly, PVN and CA3 regions were micropunched from whole brain sections from 15 and 52 week-old mice. Punched tissue was homogenised in Tri-reagent, isolated using chloroform and isopropanol, and washed in ethanol to prevent contamination. Nanodrop Spectrophotometer (Nanodrop 1000, Thermo Scientific, USA) was used to calculate concentration and purity of RNA for each sample.

cDNA synthesis was performed using TaqMan® Reverse Transcription Reagents (ThermoFisher Scientific, Basingstoke, UK) using Multiscribe Reverse Transcriptase and random hexamers. 400 ng RNA was used for each reaction. Real-time qPCR was performed using pre-validated primers of genes of interest (Table 5.1) (all: Sigma-Aldrich, UK) and SYBR Green (Bio-Rad, UK) on Roche LC480 PCR system (Roche, UK). The housekeeping genes PGK1, ATP5B and CYC1 were used to normalise GOI results. GOI data were analysed by plotting log concentration of standards against CT value and calculating mRNA expression using the CT values of the samples of interest. Positive controls of LPS-stimulated (4-6h) mouse brain tissue and plasma were used to assess inflammatory GOI qPCR results to.

Table 5.1 - **Details of genes used for relative mRNA expression analysis**

GENE	Accession No.	Primer	Sequence 5'-3'
TNF- α	NM_013693.3	Forward	5'-CGAGGACAGCAAGGGACTAG-3'
		Reverse	5'-GCCACAAGCAGGAATGAGAA-3'
IL-1β	XM_006498795.3	Forward	5'-TGTGTTTTCCTCCTTGCCTC-3'
		Reverse	5'-CTGCCTAATGTCCCCTTGAA-3'
FcγR1	NM_010186.5	Forward	5'-TACTTTGGGTTCCAGTCGGT-3'
		Reverse	5'-CCTGTATTCGCCACTGTCCT-3'
IL-6	NM_031168.2	Forward	5'-TCCAGAAACCGCTATGAAGTTC-3'
		Reverse	5'-CACCAGCATCAGTCCCAAGA-3'

5.3.4 IL-6 ELISA

IL-6 in plasma was measured using a DuoSet® ELISA Development System (R&D Systems, Abingdon, UK) according to the manufacturer's instructions with minor modifications. Briefly, after overnight plate preparation with capture antibody, the plate was washed and blocked. Appropriately diluted samples were added per well and incubated. After washing, detection antibody was added per well and incubated. Streptavidin-HRP was added to each well and then Stop Solution was added to each well and the plate gently tapped to ensure thorough mixing. The plate was immediately read at optical density 450nm. Concentrations were determined by interpolating optical density measurements from a 4 parameter standard curve, using online ELISA analysis software (Leading Technology Group, Australia).

5.3.5 Statistical Analysis

A mixed effects model was used to analyse 30 week immunofluorescence data, 52 week mRNA expression and IL-6 ELISA offspring data (see section 2.6 for details). This statistical model was used as it factored in the effects of littersize and which dam each individual originally came from. Therefore, all results are independent of dam and littersize. Results used in graphs are predicted means and SEM derived from maximal models (including diet group and littersize) applied to observed data. Depending on normal distribution, data were either not transformed or Z-transformed before mixed model statistical analysis. This made sure dependant variables were standardised allowing the effect estimates to be compared directly.

15 week mRNA expression data were statistically analysed by 2-way ANOVA since each offspring was from a different mother and littersizes were standardised, negating the need for mixed model analysis. LPS-treated mouse data difference from CC groups per sex and age were analysed by *t*-test per brain region. SPSS version 24 and GraphPad Prism was used for all analysis. 15 week cytokine (TNF-α , IL-1β, IL-6) data, which had no sex effects and low sample numbers, were displayed as both sexes combined but also split by sex to look at trends in the data in each sex.

5.4 Results

5.4.1 Inflammatory markers in 30 week-old brain

5.4.1.1 Immunohistochemical Imaging

Brain sections taken from 30 week-old male mice (Cohort A) stained for CD11b were too damaged to allow accurate quantification of inflammatory markers. Representative examples are shown in Figure 5.1 displaying damage caused by tissue collection via snap freezing process and IHC method. IHC was also performed for MHC-II and FcγR1 but data were not shown due to tissue damage similar to Figure 5.1.

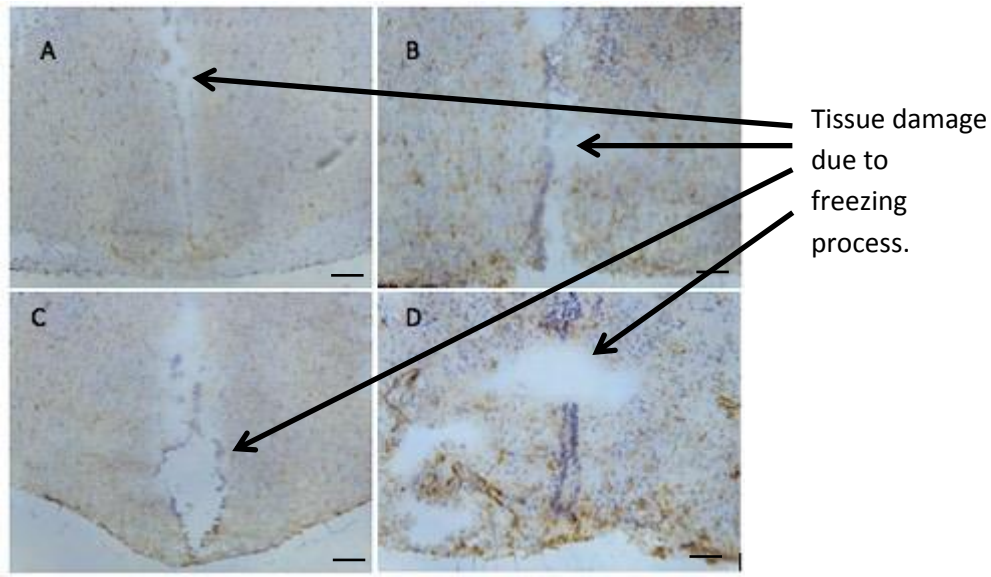

Figure 5.1 – **Representative immunohistochemical DAB stain of CD11b.** CD11b inflammatory marker staining in hypothalamic region of a representative animal from (A) CC, (B) CHF, (C) HFC and (D) HFHF groups. No statistical analysis performed. Scale bar = 100 µm.

5.4.1.2 Immunofluorescent Imaging

The least damaged brain sections taken from the same male mice (Cohort A) were then used to perform immunofluorescence in order to see whether this methodology, which utilises a briefer protocol, would reduce damage to tissue and therefore allow for more accurate quantification of staining. Brain sections were stained for CD11b, CD68 and GFAP, and hypothalamic and hippocampal areas of interest were assessed. Sections were indeed less damaged using this protocol, however there was still substantial areas of damage to the tissue.

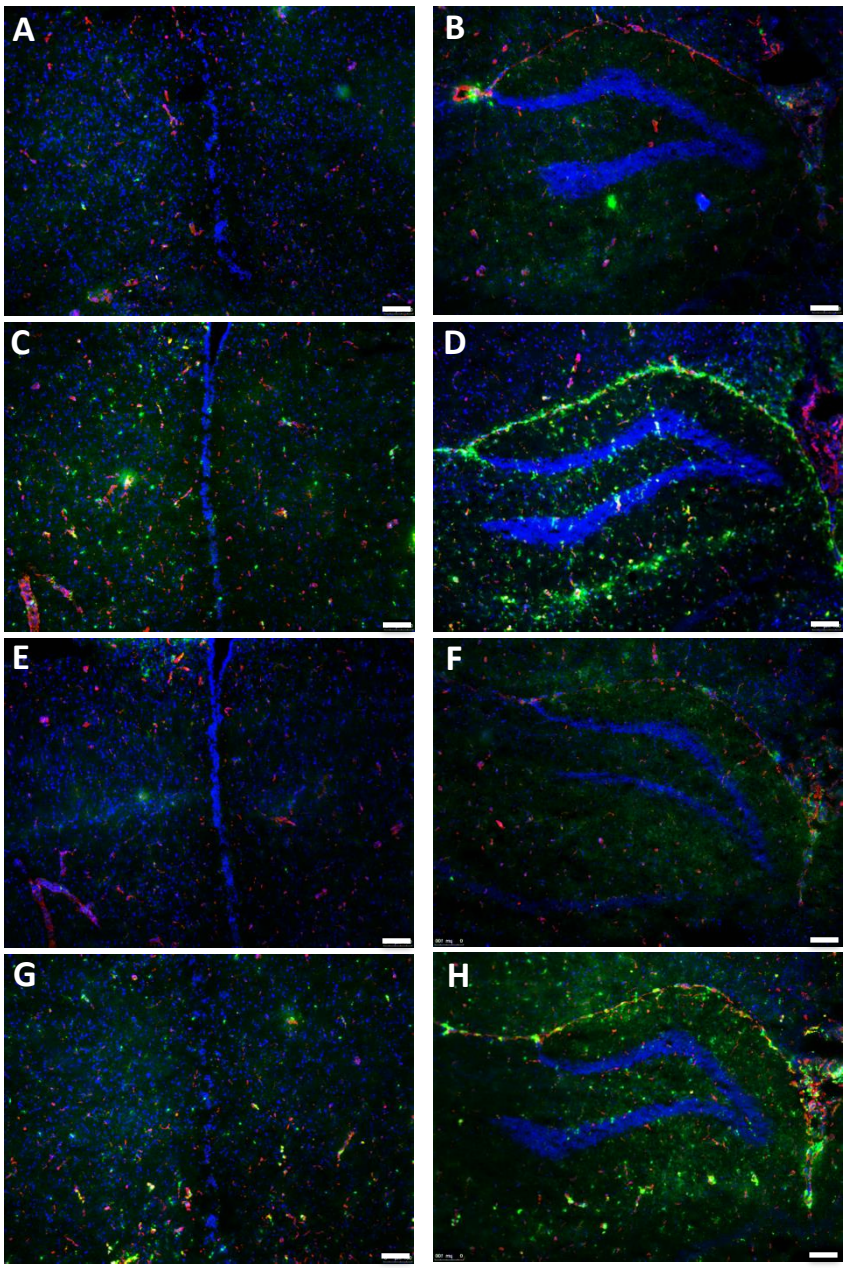

Figure 5.2 – **Representative immunofluorescent images of CD11b staining.** CD11b inflammatory marker staining in hypothalamus of a representative animal from (A) CC, (C) CHF, (E) HFC and (G) HFHF groups, and hippocampus of a representative animal from (B) CC, (D) CHF, (F) HFC and (H) HFHF groups. Sections were stained for CD11b (green), nuclei (blue) and blood vessels (red). Scale bar = 100 µm.

5.4.1.3 Immunofluorescence of inflammatory markers

Despite some damage still existent in the tissue, activated microglial markers CD11b, CD68 and astrocyte marker GFAP were quantified in hypothalamic and hippocampal areas of 30 week-old offspring. Postnatal HF diet significantly increased CD11b and GFAP in the hypothalamus (both: $P < 0.05$, Figure 5.3A and E), and maternal HF increased CD68 expression in the hippocampus ($P < 0.05$, Figure 5.3D).

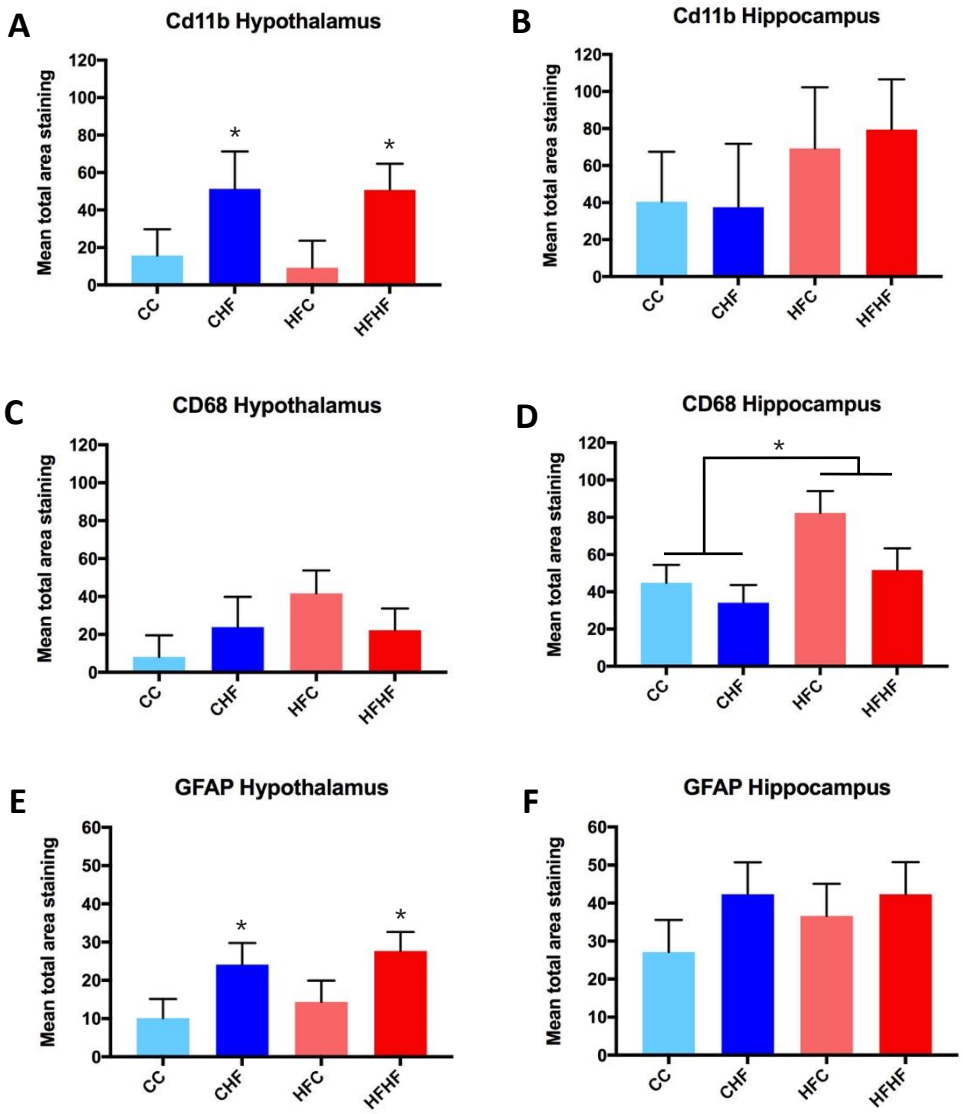

Figure 5.3 – **Quantification of CD11b, CD68 and GFAP by immunofluorescence in hypothalamus and hippocampus.** Mean intensity staining of CD11b (A, B), CD68 (C, D) and GFAP (E, F) in hypothalamus (A, C, E) and hippocampus (B, D, F) 30 week-old male brain. * $P < 0.05$. Data are displayed as predicted means ± SEM from maximal models. n = 2-3 per group (see Table 2.2 in Methods).

5.4.2 Pro-inflammatory gene expression in PVN and CA3 brain

Although IHC yielded some results, the tissue collection and IHC protocol was not ideal for quantification of inflammatory markers, therefore relative mRNA levels were assessed in order to assess inflammatory markers in the brain. These genes were measured at 15 (cohort B) and 52 weeks of age (cohort C). Data from 15 week-old offspring was shown with males and females combined as there were no overall sex differences, and fewer mice were available at this age. These data were also analysed and presented split by sex for further information (and to help compare to 52 week data). Data from 52 week-old offspring showed no effects of diet in both sexes combined for all cytokines in PVN (data not shown) but there were sex effects in CA3 and therefore 52 week data were presented in males and females separately.

5.4.2.1 IL-1β and IL-6 mRNA expression in the PVN and CA3

In 15 week-old offspring there was a significant effect of maternal HF when analysing both sexes combined for increased IL-1β and IL-6 levels in the PVN (both: $P < 0.05$, Figure 5.4A and C) and a trend for increased IL-1β levels in the CA3 ($P < 0.1$, Figure 5.4B) . There was an interaction between maternal and postnatal HF such that the HFHF group was significantly higher than with either maternal or postnatal obesity alone in the PVN for both IL-1β and IL-6 (interaction; $P < 0.0001$ and $P < 0.001$ respectively, Figure 5.4A and C). The HFHF group was significantly different from all groups for IL-1β in PVN (CC, $P < 0.01$; CHF, $P < 0.001$; HFC, $P < 0.0001$) and IL-6 in PVN (CC, $P < 0.05$; CHF, $P < 0.0001$; HFC, $P < 0.01$), and this was only true in females when split by sex. There was no interaction effect in the CA3.

When split by sex, there was a significant effect of maternal HF to increase IL-1β levels in 15 week-old female PVN and CA3 tissue (both: $P < 0.05$, Figure 5.5C and G) but not males (Figure 5.5A and E). However, there was an interaction between maternal and postnatal HF on IL-1β levels in the PVN at 15 weeks such that the HFHF group was significantly higher than with either maternal or postnatal obesity alone in males ($P < 0.01$, Figure 5.5A) and females ($P < 0.05$, Figure 5.5C). At 52 weeks of age, the only effect observed was in the CA3 in females; postnatal HF increased IL-1β levels ($P < 0.05$, Figure 5.5H) but only the HFHF group was significantly increased from CC ($P < 0.05$). There was also an effect of sex in 52 week CA3 where female IL-1β levels were higher than in males ($P < 0.05$, Figure 5.5F and H).

There was a similar pattern in the data for effects of diet on IL-6 in the brain. At 15 weeks of age there was again a significant effect of maternal HF to increase IL-6 levels in female PVN ($P < 0.05$, Figure 5.6C) and this was a trend in the CA3 ($P = 0.081$, Figure 5.6G). There was no effect of either maternal or postnatal HF on IL-6 levels in males at this age. Similarly to IL-1β data, there was an

interaction between maternal and postnatal HF such that the HFHF group was significantly higher than with either maternal or postnatal obesity alone in male ($P < 0.01$, Figure 5.6A) and female ($P < 0.05$, Figure 5.6C) PVN at 15 weeks. At 52 weeks, the only effect observed was in female CA3; maternal HF tended to increase IL-6 levels ($P = 0.058$, Fig. 5.6H) and postnatal HF also weakly tended to increase IL-6 levels ($P = 0.099$, Fig. 5.6H) such that only the HFHF group was significantly increased from CC ($P < 0.05$). There was also an effect of sex in 52 week CA3 where female IL-6 levels were higher than in males ($P < 0.05$, Figure 5.6F and H).

Data from LPS-treated mice did not show significantly increased inflammatory markers compared to CC mice for IL6 PVN [15 week; CC: 1.61 ± 0.57, 52 week male; CC: 0.76 ± 0.22, female: 0.73 ± 0.38, LPS: 0.98 ± 0.22], IL6 CA3 [15 week; CC: 1.06 ± 0.16, 52 week male; CC: 1.23 ± 0.4, female: 0.87 ± 0.3, LPS: 1.18 ± 0.45], IL-1β PVN [15 week; CC: 1.12 ± 0.54, 52 week male; CC: 0.52 ± 0.16, female: 0.39 ± 0.28, LPS: 0.51 ± 0.04], IL-1β CA3 [15 week; CC: 0.91 ± 0.13, 52 week male; CC: 0.81 ± 0.26, female: 0.87 ± 0.21, LPS: 0.73 ± 0.11].

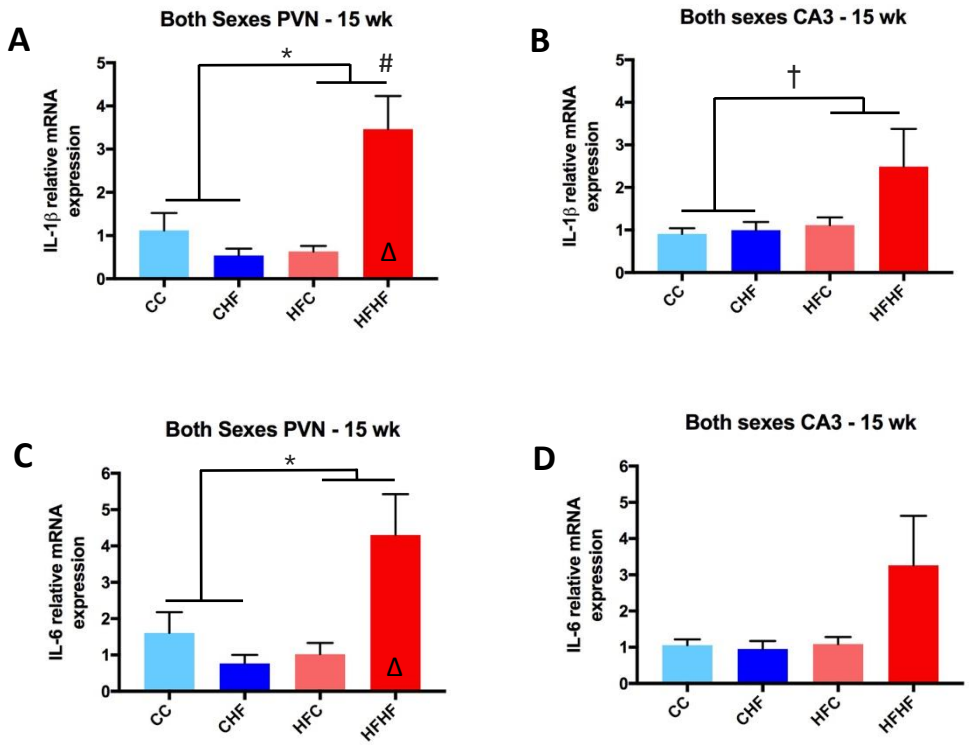

Figure 5.4 – **IL-1β and IL-6 mRNA levels in 15 week-old PVN and CA3.** Effect of maternal and postnatal HF diet in both sexes combined on IL-1β (A, B) and IL-6 (C, D) relative mRNA levels in the PVN and CA3 . * $P < 0.05$, † $P < 0.1$. # $P < 0.05$ pre*postnatal interaction group affected. Δ, HFHF group significantly different from CC. Data are displayed as predicted means ± SEM from maximal models. n = 7-9 per sex per group (see Table 2.2 in Methods).

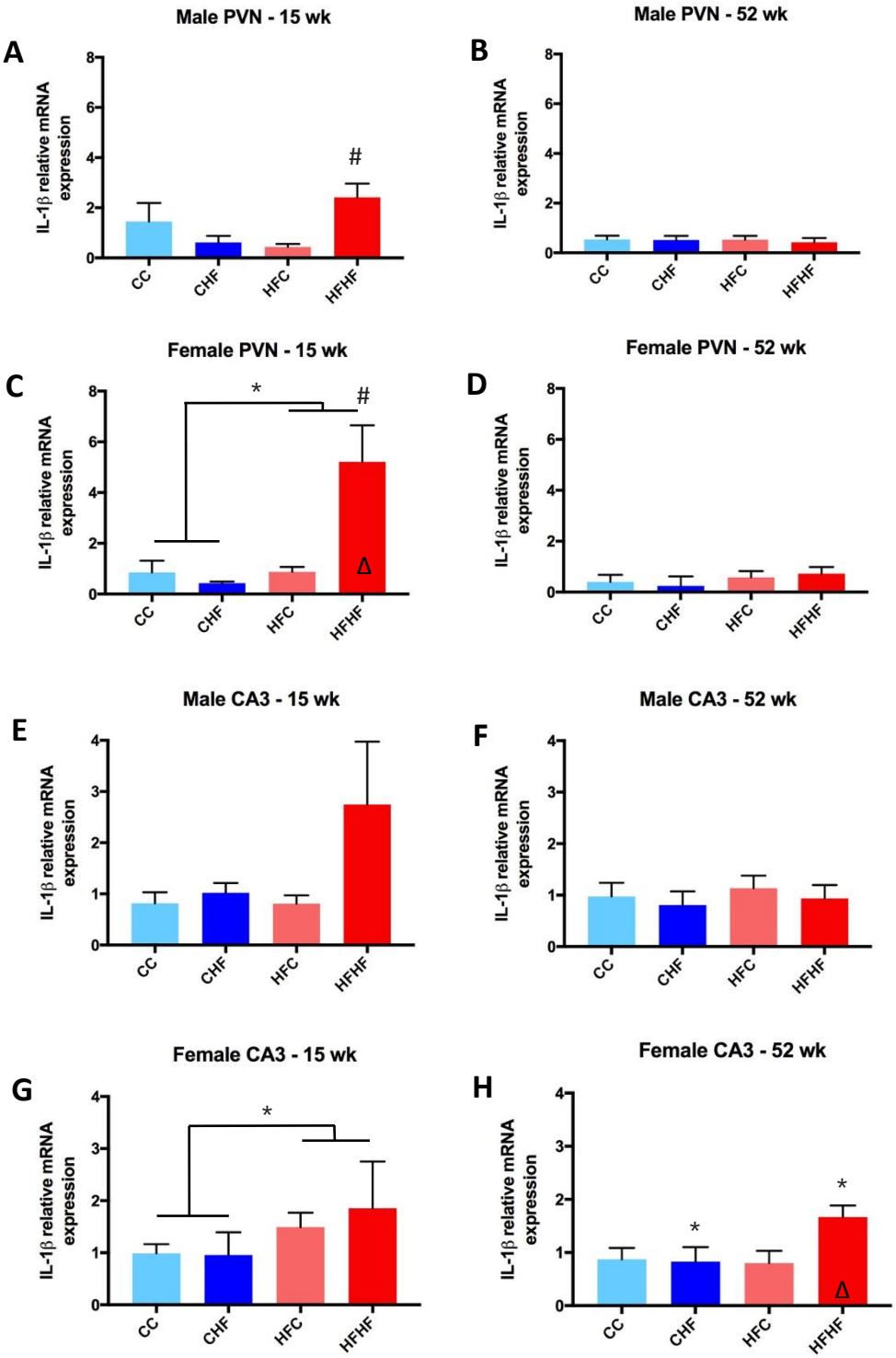

Figure 5.5 – **IL-1β mRNA levels in PVN and CA3.** Effect of maternal and postnatal HF diet in male (A, B, E, F) and female (C, D, G, H) offspring on IL-1β relative mRNA levels in the PVN and CA3 (15 weeks; [PVN: A, C] [CA3: E, G] and 52 weeks; [PVN: B, D] [CA3: F, H]). * $P < 0.05$. # $P < 0.05$ pre*postnatal interaction group affected. Δ, only HFHF group significantly different from CC. Data are displayed as predicted means ± SEM from maximal models. n = 3-9 per sex per group (see Table 2.2 in Methods).

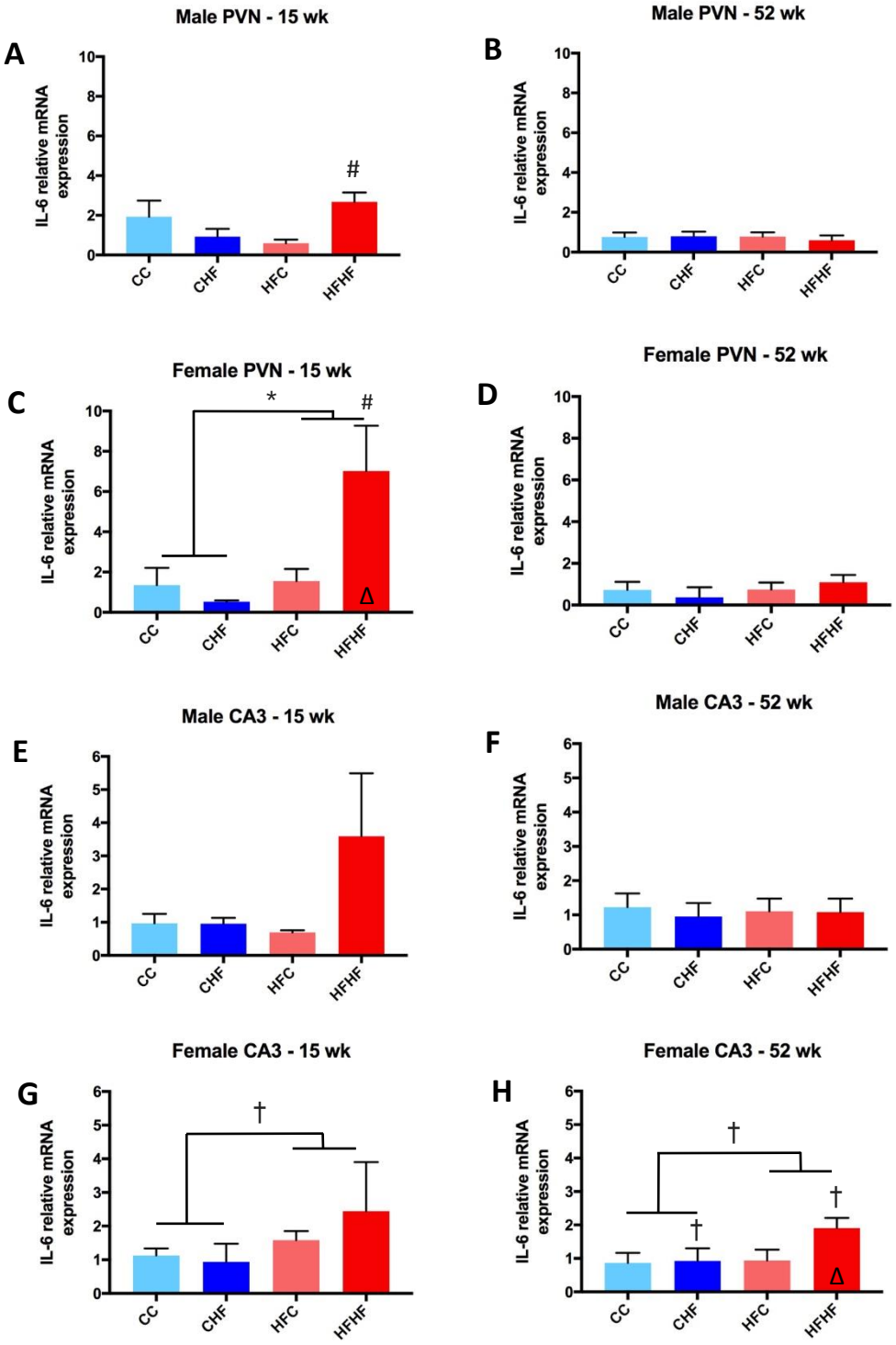

Figure 5.6 – **IL-6 mRNA levels in PVN and CA3.** Effect of maternal and postnatal HF diet in male (A, B, E, F) and female (C, D, G, H) offspring on IL-6 relative mRNA levels in the PVN and CA3 (15 weeks; [PVN: A, C] [CA3: E, G] and 52 weeks; [PVN: B, D] [CA3: F, H]). * $P < 0.05$, † $P < 0.1$. # $P < 0.05$ pre*postnatal interaction group affected. Δ, only HFHF group significantly different from CC. Data are displayed as predicted means ± SEM from maximal models. n = 3-9 per sex per group (see Table 2.2 in Methods).

5.4.2.2 TNF-α mRNA expression in the PVN and CA3

In 15 week-old offspring PVN, when analysing both sexes combined, there was an interaction ($P < 0.001$, Figure 5.7A) between maternal and postnatal HF such that the HFHF group was significantly increased from all other dietary groups (from CC: $P < 0.01$, from CHF and HFC: $P < 0.001$). There was no interaction effect in the CA3.

When split by sex, there was a significant effect of maternal HF to increase TNF-α levels in 15 week-old female the PVN and CA3 (both: $P < 0.05$, Figure 5.8C and G). There was no significant effect of either maternal or postnatal HF on TNF-α levels in males at 15 weeks of age (Figure 5.8A and E). Similar to the interleukin data, there was an interaction between maternal and postnatal HF such that the HFHF group was significantly higher than with either maternal or postnatal obesity alone in male ($P < 0.05$, Figure 5.8A) and female ($P < 0.05$, Figure 5.8C) PVN at this age. At 52 weeks of age, the only effect of diet was in female CA3; there was significant effect of maternal HF to increase TNF-α levels ($P < 0.05$) and postnatal HF tended to increase TNF-α levels also ($P = 0.08$, Figure 5.8H) such that only the HFHF group was significantly increased from CC ($P < 0.01$). There was also an effect of sex in 52 week CA3 where female TNF-α levels were higher than males ($P < 0.05$, Figure 5.8F and H).

Data from LPS-treated mice did not show significantly increased inflammatory markers compared to CC mice for TNF-α PVN [15 week; CC: 1.09 ±0.41, 52 week male; CC: 0.55 ±0.18, female: 0.36 ±0.45, LPS: 0.68 ±0.04], TNF-α CA3 [15 week; CC: 0.68 ±0.10, 52 week male; CC: 0.95 ±0.27, female: 0.59 ±0.24, LPS: 0.91 ±0.16].

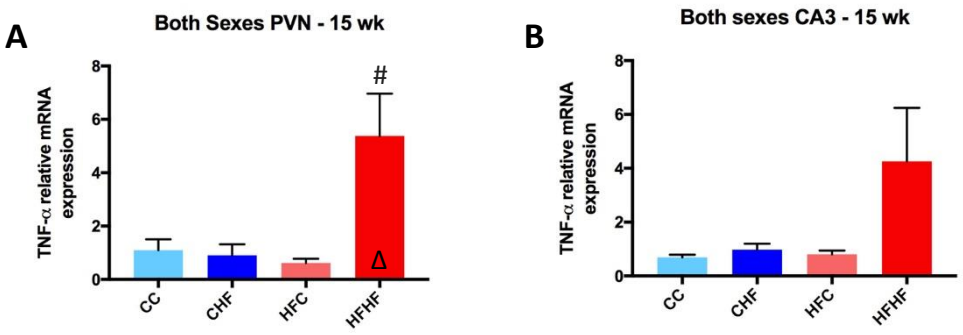

Figure 5.7 – **TNF-α mRNA levels in 15 week-old PVN and CA3.** Effect of maternal and postnatal HF diet in both sexes combined on TNF-α relative mRNA levels in the PVN and CA3. # P < 0.05 pre*postnatal interaction group affected. Δ, only HFHF significantly different from CC. Data are displayed as predicted means ± SEM from maximal models. n = 7-9 per sex per group (see Table 2.2 in Methods).

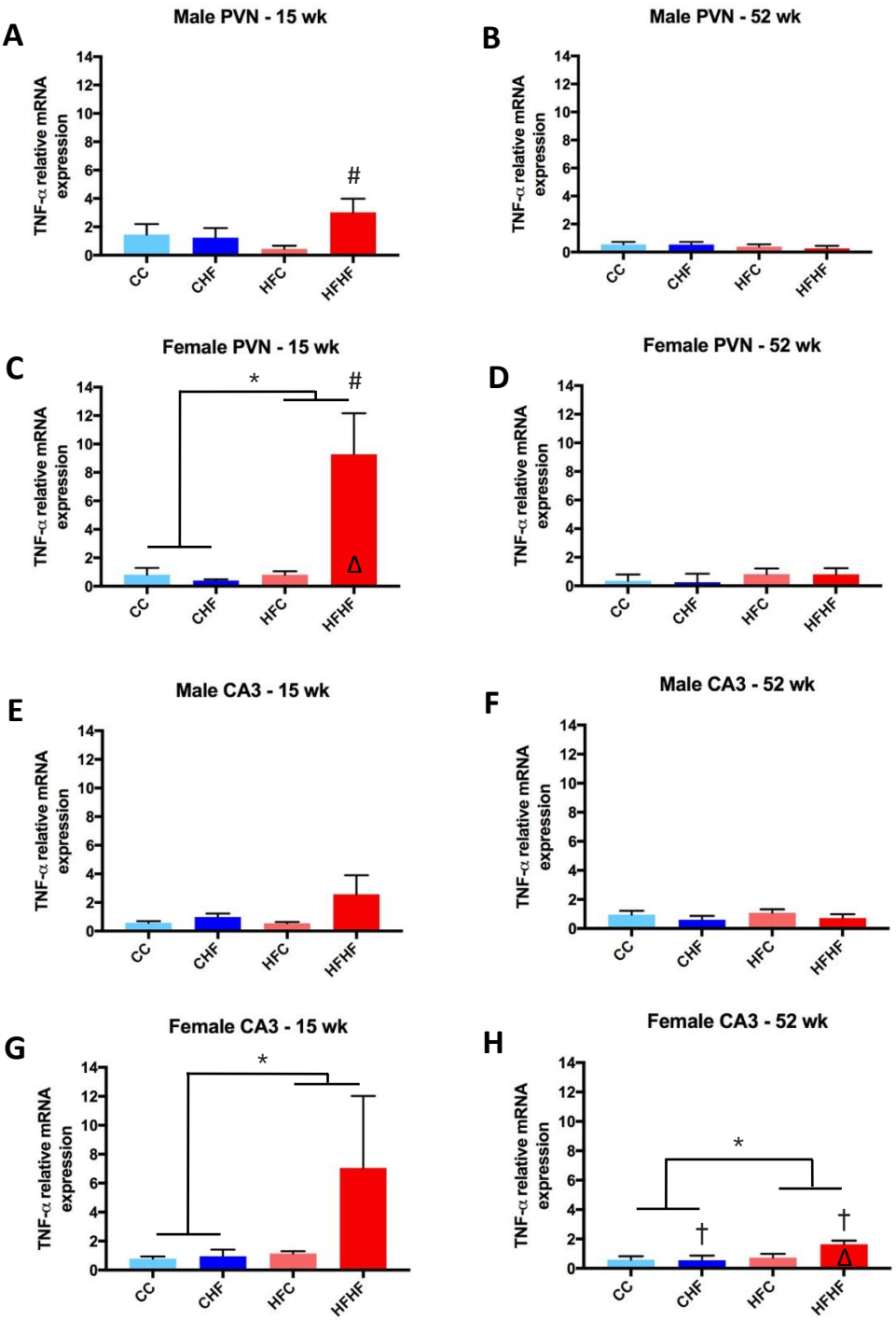

Figure 5.8 – **TNF-α mRNA levels in PVN and CA3.** Effect of maternal and postnatal HF diet in male (A, B, E, F) and female (C, D, G, H) offspring on TNF-α relative mRNA levels in the PVN and CA3 (15 weeks; [PVN: A, C] [CA3: E, G] and 52 weeks; [PVN: B, D] [CA3: F, H]). * $P < 0.05$, † $P < 0.1$. # P < 0.05 pre*postnatal interaction group affected. Δ, only HFHF significantly different from CC. Data are displayed as predicted means ± SEM from maximal models. n = 3-9 per sex per group (see Table 2.2 in Methods).

5.4.2.3 FCγR1 mRNA expression in the PVN and CA3

In 15 week-old offspring PVN, when analysing both sexes combined, there was a significant effect of postnatal HF diet to decrease FCγR1 levels ($P < 0.05$, Figure 5.9A), and in the CA3 there was a trend for maternal HF to increase FCγR1 levels ($P = 0.055$, Figure 5.9B). There was an effect of sex at 15 weeks of age such that general FCγR1 levels in the females were elevated from males in both the PVN and CA3 (both: $P < 0.0001$, Figure 5.10A, C, E, G).

There was no effect of diet on FCγR1 mRNA expression in the 15 week-old female offspring. In the males however, there was a significant effect of maternal HF to increase FCγR1 mRNA levels in both PVN and CA3 (both: $P < 0.05$, Figure 5.10A and E) and an additional effect of postnatal HF in the PVN to decrease levels ($P < 0.05$, Figure 5.10A). In the 15 week male CA3 only the HFHF group was significantly increased from CC ($P < 05$). At 52 weeks of age in the males, there was an effect of maternal HF to decrease FCγR1 levels in both PVN ($P < 0.05$, Figure 5.10B) and CA3 ($P < 0.01$, Figure 5.10F) and there was an additional effect of postnatal HF to decrease levels in the CA3 ($P < 0.0001$, Figure 5.10F) such that HFHF was significantly decreased from all other diet groups (CC, $P < 0.0001$; CHF, $P < 0.004$; HFC, $P < 0.002$). In 52 week-old females there was no effect in PVN tissue (Figure 5.10D), but there was an effect of postnatal HF to decrease FCγR1 levels in CA3 ($P < 0.01$, Figure 5.10H). Overall FCγR1 levels were decreased in females at 52 weeks of age compared to males in both PVN and CA3 tissue (both: $P < 0.0001$, Figure 5.10B, D, F, H).

Data from LPS-treated mice showed significantly increased FCγR1 for all ages, sexes and brain regions compared to CC mice (all: $P < 0.0001$); FCγR1 PVN [15 week male; CC: 0.59 ±0.09, 15 week female; CC: 0.9 ±0.12, 52 week male; CC: 1.06 ±0.12, female: 0.46 ±0.12, LPS: 2.45 ±0.11], FCγR1 CA3 [15 week male; CC: 0.6 ±0.1, 15 week female; CC: 0.94 ±0.09, 52 week male; CC: 1.2 ±0.1, female: 0.72 ±0.07, LPS: 3.17 ±0.15].

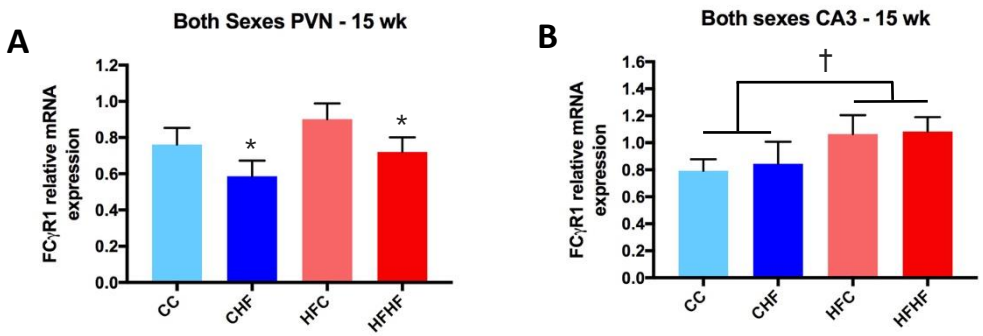

Figure 5.9 – **FCγR1 mRNA levels in 15 week-old PVN and CA3.** Effect of maternal and postnatal HF diet in both sexes combined on FCγR1 relative mRNA levels in the PVN and CA3. * $P < 0.05$, † $P < 0.1$ Data are displayed as predicted means ± SEM from maximal models. n = 7-9 per sex per group (see Table 2.2 in Methods).

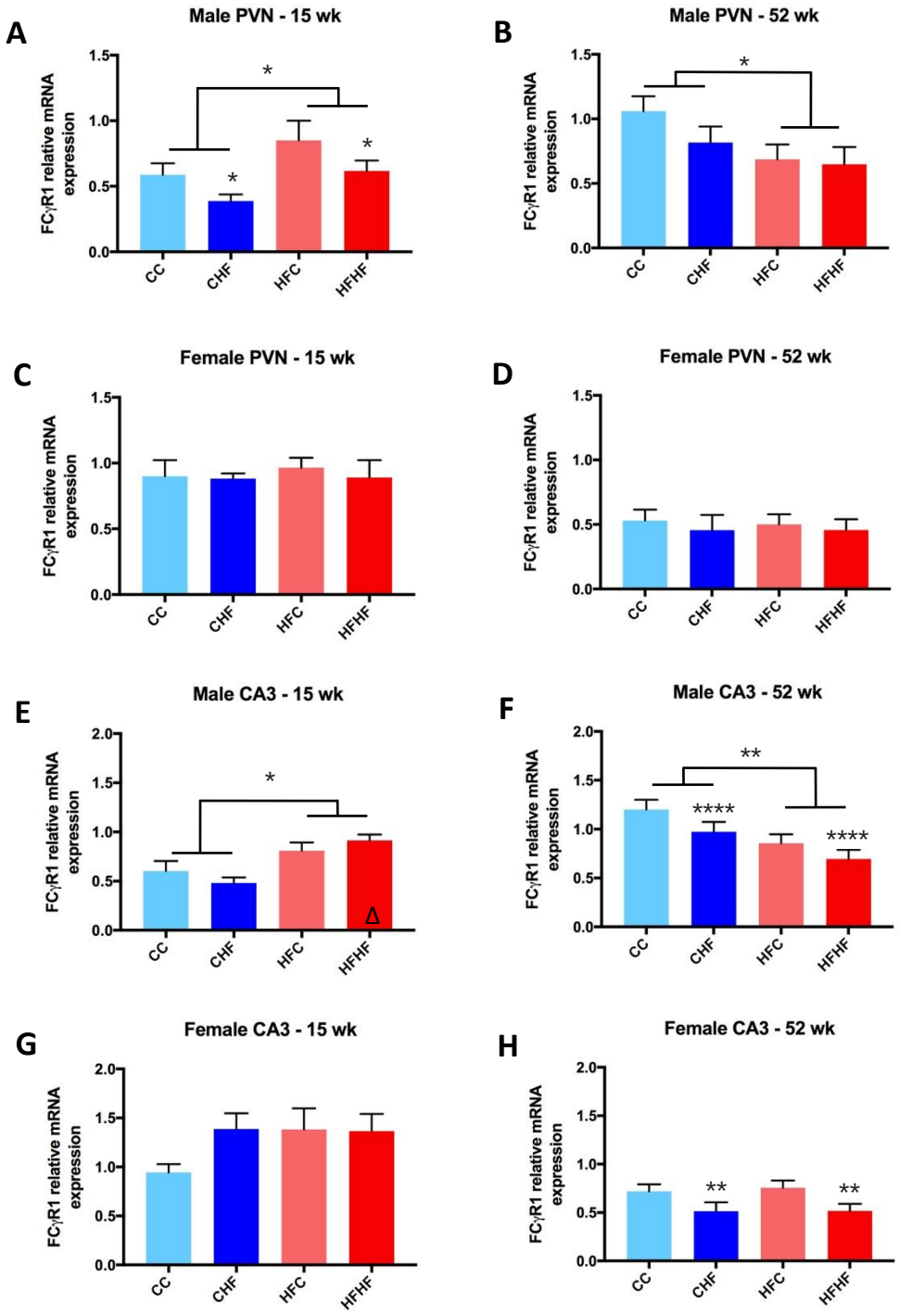

Figure 5.10 – **FCγR1 mRNA levels in PVN and CA3.** Effect of maternal and postnatal HF diet in male (A, B, E, F) and female (C, D, G, H) offspring on FCγR1 relative mRNA levels in the PVN and CA3 (15 weeks; [PVN: A, C] [CA3: E, G] and 52 weeks; [PVN: B, D] [CA3: F, H]). **** $P < 0.0001$, ** $P < 0.01$, * $P < 0.05$. Δ, only HFHF significantly different from CC. Data are displayed as predicted means ± SEM from maximal models. n = 3-9 per sex per group (see Table 2.2 in Methods).

5.4.3 Circulating IL-6 concentrations

Although IL-6 mRNA expression in the PVN and CA3 was largely unaffected by diet at 52 weeks, circulating IL-6 concentrations were measured in plasma collected at post mortem in a subset of offspring at 52 weeks of age (Cohort C). In both male and female offspring there was a significant effect of postnatal HF diet to increase circulating plasma IL-6 concentrations (both sexes; $P < 0.05$). There was no effect of maternal HF diet.

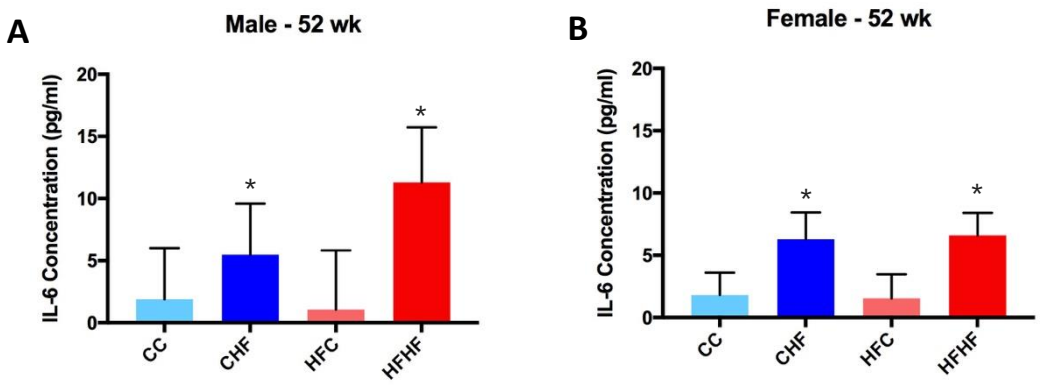

Figure 5.11 – **Plasma IL-6 plasma concentrations at 52 weeks.** Effect of maternal and postnatal HF diet in male (A) and female (B) on IL-6 plasma concentrations. * $P < 0.05$. Data are displayed as predicted means ± SEM from maximal models. n = 5-9 per sex per group (see Table 2.2 in Methods).

5.5 Discussion

In Chapter 3 I showed that HF diet influenced behaviours such as hyperactivity, anxiety and memory in an age and sex-dependent manner and in Chapter 4 I showed that HPA function was altered due to HF diet in both a sex and age-dependent way also. This chapter assessed whether these behavioural and HPA changes were associated with neuro-inflammatory effects of maternal and postnatal HF diet. The first approach was to assess markers of microglial activation via IHC in brains from a cohort of 30 week-old animals. Subsequently, specific tissue regions of the hypothalamus and hippocampus (PVN and CA3, respectively) were micropunched from snap frozen 15 and 52 week-old whole brains and qPCR performed to assess relative mRNA expression of pro-inflammatory markers.

5.5.1 Preliminary immunohistochemistry staining in 30 week-old male brain

Preliminary results from immunohistochemical staining of brain sections showed that the hypothalamic area was often severely damaged and therefore would not be able to be quantified accurately. This was due to the process of tissue collection as the banked whole brains were snap frozen, rather than perfused, which causes the tissue to be more susceptible to damage during sectioning and staining. Regardless, least damaged sections were chosen for immunofluorescent staining (briefer protocol) of hippocampal and hypothalamic regions and it was possible to perform some quantitative analysis. There was significantly increased CD11b and GFAP due to postnatal HF diet which is consistent with current literature that show elevation of microglial activation (including these 2 markers) in animals subject to diet-induced obesity (312, 487-489). In particular, a similar study to mine on the effects of maternal and postnatal HF together showed a significant effect of postnatal but not maternal HF to increase microglial activation, as measured by Iba1 and GFAP, in the 20 week-old rat cortex (278). Maternal HF diet increased the presence of CD68 in the hippocampus, which is also consistent with a pivotal paper by Bilbo *et al.* showing that a maternal 60% HF diet increased microglial expression in the hippocampus of adult offspring (277). Although it was not significant, there appeared to be an increase in CD11b expression in the hippocampus due to maternal HF which may have been confirmed with larger sample numbers. These changes to microglial activation could be causing injury to the brain and (in the context of this thesis) subsequently affecting long-term functioning of these areas, such as regulation of HPA axis function and behaviour (hypothalamus), and memory deficits and alteration to negative feedback of the HPA axis (hippocampus).

There was no interaction between maternal and postnatal HF diet, despite changes to both maternal and postnatal HF separately, which would suggest my hypothesis that further HF-

feeding post maternal HF may worsen neuroinflammation is not supported. However, I am unable to make a robust conclusion from these preliminary results in a small sample of animals, with tissues of poor quality. It was therefore decided not to pursue this immunohistochemical approach further. Instead, I decided that molecular analysis of gene expression (qPCR) would provide more quantitive information on the effects of maternal and postnatal HF diet on offspring neuroinflammation in the PVN and hippocampus (CA3). Analysis of inflammatory genes was performed on 15 and 52 week-old offspring: these ages match the ages of behavioural testing (Chapter 3) and HPA analysis (Chapter 4) so any changes in inflammatory genes of interest can be linked to behavioural test outcomes.

5.5.2 Effect of maternal and postnatal obesogenic HF diets on mRNA expression of inflammatory markers

5.5.2.1 Maternal Obesity

5.5.2.1.1 Cytokines

There were generally no effects of sex on cytokine data but I was interested in effects of each sex due to sex differences observed in HPA (Chapter 4) and behaviour (Chapter 3), therefore data were presented as both sexes combined and separately. Discussion of effects will mainly comprise of combined effects; however, I will highlight potential sex differences.

Results from cytokine mRNA level analysis showed that there was an effect of maternal obesity to increase both IL levels across the PVN and IL-6 in CA3 and while there was a similar pattern in TNF-α data no significance was found. This pattern generally appeared to be due to the large increase in cytokine levels in the HFHF group, and in the PVN (but not CA3) there was a significant interaction between maternal and postnatal HF such that the HFHF group was increased. Maternal HF diet on its own (HFC) was not enough to stimulate any change in cytokine expression from the control group (CC). The interaction effects suggest that maternal obesity may prime offspring for an inflammatory response to further postnatal obesogenic HF-feeding and this aligns with my hypothesis that maternal obesity coupled with further postnatal obesity may exacerbate inflammation. No other inflammation interaction effects were found in my study suggesting this phenomenon only occurs in cytokines. Research by Bilbo *et al*. has shown a marked increase in hippocampal IL-1β following LPS-treatment in 13 week-old offspring from mothers fed 60% HF diet throughout pregnancy and lactation (277), indicating that maternal obesity may also program increased inflammatory response to later stress such as infection. I was unable to assess inflammatory response to restraint stress, however this would be interesting to match to my Chapter 4 restraint data in future studies and to assess the extent of maternal priming. Few

studies have measured basal cytokine levels in the brain of offspring from obese mothers, however in non-human primates there has been shown to be elevations of hypothalamic IL-1β and IL-1 receptor levels compared to offspring from C-fed mothers (117). Even fewer studies have assessed the effects of both maternal and postnatal obesity on offspring neuroinflammation. However in one study using 58% HF to induce maternal and postnatal obesity showed no maternal effect on hippocampal TNF-α levels in 17 week-old rat offspring (both sexes) (400) and a similar experimental rat study using 60% HF diet also showed no effect of maternal diet on TNF-α levels in 20 week-old rat cortex (males) (278). The latter study by White *et al.* also showed an elevation of IL-6 due to maternal HF, similar to my results in the PVN. Neither of these studies show interactions between maternal and postnatal obesity suggesting that there may be experimental variations that may account for altered outcomes between studies and further work is necessary to determine the strength of the effect of maternal diet to prime offspring brain.

Interestingly, this effect of maternal obesity and interaction with postnatal obesity did not persist into mature adulthood at 52 weeks of age which suggests that the brain may be able to attenuate the inflammation due to HF diet exposure over time, potentially via reductions in microglia or increased BBB integrity. It is understood that maternal obesity is linked to compromised BBB integrity in gestational offspring (490), but no studies of the long-term effect on offspring have been performed. In addition, while there may be few effects of diet on cytokine levels in the PVN and CA3 at 52 weeks, permanent damage may have been caused by elevated inflammation at young adult age that may account for some of the subtle exacerbations of HPA dysfunction and behaviour in the HFHF group that were seen in the previous chapters. Further studies could assess whether this hypothesis is true by measuring neuronal injury in these mice.

There was an effect of maternal obesity to increase IL-6 and TNF-α expression in the female CA3 at 52 weeks. Maternal obesity was also associated with an increase in hippocampal CD68 (microglial marker) expression at 30 weeks as measured by immunofluorescence which may suggest activated microglia may be mediating the elevated cytokines in the CA3 at 52 weeks. However, this immunofluorescence data was performed in males which was not associated to mRNA changes in males at 52 weeks. Interestingly, for both cytokines only the HFHF group was significantly elevated from CC in both sexes, similar to the overall effects of HFHF at 15 weeks. This persistent elevation could be affecting hippocampal function and may be influencing HPA negative feedback. Circulating IL-6 was not affected by maternal obesity, suggesting the elevated inflammation in the CA3 at 52 weeks may be specifically programmed *in utero,* affecting long-term function of the hippocampus. There was no effect of maternal obesity on the hypothalamus, suggesting region-specific effects. In future studies it would be beneficial to measure the cytokines via IHC to match it to molecular data. To my knowledge there are no studies assessing

the effect of both maternal and postnatal obesity on neuroinflammation in older mice. However, its well-known there is an effect of aging to increase inflammation in the brain in humans (491) and rodents (492) which may potentially be masking effects of inflammation in the PVN due to earlier dietary challenges.

5.5.2.1.2 Microglial Activation

As mentioned previously FCγR1 is an IgG receptor expressed on microglia that is upregulated in microglial activation and expressed at low levels in periods of low inflammation (surveying phase) (493). In my study there were highly significant effects of sex so both combined and split data will be discussed. When both sexes were analysed together there was only a trend for increased FCγR1 in the CA3 of the maternal HF groups. However when split by sex, maternal HF only affected male offspring; FCγR1 mRNA levels were increased at 15 weeks of age but decreased at 52 weeks of age in both brain regions.

The increase in FCγR1 at 15 weeks is associated with the increase in cytokine levels, however this is only true in males. This may suggest separate methods of cytokine production in the males and females (such as via astrocytes or neurons, or weakening of the BBB in females). The effect of maternal obesity to increase microglial and immunohistochemical expression has been previously reported by Bilbo *et al*. (as mentioned above). The study by Bilbo *et al.* used maternal obesogenic 60% HF diet in rats and observed an increase of hippocampal CD11b mRNA expression and Iba-1 immunohistochemical expression in both sexes of 13 week-old offspring (277). Although changes were seen in both sexes in the study by Bilbo *et al.*, in my study only the males were significantly affected by HF diet, and this in part may be due to experimental variation (such as the lower percentage fat used or species). This potential sex difference warrants further investigation. The effect of maternal HF diet to increase microglial activation may also be modulated by age. Maternal 60% HF has previously been shown to have no effect on cortex Iba-1 expression in 20 week-old offspring, regardless of further postnatal HF-feeding (278). To my knowledge there is currently no research on the long-term effects of maternal obesity on microglial activation in aged adult offspring. I speculate that the decrease in FCγR1 at 52 weeks of age following maternal HF diet in my study may be due to downregulation of FCγR1 due to chronic elevation of inflammation. When microglia are subjected to the same stimulus over an extended period of time, the cells adjust to this new environment in their overall efforts to maintain homeostatic balance. For example this has been shown to occur for repeated exposures of microglia to LPS (494) and Alzheimer's Disease brains microglia are unable to stimulate phagocytosis of plaques that they are in contact with (494). Further analysis of pro- and anti-inflammatory cytokines would be necessary to assess this hypothesis. No interactions between maternal and postnatal

obesity were observed to affect FCγR1 levels, indicating that maternal or postnatal obesogenic HF-feeding alone were enough to stimulate an effect. Additionally, in my study, assessment of an age between 15 and 52 weeks may help to elucidate how and when this reversal of FCγR1 expression due to maternal obesity may come about.

5.5.2.2 Postnatal Obesity

5.5.2.2.1 Cytokines

There were no overall effects of postnatal HF on cytokine levels at either age except a tendency to increase levels of all 3 cytokines in 52 week female CA3, with only IL-1β a significant effect. These effects were largely due to the HFHF group as this was the only group significantly increased from CC. These results were unexpected given the strong effect of postnatal HF diet on HPA and behaviour in the previous chapters. Circulating IL-6 however, was significantly increased in both obese male and female plasma at 52 weeks of age, an effect well documented in the literature (495, 496) and confirms that mice fed postnatal HF diet in this study were in an inflammatory state. However, the lack of effect of maternal obesity to cumulatively add to the basal circulating IL-6, in the HFHF group in particular, goes against my hypothesis that postnatal HF would worsen effects of maternal HF for this particular outcome. In previous literature, cytokine levels in the brain following postnatal HF-feeding have been consistently shown to be increased. For example 20 weeks of 60% HF-feeding has shown to both increase levels of the same 3 cytokines as measured in my study in the hypothalamus by as much as 50% in male rats compared to controls (312), and also increase TNF-α levels in the hippocampus of mice also (497). Another study however found no change to basal hippocampal IL-1β (protein) following 20 weeks of 42% HF-feeding in male rats (498), but the mice displayed an elevated response to stress indicating that obesity may be priming inflammatory stress response. Further work to assess whether this may be occurring in my study would be beneficial.

5.5.2.2.2 Microglial activation

Although there were no postnatal HF diet-related changes to cytokines in my study, there was an increase in both CD11b and GFAP in the hypothalamus but not hippocampus in 30 wk IHC data. These changes indicate microglial and astroglial activation in the hypothalamus and thus increased inflammation. Considering that activated microglia and astrocytes often both respond to, and secrete pro-inflammatory cytokines, it is unexpected that no association was found with cytokine levels at either 15 or 52 weeks of age. Astrocyte activation is associated with neurodegeneration and the increase observed due to postnatal HF diet may well be associated with changes to stress and stress response. Astrocyte activation is induced by a number of

molecules including IL-6, and injury to the brain, which could suggest that there may be an effect of diet on cytokine levels that were not able to be measured in this study. Previous research has shown an upregulation of GFAP in the hypothalamus in female obese mice (both genetic and HF-fed) that was most pronounced in the PVN (499). Astrocytes are also involved in maintenance of the BBB and increased GFAP in obese mice has been associated with potential changes to the BBB via alterations to microvessels (499). As astrocytes are also known to functionally regulate synaptic plasticicity via interaction with neurons, the increased expression in the hypothalamus in my study may potentially be involved in modulating changes to HPA function, however further research would be necessary to confirm this.

Increased hypothalamic microglial activation measured by CD11b in the postnatal HF groups at 30 weeks was not positively associated with microglial activation measured by FCγR1 at either age. Interestingly, postnatal obesity decreased FCγR1 in the 15 week-old PVN when both sexes were combined but when data were split by sex this effect was only true in the males, and this reduction conflicts with 30 week data. This reduced FCγR1 is interesting as microglial activation has consistently shown to be increased in the PVN, ARC and generally in the hypothalamus in mouse models of obesity (487, 488, 500, 501). However studies of inflammation are most commonly assessed by IHC, and measurement of the gold standard of microglial activation Iba-1, which I decided against due to the condition of tissue available (but would be beneficial to measure in future studies). One possible reason for this effect of reduced FCγR1 due to postnatal obesity in my study could be downregulation due to potential overexpression of IgG. However, this conflicts with exisiting showing that diet induced obesity in C57BL/6 mice is associated with increased hypothalamic accumulation of IgG and that this is linked with increased activated microglia (as measured by Iba1) (501). In order to reach a more conclusive result on why this microglial activation marker may be decreased in my male mice it would be beneficial to measure mRNA levels of other markers of microglial activation and inflammation, including IgG. Interestingly this effect of postnatal HF diet was not observed in 15 week-old male hippocampal CA3 or in 15 week-old females in either brain region. This could suggest that the hypothalamic PVN in particular may be susceptible to postnatal HF induced obesity in young adulthood and this has a sex-specific effect in males only.

At 52 weeks of age, postnatal obesity also decreased FCγR1 in the CA3 in both male and females. Previously, microglial activation (measured by Iba1 by IHC) has been shown to be elevated in the hippocampus of aged (15-16 month-old) mice fed a 60% HF diet (from 2 months), but not before this age (447). And this research is supported by another study that also shows 60% HF diet given from the age of 2 months is able to alter FCγR1 mRNA expression at 24 months but not at 7 months of age (489). That study also showed an age related increase in IgG and other markers of

microglial inflammation, Iba1 and CD68, in the 24 but not 7 month-old mice which was exacerbated when fed a HF diet. They also indicated a disruption of the BBB in aging that appears to be worsened with obesity (489). As mentioned previously, it would be beneficial to measure BBB integrity in future research. However from my study, BBB integrity may be intact at both 15 and 52 weeks of age due to the unaffected or reduced mRNA expression of FCγR1 and cytokines. As stated above, this effect of reduced FCγR1 may be a modulating effect to reduce inflammation, however further investigation into the role of maternal and postnatal obesity on neuroinflammation is warranted.

5.5.3 Limitations

Conclusions about IL-1β, IL-6 and TNF-α gene mRNA expression data are uncertain because these markers were not shown to be elevated in LPS-treated mouse compared to untreated CC groups. This was unexpected. It is possible the primers used were not optimal (although the qPCR appeared to work appropriately). In order for successful analysis of these genes, the work would best be repeated with new primers created using the primer design protocol in Chapter 2 (Methods). Interestingly though, the results from these 3 genes show a very similar pattern, with the same effects of maternal and postnatal HF diet seen across the same brain regions, sex and age groups. This, alongside the < 10% co-efficient of variation for these analyses indicates that the lack of response of positive controls in these samples is not due to pipetting error. The lack of effect of postnatal HF diet to influence cytokine levels may also be due to my uncertainty over the measurement of these markers. Next steps to test this would be to assess inflammatory markers in the perfused brains of my Cohort C mice using IHC techniques for comparison to my cytokine mRNA data.

5.5.4 Conclusion

In this chapter, I found evidence of altered microglial activation in the hypothalamus and hippocampus and systemic inflammation, dependent on time of exposure to the HF diet and sex and age of the offspring. Changes in cytokine mRNA levels in the 15 week-old offspring hypothalamus and hippocampus following maternal obesity, which were most pronounced in the HFHF group, suggest nutrition in this period may prime offspring for elevated response to further postnatal HF-feeding, particularly in the PVN. The similar results between all 3 cytokines is intriguing and may indicate a similar mechanism of upregulation/maternal priming at 15 weeks. Strongest effects were seen in the females despite no significant overall sex differences at 15 weeks. This interaction supports my hypothesis that effects of maternal HF diet will be exacerbated by further postnatal HF-feedings. However, these effects were obscured at 52 weeks.

Interestingly, increased systemic inflammation in the postnatal obese groups at 52 weeks did not match neuroinflammation or microglial activation. More investigation is required in order to elucidate the potential priming effects of maternal HF diet, and further assessment of cytokine activity is required, such as inflammatory response to acute stress. In terms of microglial activation, the sex-specific changes, with males most affected by maternal obesity, could further support my previous findings from Chapter 3 and Chapter 4 that female offspring may be relatively protected from the negative consequences of maternal obesity.

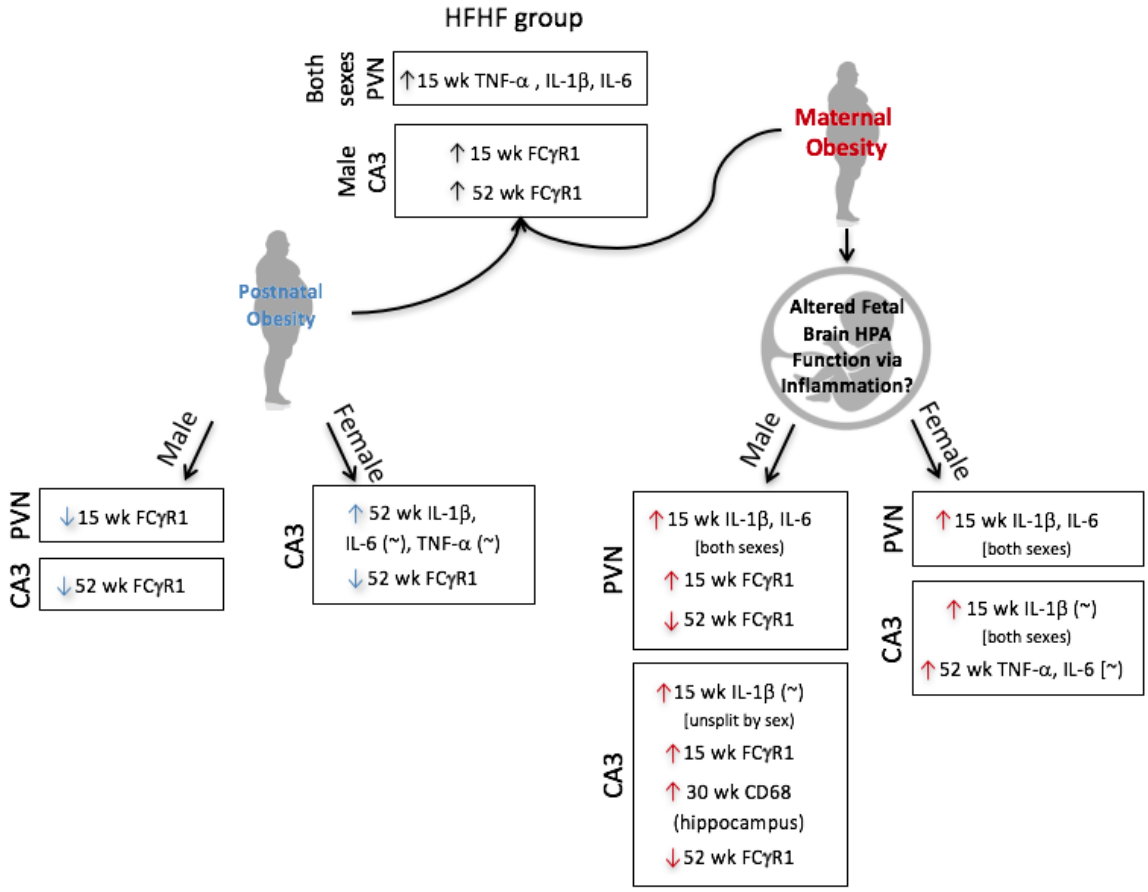

Figure 5.12 – **Schematic diagram of overarching results from Chapter 5.** Overall independent effects of maternal (red) and postnatal (blue) obesity on behaviour and memory in male and female offspring at 15 and 52 weeks of age. Combined box shows effects that were exacerbated in the HFHF group (significantly different from CC group). Results from 15 week-old offspring cytokine data not split by sex due to lack of sex effect. ~ = trend.

Chapter 6 General Discussion

Research in the field of developmental origins of health and disease initially focussed on undernutrition but more recently is investigating overnutrition. There is a current worldwide epidemic of obesity; currently 20% of child-bearing women are obese and this could be leading to intergenerational obesity. The long-term consequences of maternal obesity on offspring, and in particular the effects of further postnatal obesity require further elucidation. Emerging research shows that maternal obesity during pregnancy and lactation negatively affects offspring behaviour and cognition (74, 277) and that this may be related to changes in HPA axis function (75, 273) and brain inflammation (277). However, the mechanisms behind this are unclear, and reported effects are contradictory. Understanding the effects of maternal obesity would not only contribute to understanding the long-term consequences of the public health crisis, but will also allow for developing strategies to treat future offspring. In light of this, the main aim of this thesis was to investigate and understand the main impact of maternal obesity and further postnatal obesity on anxiety, memory and HPA axis function, and whether inflammation may be the underlying mechanism.

6.1 Overview of findings

In Chapter 3, using an established mouse model of maternal and postnatal obesity via obesogenic HF-feeding, changes in locomotor and anxiety behaviour and memory were explored. Changes to anxiety were observed to be sex- and age-specific. Effects of maternal obesity were generally observed in the males, with changes to anxiety behaviour from young age for example pup calls were increased in offspring from obese mothers. There were few effects of maternal obesity on anxiety at 15 weeks, but at 52 weeks there was a trend for decreased anxiety on the EPM test in males but not females. Maternal obesity generally decreased locomotor activity at 52 weeks of age (but not 15 weeks) which implies that the effect of maternal obesity on locomotor and anxiety behaviours are influenced by age. Additionally, memory was affected by maternal obesity and age in a sex-specific manner. Male offspring showed impaired non-spatial recognition memory at 15 weeks but not 52 weeks of age, and the opposite was observed in females where maternal obesity had an effect on improving memory at 52 weeks of age but had no effect at 15 weeks of age.

Postnatal obesity also influenced behaviour which was apparent in both sexes. Some measures of locomotor activity were decreased in both sexes at 15 weeks of age which became exacerbated by age, and in males only anxiety appeared to be decreased due to postnatal obesity at 15 weeks of age as measured by EPM. At 52 weeks of age both sexes exhibited increased anxiety in the OF test due to postnatal obesity but only the females had associated anxiety on the EPM. In terms of the hypothesis for this chapter, I did observe effects of maternal obesity but there were few interactions with postnatal obesity. Despite few interactions, evidence suggests that in some parameters of behaviour maternal and postnatal obesity had a cumulative detrimental effect such that HFHF was the group only or most significantly different from the CC group, such as on 15 week-old male memory and OF anxiety for 52 week-old offspring. This suggested that maternal obesity could indeed alter behaviour in offspring in later life, but effects may be too subtle to cause a significant interaction between maternal and postnatal obesity.

In Chapter 4, the same mouse model was used to assess HPA axis function that could be linked with changes in behaviour. Banked tissue was used for 15 week assessment, but the same mice as used in Chapter 3 were used for 52 week assessment. Similar to Chapter 3 there were sex-specific and age-dependent effects on the HPA axis. Effects of maternal obesity on HPA function were mainly seen in the male offspring. In 15 week-old male offspring, maternal obesity tended to increase basal CORT and this was due to the HFHF group in particular suggesting that maternal obesity may be able to program offspring for altered HPA function that can be worsened by further postnatal obesity. This elevation of basal CORT corresponded to increased CRH output due to maternal obesity, but ACTH concentrations were not measured at this age. Maternal obesity also increased MR levels in both brain regions in 15 weeks-old males. In 52 week-old males however, there was an effect of maternal obesity to increase the response to stress. Maternal obesity decreased MR levels in the male CA3, but not the PVN, which was linked to increased FKBP51 in the same mice, potentially impairing negative feedback in this brain region only which could indicate the CA3 may be more sensitive to changes in diet at this age.

Postnatal obesity generally had an effect at 15 weeks in males and 52 weeks in both sexes. In the 15 week male CA3, postnatal obesity decreased MR and increased CRH, an effect that would impair negative feedback. FKBP51 was increased in the CA3 also which could reduce steroid receptor binding affinity to CORT and cause glucocorticoid resistance, further impairing negative feedback of the axis. At 52 weeks of age, postnatal obesity increased basal CORT and decreased stress-induced CORT, however due to the additional effect of maternal obesity to increase CORT AUC, the HFHF group was comparable to CC suggesting over a period of time maternal obesity may have a protective effect for further postnatal obesity when exposed to a stressor. Interestingly, the elevations in basal CORT levels were not driven by enhanced ACTH or CRH,

indicating potential changes to pituitary and adrenal receptor expression and/or function. In 52 week-old females, there was increased plasma basal and decreased stress-induced CORT, and ACTH data were correlated to both results, however, CRH mRNA levels were unchanged in the PVN. MR was altered differentially in the PVN and CA3 regions (increased and decreased respectively) which may also be linked to the increased FKBP51 observed in the PVN only which is able to decrease CORT binding affinity to steroid receptors. FKBP51 is also associated with anxiety disorders (359), and this links to the increased anxiety in 52 week-old females due to postnatal obesity expressed consistently across behavioural tests in Chapter 3. These data overall further strengthen the idea in Chapter 3 that females may be relatively protected from the long-term detrimental effects of maternal obesity, but males are susceptible from a young age. In terms of the hypothesis of Chapter 4, I can conclude that maternal HF, in male offspring only, alters HPA function at both young and adult ages but that the effects of postnatal HF to worsen these effects occur primarily at 15 weeks of age, indicating that the effect diminishes with age when faced with further postnatal HF-feeding.

In Chapter 5, the same mouse model of obesity was used to assess the effect of HF diet on inflammation in the brain, looking at cytokine mRNA levels and markers of microglial activation, that could influence HPA function. Banked tissue was used for 15 week-old mouse assessment, but the same mice as used in Chapter 3 were used for assessment at 52 weeks. Similar to the previous two chapters there were sex-specific and age-dependent effects of HF diet. There were effects of maternal obesity and interaction with postnatal obesity on 15 week-old PVN cytokine mRNA levels such that the HFHF group was the only diet group increased from CC across sexes predominantly in the PVN. There were no significant effects of diet in CA3. The only changes to cytokine levels at 52 weeks of age were in female CA3 where again, despite overall maternal and postnatal diet effects, it was only the HFHF group that was the significantly elevated from CC. From assessing microglial activation data, maternal obesity seemed to influence male but not female offspring FCγR1 mRNA levels at both 15 and 52 weeks (increased and decreased respectively). Maternal obesity also showed an increase in CD68 microglial expression in the 30 week-old male hippocampus immunofluorescence data, although there was no female data for comparison between sexes. The lack of effect of maternal obesity to affect microglial CD11b and astrocytic GFAP staining in the hypothalamus and hippocampus at 30 weeks may indicate that the change of expression direction between 15 and 52 week takes place around this 30 week age, although the immunofluorescence data takes into account the entire hypothalamic/hippocampal region instead of a target analysis area (i.e. PVN or CA3).

Postnatal obesity had the effect of reducing FCγR1 mRNA expression in the CA3 in both sexes at 52 weeks, and in 15 week male PVN. Due to the lack of effect of postnatal obesity in the PVN at 52

weeks of age, this strengthens the position that the PVN may mediate the detrimental effects of HF diet, although overall expression in the hypothalamus at 30 weeks indicates that postnatal HF increases expression of activated microglia and astrocytes. In relation to this Chapter's hypothesis, I can conclude that maternal obesity influences male offspring from young to old adulthood, but postnatal obesity may only be able to worsen these effects at 15 weeks of age.

6.2 Are maternal and postnatal obesity-related changes in anxiety and memory linked to changes in HPA function?

The initial investigations in Chapter 3 into the impact of maternal and postnatal obesity on behaviour were fundamental in providing evidence that anxiety and memory were negatively affected by both maternal and postnatal obesity in a sex-specific and age-dependent manner. Results from Chapter 4 on the HPA axis also show similar changes that may be associated with the changes in behaviour and cognition as can be seen in Figure 6.1 and 6.2.

6.2.1 1 week-old pups

As previously mentioned, the results from this study are, to my knowledge, the first to demonstrate changes in pup USVs due to maternal obesity. The effect of maternal obesity on male pup anxiety behaviour was observed from the age of 1 week and persisted into adulthood. It was not possible to measure HPA axis function at 1 week in my study, and future work assessing early changes to HPA in this obese mouse model would be beneficial to understand whether maternal obesity is able to alter stress output at this age. Emerging research using a cross-fostering model suggests there may be a bi-directional effect between poor maternal care and pup behaviour resulting from maternal obesity (290). Analysis of HPA function in the late gestation fetus would allow us to determine whether poor maternal care behaviours observed in the obese mothers may influence or exacerbate any potential changes.

6.2.2 15 week-old offspring

6.2.2.1 Maternal Obesity

Maternal obesity overall, impacted male offspring behaviour and HPA axis at both ages more frequently than females. At 15 weeks of age there were no effects of maternal obesity on exploratory/locomotor activity or anxiety in either sex despite an effect of maternal obesity to increase basal HPA activity (via CORT and CRH measurements) in males. In females, the reduction

due to maternal obesity in FKBP51 mRNA levels observed in CA3 may be increasing steroid receptor affinity for CORT, which may explain why CA3 MR expression is reduced in these same mice and why we see no effects of maternal obesity on behaviour or memory if negative feedback is functioning to maintain normal HPA homeostasis. The lack of maternal obesity-related anxiety in males may be due, in part, to the elevated MR levels seen in both brain regions which may be enhancing negative HPA feedback in the maternal obese groups.

Maternal obesity did however tend to impair memory in males, but not females, at 15 weeks of age. However, as overexpression of MR is associated with improved spatial and non-spatial memory (502), it is unlikely the reduction in MR due to maternal obesity is involved in the impaired memory in males. Memory impairment was most prominent in the HFHF group which was the only diet group significantly reduced from the CC group. Interestingly at this age, it was also only male offspring that displayed hyperactivity of the HPA axis as reflected by increased (trend) basal plasma CORT levels and CRH mRNA levels (in both PVN and CA3) due to maternal obesity. CRH has been implicated in the formation of hippocampal-mediated memory and in stress-induced activation of neurons and hippocampal pyramidal cells (503). CRH contributes to the effects of acute stress on synaptic plasticity and memory (179, 184), and chronic exposure to CRH in the hippocampus has shown to cause dendritic atrophy and persistent memory impairments (223) which can be alleviated by blocking CRFR1 (182). It is for these reasons that I postulate that the elevation in CA3 CRH mRNA levels due to maternal obesity in 15 week-old males may be influencing the memory impairments in my study, and as before, the only significant difference from the CC group is the HFHF group which leads me to conclude that this group in general is the most affected. It would be beneficial to measure hippocampal CRH receptors in my study to investigate whether changes in CRH sensitivity may be involved in memory deficits. My results indicating that the male HFHF group has the most detrimental memory impairment are consistent with previous literature assessing spatial memory via Morris water maze testing in rats ages between 16.5-19.5 weeks subject to 58-60% HF maternal and postnatal diet (278, 400). Neither of these studies of maternal and postnatal obesity however looked at related changes to the HPA axis that may be influencing these changes, therefore, to my knowledge, this part of my thesis is novel.

6.2.2.2 Postnatal obesity

At 15 weeks of age there were some reductions to locomotor activity such as vertical movement and velocity in both sexes which was likely linked to weight. The only effect on anxiety behaviour was a tendency for reduced anxiety in males from postnatal obese groups, of which the HFHF group alone was significantly increased from the CC diet group.

In the male offspring FKBP51 mRNA levels in the CA3 region were upregulated by postnatal obesity with the most increased diet group being the HFHF group due to an interaction between maternal and postnatal HF diet. Little is known about the effects of increased FKBP51 on memory. Previous research has shown that FKBP51 knockout mice (13-17 week-old females) do not show any memory deficits (361), however it would be beneficial to repeat that study with male mice, as changes to stress response and HPA-function are well known to have differential sex effects, as can be seen in this thesis. FKBP51 is not altered in the PVN of the same males, which concurs with the lack of anxiety behaviour in males at 15 weeks. However, FKBP51 has been shown to be significantly increased in the hippocampal CA1 and dentate gyrus of stressed animals (350), which leaves the decreased anxiety seen in postnatal HF-fed male offspring largely unexplained within the realms of this thesis. There was also an effect of postnatal obesity to increase CRH in the CA3, in addition to the maternal effect mentioned above, which may be contributing to the increase in HFHF from CC and impairing memory (although there were no postnatal diet effects on memory). Considering the role of MR overexpression in improving memory (502, 504) and reducing anxiety (505), it is interesting that we see no negative consequences on memory due to the reduction of MR levels in postnatal obese males.

Although there were no apparent effects of diet on anxiety in females at 15 weeks, there are some changes to the HPA axis, such as a trend for increased CRH due to postnatal obesity that might be preliminary changes to the HPA hyperactivity and anxiety we observe in later age.

6.2.3 Conclusion

Although both maternal and postnatal obesity are separately associated with changes in HPA function, anxiety and memory, there is a clear theme in 15 week-old male data that it was the HFHF group that I generally saw significant differences from the CC group, indicating that this group was the most affected. The changes to basal HPA function may be associated with impaired memory that was generally most pronounced in the HFHF group. While chronic activation of the HPA axis has been known to be associated with memory deficits (506), it is also associated with increased anxiety disorders rather than decreased (201, 507, 508), therefore the association between HPA function and the trend of decreased anxiety in males in my study is unclear and warrants further investigation. The sex differences observed between male and female anxiety at this age show that females may be relatively protected from the effects of obesity, both maternal and postnatal, in young adulthood. Further work in understanding this ‘protective’ effect would be valuable.

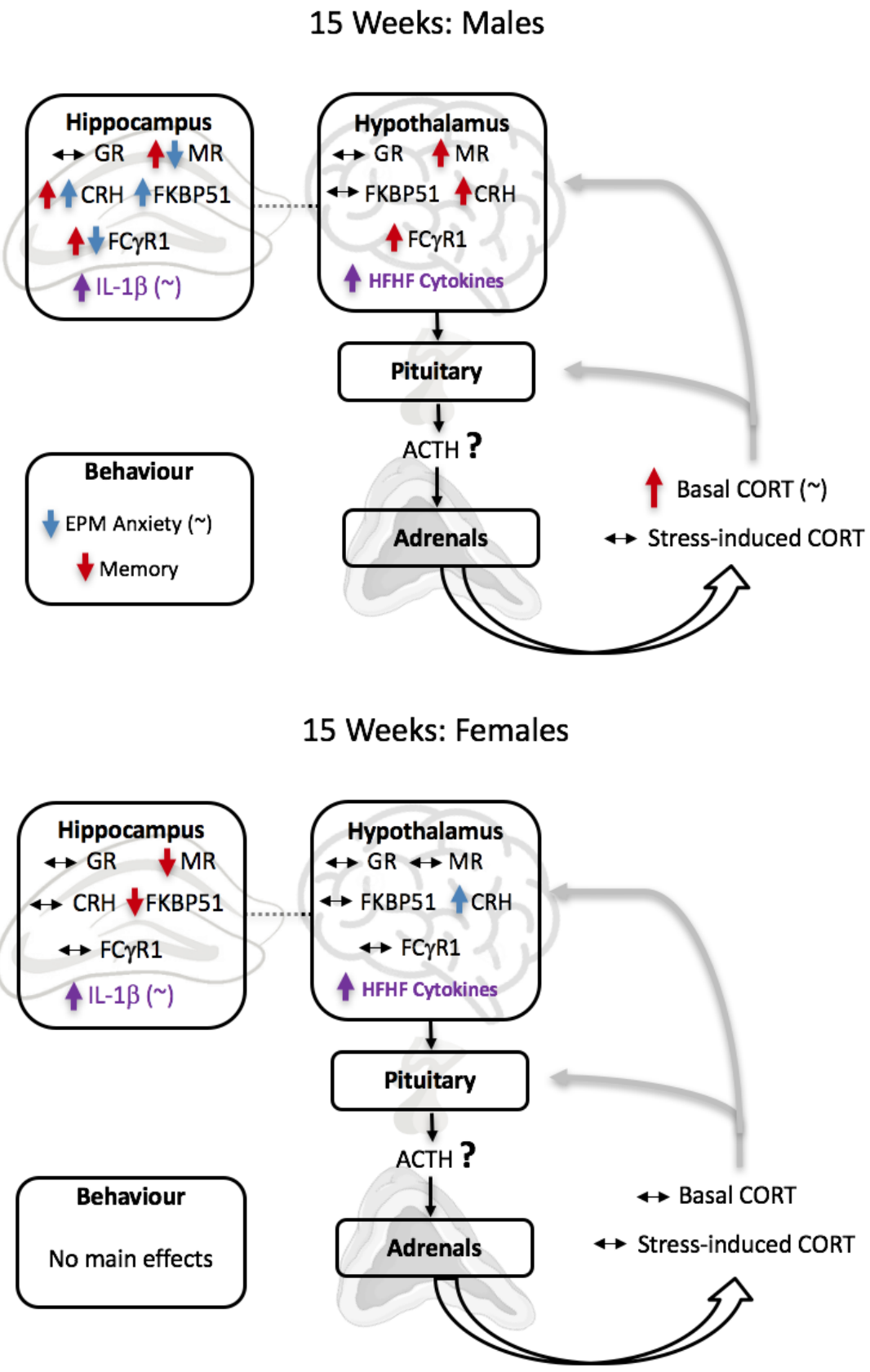

Figure 6.1 – **Summary diagram of results from 15 week-old male and female offspring.** Overall independent effects of maternal (red) and postnatal (blue) obesity on behaviour and memory, HPA activity and neuroinflammation in male and female offspring at 15 weeks of age. Results from 15 week-old offspring cytokine data not split by sex due to lack of sex effect (purple). ~ = trend. Arrows indicate direction of activity, ◄► = no effect. EPM = elevated plus maze.

6.2.4 52 week-old offspring

6.2.4.1 Maternal Obesity

In the mature adult offspring there were mixed effects of maternal obesity on anxiety and no effect on memory in males. In both sexes maternal obesity increased anxiety in the OF test but in males only there was a trend for decreased anxiety when assessing percentage of time on the EPM open arm. OF thigmotaxis and EPM open arm behaviour are routinely used to assess anxiety phenotypes in mice and exploit the natural aversion of rodents to open spaces. Data obtained from these two tests in the same samples of mice have shown to be comparable (509), therefore the differing outcomes on the OF and EPM tests in the same 52 week-old male mice, and the lack of effect of maternal obesity in the EPM in females, in my study are unexpected. Although there was an increased in stress-induced CORT due to maternal obesity, the reduced EPM anxiety in the males may be associated with the decrease of CRH in the PVN of the same animals as this was the only significant effect of maternal obesity to reduce basal stress genes. Previously, a study using CRH knockout mice showed significantly reduced CRH particularly in the PVN (but not brainstem), and this was associated with increased percentage of time on the open arms of the EPM alongside other measures of anxiety (510). However, a 60% maternal HF diet has been shown to increase EPM anxiety at 12 months in C57Bl/6 mice (in males), this was not associated with changes to CORT and the only measure of GR and FKBP51 was performed following chronic social defeat stress (75). Although there was an effect of maternal obesity diet to significantly decrease one parameter of thigmotaxis behaviour (% distance travelled in the centre area) in both sexes, there were no associated changes to HPA function that occurred in both sexes within my study that may explain this. Maternal obesity did not affect FKBP51 levels in the PVN of either sex but there was an increase in male CA3. FKBP51 knockout mice have shown no effects on memory therefore this increase is not associated with changes in behaviour. Few studies overall have assessed the long-term effect of maternal obesity on FKBP51 levels in the brain. One study using maternal 60% HF-feeding has previously been shown to increase FKBP51 in the PVN at 3 and 12 months of age (the latter following chronic social defeat stress), but this was associated with decreased anxiety at 3 months and increased anxiety at 12 months of age (75). These results may not show a true association between anxiety and FKBP51 levels however as that study included testing involving physical activity and a period of fasting which could be causing this increase in FKBP51 (and also FKBP51 expression was measured via in situ hybridization). Further assessment of FKBP51 levels in offspring from obese mothers is warranted. Overall, this effect of maternal obesity to increase anxiety in the OF is unexplained by my thesis and further work assessing genes involved in HPA function, such as CRH (CRFR) and ACTH (MC2R) receptors, may elucidate this effect. In general,

the literature assessing the long-term effects of maternal obesity in mature mice, particularly females, is lacking.

At 52 weeks of age in my study, maternal obesity no longer appeared to have an effect on impairing memory in males and there was no difference in non-spatial recognition memory between the dietary groups. As mentioned previously elevated CRH has an effect to impair memory, and in the CA3 at this age there is no effect of diet on CRH expression. This further strengthens the idea that increased CRH may be linked to the memory deficits seen in earlier age in males due to maternal obesity.

Interestingly, memory in the females at 52 weeks of age appeared to be improved due to maternal obesity which was largely due to the HFC group which was the only diet group significantly increased from CC. Interestingly, the only change to hippocampal CA3 due to maternal obesity was a trend for increased CRH. However as mentioned previously, CRH is implicated in poorer memory, therefore further work in the hippocampus is necessary to assess whether changes to HPA axis are able to influence this improved memory in my study.

6.2.4.2 Postnatal Obesity

Postnatal obesity understandably decreased all outputs of locomotor activity in both sexes which were likely due to weight, however they could also be related to anxiety but I was unable to conclude this in my study. Zone data from the OF test served as a better measure for anxiety at this age. The increased anxiety in both sexes due to postnatal obesity observed in the OF test is linked to the similar increase in basal CORT. As the OF is a habituated test, the CORT response to the restraint stress test is not directly comparable. Basal CORT however, is the only measure to be associated with OF anxiety in the males, as basal plasma ACTH is not significantly increased, but tended to be decreased due to postnatal obesity. This is interesting and could indicate that despite a reduction in basal ACTH, basal CORT alone may be able to increase anxiety in the OF test in mature adult male offspring. The lack of anxiety behaviour in the EPM in males but not females, could indicate that this measure of anxiety is less related to basal CORT levels in my study. In addition, the lack of influence of postnatal (or maternal) obesity to alter FKBP51 mRNA levels in the male hypothalamic PVN region also supports a differential effect of diet on anxiety, as FKBP51's role in neuropsychiatric disorders has been well validated (511, 512). Considering increased MR expression has been shown to play a role in anxiety (513), it is surprising to see no changes to MR levels in PVN either. Although there was no change to FKBP51 in the PVN, there was an increase in the CA3 due to postnatal obesity, which when coupled with the increase due to maternal obesity, made the HFHF group the most significant from CC. However, this was not

associated to changes in memory and may instead be impairing negative feedback of the HPA axis.

In the females, the consequences of long-term postnatal obesity were more consistently altered and understandable. Increased anxiety in the OF correlated to results in the EPM and both basal CORT and ACTH were upregulated basally (despite no change in CRH levels). Increased anxiety in the females was also associated with increased FKBP51 levels in the PVN in the postnatal obese groups which fits with the previous literature showing FKBP51 to be elevated in anxiety and other neuropsychiatric disorders (511). Interestingly, despite overexpression of MR to generally be associated with reduced anxiety (505), the overexpression of MR in the postnatal obese groups in my study were associated with increased anxiety. It could be plausible that the increased FKBP51 in the same mice PVN may be reducing MR sensitivity which may account for the increase in my study. At this age, the only output where HFHF was the only group significantly increased from CC was in basal ACTH which showed an interaction. As this interaction only corresponded with anxiety and locomotor effects that were observed in both sexes (and ACTH trended to be reduced in the postnatal HF groups in males) it is unlikely to have had an effect on behaviour.

Interestingly, no general changes to GR expression were seen in my study. This is interesting considering that HF diet was able to alter both anxiety and memory (mediated by age and sex) and HPA axis regulation. However, this may be due to diurnal effects of obesity on GR as a similar 45% HF diet fed to mice for 12 weeks has shown to reduce GR expression in PVN in the AM (9:00) but not the PM (18:00) (454). As sacrificing took place in the PM in my study it is plausible that the effects of HF diet may be too subtle to detect and not observed at this time. CORT also acts on the MR in both the PVN and CA3. The overexpression of MR is generally associated with a reduction in anxiety (461) and improved memory (502, 504) due to functional negative feedback, however none of the changes in MR mRNA levels in my study at either age were associated with changes in anxiety or memory. This may suggest that obesity may be working to impair or improve memory using a different pathway or mechanism.

6.2.5 Conclusion

Overall, taking the changes to anxiety and memory in Chapter 3 and HPA-function in Chapter 4 into account, there are clear associations between HPA-dysregulation and anxiety and memory in 15 week-old males, and anxiety in 52 week-old females, but the role of HPA function in 52 week-old male anxiety and female memory following exposure to obesity remains unclear. Further assessment of HPA-related markers would be beneficial to elucidate how these behavioural changes occur. The findings in this thesis suggest a sex-specific and age-dependent role of

maternal and postnatal obesity, and that both combined may, in some cases, lead to more severe outcomes (particularly in the males), indicating an increased susceptibility to HPA and behavioural changes following maternal obesity on the HPA axis.

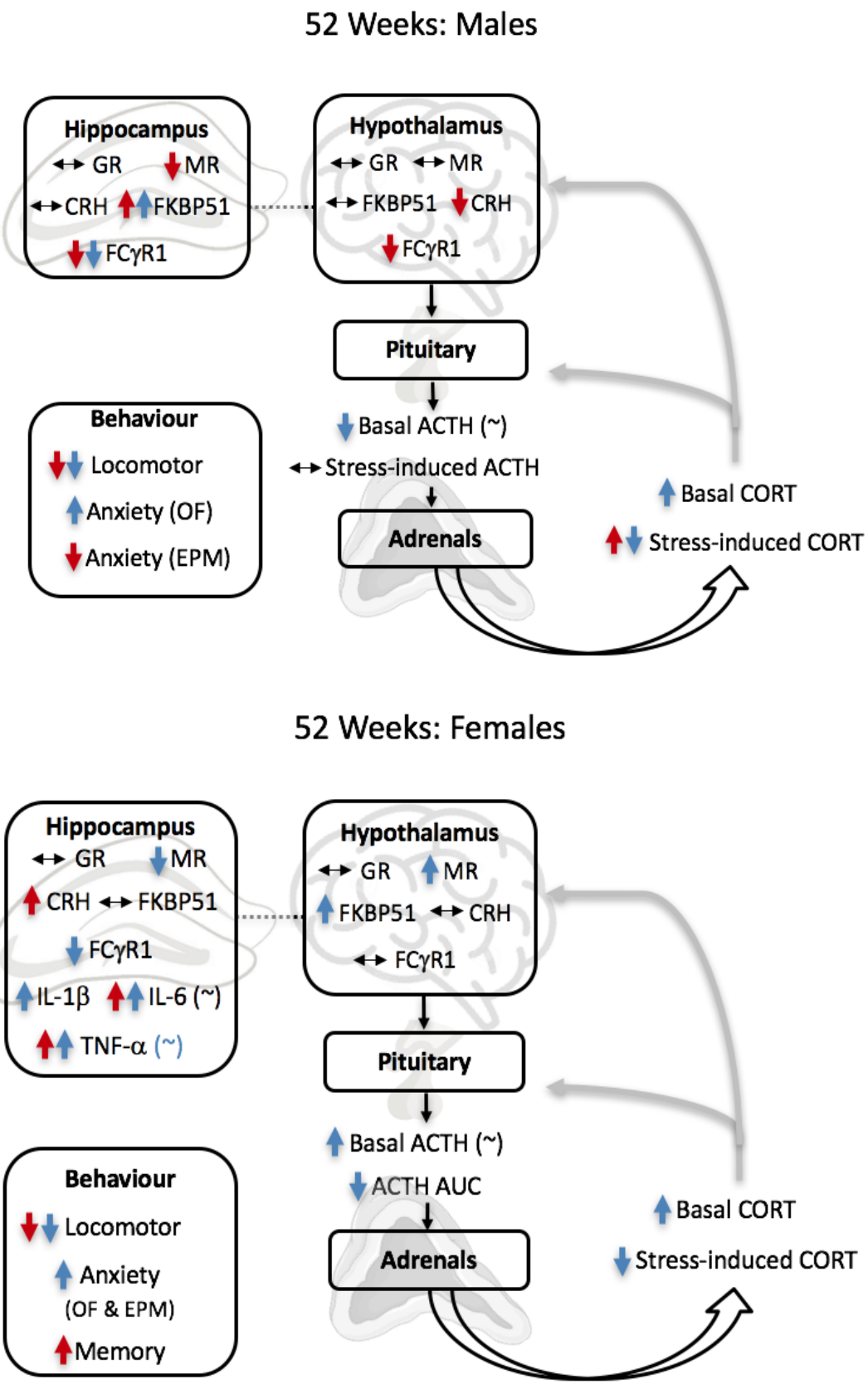

Figure 6.2 – **Summary diagram of results from 52 week-old male and female offspring.** Overall independent effects of maternal (red) and postnatal (blue) obesity on behaviour and memory, HPA activity and neuroinflammation in male and female offspring at 52 weeks of age. ~ = trend. Arrows indicate direction of activity, ◄► = no effect. OF = open field, EPM = elevated plus maze.

6.3 Could neuroinflammation be a potential mechanism causing changes to HPA function?

The role for neuroinflammation as a potential mechanism of changes to HPA function yielded mixed results. Similar to the previous Chapter results there was once again sex-specific and age-dependent effects on inflammation. Maternal obesity may prime both sexes of offspring for increased neuroinflammation when fed further postnatal HF, but in general neuroinflammatory effects were largely seen in males in later age as can be seen in Figures 6.1 and 6.2. Changes in inflammation at both ages were generally associated with behaviour and HPA changes in males but less in females. Further investigation into neuroinflammation in this mouse model would be beneficial to associate changes with effects seen in Chapters 3 and 4.

6.3.1 15 week-old offspring

6.3.1.1 Maternal Obesity

Although there were effects of maternal obesity on IL-1β and IL-6 in the PVN, this was largely the product of an interaction between maternal and postnatal diet to increase cytokine levels in the HFHF group of all 3 cytokines measured IL-1β, IL-6 and TNF-α. These pro-inflammatory cytokines (produced by activated microglia) have long been known to induce CRH secretion (514, 515) and in my study the upregulation of all three of these cytokines in the PVN may be linked to the increased CRH expression, particularly in the HFHF group in the PVN of both male and female offspring, which was the only group significantly elevated from CC. This increase in cytokines in the HFHF group may be associated with the elevations of HPA genes in the HFHF group observed in the males (but not females) at this age outlined in section 6.2. There were no significant effects of diet on cytokine levels in the CA3, except for a trend in maternal obese groups to increase IL-1β, which may be weakly associated with the increases to CRH observed in the male mice which may be modulating memory impairment. Further assessment of cytokine activity in the hippocampus must be performed to determine the involvement on hippocampal CRH and memory.

In addition to cytokine expression, FCγR1, a marker of microglial activation, was increased due to maternal obesity in both brain regions of male offspring and this was also associated with male CRH levels in both brain regions. Interestingly, despite increased cytokine levels in the HFHF groups of both sexes, there was no effect of maternal obesity or an interaction effect on microglial activation in females, indicating that cytokine regulation in these brain regions may be sex-specific. The linking of inflammation and HPA output in maternal and postnatal HF-fed

offspring is novel therefore there are no studies for direct comparison. As well as CRH, FCγR1 levels was associated with CORT in the male offspring only. Maternal obesity increased both FCγR1 and plasma CORT levels which was most exacerbated in the HFHF group similarly to cytokine expression. These data further suggest potential inflammatory modulation on the HPA axis may be sex-specific. These combined effects in the male hippocampus, alongside elevated CORT, may be associated with the memory impairment observed in the males due to maternal obesity as inflammation in the hippocampus has been widely shown to impair memory and is involved in diseases featuring memory loss such as Alzheimer's Disease (516).

Elevated FCγR1 due to maternal obesity was also linked to elevated MR in both brain regions of male but not female offspring. Inappropriate MR activation (such as by high salt intake) and binding with aldosterone is associated with an inflammatory response as shown in receptor blockade studies (517) and glucocorticoids are able to induce activation of MR (518). However, recent research shows that MR is also able to be transactivated by cytokines such as IL-6 (519). In my study, the elevated MR may play a dual role in the increased inflammation observed; by being activated by elevated inflammation and also exacerbating it in a positive feedback loop. It would be interesting to measure aldosterone in future studies to assess whether this hormone is upregulated and is contributing to the increased inflammation in these mice. Future work would also benefit from the measurement of 11β-HSD2, an enzyme which alters availability of CORT on MR by inactivating glucocorticoids, to assess whether any changes in basal inflammation may be contributing to this dysregulation of the HPA axis. It is thought cytokines including IL-6 and IL-1β are able to inhibit 11β-HSD2 (520, 521), and this may contribute to further HPA axis activation that is particularly seen in male offspring at 15 weeks of age.

Lastly, increased FKBP51 levels in the male CA3 HFHF group due to an interaction effect was postulated to be associated with cytokine expression, although this effect was not seen in the females. FKBP51 knockout studies show that FKBP51 has a role in increasing pro-inflammatory cytokines (522), however few studies have assessed the effects that inflammation may have on FKBP51 expression. One recent study showed that FKBP51 expression may be negatively influenced by sustained IL-1β *in vitro* (362), and this may be a reason why we see no effect of significantly increased inflammation in the HFHF group on FKBP51 in the PVN at this age, but this does not explain the effect seen in CA3. In my *in vivo* study, the LPS-treated mice significantly increased FKBP51 expression in the PVN, but not CA3, indicating that an inflamed state may be associated with elevated FKBP51 expression which is brain region-specific. If indeed the effects of inflammation on FKBP51 are brain region-specific, it is unclear why the increase of FKBP51 levels in the CA3 are observed in male HFHF group. Further work to determine the effect of inflammation on FKBP51 expression, and whether effects are limited to the PVN are necessary. In

keeping with the effect of sex, the female offspring may be protected from the effects of inflammation on HPA at this age which is why we see no change to FKBP51 expression or behaviour and memory at 15 weeks of age.

6.3.1.2 Postnatal Obesity

Interestingly, the only effect of postnatal obesity on neuroinflammation at 15 weeks of age was to decrease FCγR1 levels in the PVN of males but not females and there was no association with cytokine levels in the same mice. Considering both hypothalamic IgG and microglial expression of Iba-1 and GFAP have been shown to be upregulated in mice fed 58% HF diet for 16 weeks (501), my data does not fit with current literature, especially as FCγR1 is a microglial IgG receptor. This reduction of microglial activation is not associated with postnatal obesity-related changes in the HPA at this age, but may be associated with the reduced anxiety we see in the males on the EPM at 15 weeks as generally an inflammatory state induces anxiety on the EPM (523).

6.3.2 30 week-old offspring

Although microglial levels measured via FCγR1 levels were decreased in male PVN at 15 weeks, there was an upregulation in the whole hypothalamus at 30 weeks as measured by CD11b and also astrocytic activation measured by GFAP. These outcomes better match existing literature in obese mouse models. Unfortunately, there were no measures of behaviour or HPA function at 30 weeks to compare these changes with. However, increases in hypothalamic inflammation in postnatal obese groups at 30 weeks were associated with changes observed at 52 weeks in systemic inflammation, HPA dysregulation and anxiety. The increased in CD68 levels in the hippocampus due to maternal obesity relates to FCγR1 levels in the CA3 both of which may be contributing to memory impairment observed in 15 week-old males. IHC assessment of these markers in both 15 and 52 week-old brain would be beneficial in case there are inconsistencies in inflammatory expression due to differing methodology.

6.3.3 52 week-old offspring

6.3.3.1 Maternal obesity

At 52 weeks of age, there were no effects of maternal diet or interaction with postnatal diet on cytokine mRNA expression in the PVN of both sexes of offspring, or CA3 in the males. This may suggest that the effect of maternal priming for increased inflammatory response at 15 weeks may be ameliorated in later age. In males, the only change in inflammation due to maternal obesity

was a decrease of microglial activation (FCγR1) in both brain regions. This reduction in microglial activation directly contradicts the general consensus of existing literature which shows consistent upregulation of microglial activation in mouse models of maternal obesity (277, 324) which were associated with changes to anxiety. However, this reduced microglial activation may be associated with the reduction in anxiety in the EPM which was seen in males only. This reduction in FCγR1 may also be associated with the reduction in CRH observed in the male PVN. As microglia express CRFR, CRH is able to activate microglia and is implicated in neurodegenerative disorders (524). In my study, the long-term effect of maternal obesity may be downregulating microglial activation via reduced CRH although the mechanisms underpinning this potential action require investigating, such as CRFR assessment in the same brain regions. Reduction in MR levels in the hippocampus may also be associated to the reduced FCγR1 levels due to maternal obesity. MR's are expressed at low levels on microglia and act in a stimulatory way when binding to CORT (as opposed to GR's which act as an inhibitor) (525), however this reduction was not associated with changes in memory impairments. These effects are not observed in the females, which further suggests males are more susceptible to the effects of maternal obesity throughout life.

As the LPS-treated mouse data showed elevations of FCγR1 but no change to FKBP51 in the CA3 it is unlikely that the elevation in FKBP51 in the male CA3 due to maternal obesity are related, although further research into the brain region-specific effects of inflammation on FKBP51 are required to confirm this.

Although the effect of maternal obesity to increase IL-6, TNF-α (which was most increased in the HFHF group) was associated with memory improvement despite existing literature showing the opposite effect (526), further analysis showing that the HFC group was largely responsible for the improvement in memory, therefore on a diet group basis, changes to CA3 cytokine expression did not contribute to memory outcomes.

6.3.3.2 Postnatal Obesity

There were few effects of postnatal obesity on cytokine levels and this was interesting due to the strong effects of postnatal diet on both HPA dysregulation and anxiety in both sexes at this age. In addition, aging is known to weaken the blood brain barrier, a hallmark of which is increased neuroinflammation (527).

In males, decreased FCγR1 in the CA3 due to maternal obesity was further decreased by postnatal obesity such that the HFHF group was the most significantly decreased from CC. Like maternal obesity studies, postnatal obesity has also been shown to increase microglial expression in the

brain which is associated with changes to behaviour and/or HPA regulation (312). Similar to the effects of maternal obesity to decrease FCγR1, this could be associated with the lack of effect of diet on cytokine expression. However, recent research shows that HF-feeding in rats (60% HF for 8 weeks) upregulates CNS microglial activation but may actually downregulate pro-inflammatory gene expression and upregulate anti-inflammatory gene expression (528). In addition, it is important to note that mRNA expression levels may not reflect protein expression levels as post-transcriptional and post-translational regulation will also have an impact on protein levels (529). It would be interesting to measure anti-inflammatory genes and protein levels at both ages in future work.

Although there was an effect to increase all 3 cytokines and reduce FCγR1 in CA3 female postnatal obese groups, there were no accompanying effects of postnatal obesity on memory. Although other reports have indicated that an increase in these cytokines may impair memory function (526). However, to my knowledge, no studies on the effect maternal and postnatal obesity on HPA and inflammatory outcomes have been performed, particularly in aged females, therefore there is no basis for comparison. The hippocampus is responsible for multiple types of memory and learning, therefore further investigation of whether this microglial reduction due to postnatal obesity in females is related to specific memory outcomes is necessary to conclude whether memory is affected by diet.

The reduction of FCγR1 levels in the CA3 was common to both sexes and may be being downregulated due to the anti-inflammatory action of basal CORT which was elevated in the same mice. This could indicate that as there were no reductions of FCγR1 in the PVN, this region may be resistant to CORT or that the CA3 is more susceptible to CORT, however further studies would be required to confirm this.

Although there were few changes to neuroinflammation at this age due to postnatal obesity, systemic IL-6 production was increased in both sexes. Although the primary mechanism of glucocorticoid release is via CRH in the PVN, systemic cytokines may also function on the pituitary and adrenal glands to produce glucocorticoids also. Cytokine receptors have been detected at all HPA axis levels, and therefore, each level can serve as an integration point for immune and neuroendocrine signals. In addition, cytokines are synthesized in the brain, the anterior pituitary, and the adrenal gland. The production of local cytokines in these tissues may function in a paracrine manner to amplify and maintain elevated HPA activity during chronic inflammation (530). This may explain why elevated basal CORT was seen in both males and females despite no effects of postnatal obesity CRH levels in the PVN. Chronic systemic inflammation has been well established to cause changes to anxiety (531, 532), memory (533) and dysregulation of the HPA

axis (130). As low-grade chronic inflammation is a hallmark of obesity, the upregulation of IL-6 in postnatal obese offspring is not surprising. These changes in systemic IL-6 were linked to elevated basal CORT in both sexes and ACTH in the females, and also increased OF anxiety observed in both sexes and EPM in the females due to postnatal obesity. There were no corresponding effects of inflammation in brain tissue, even though, as mentioned previously, aging is known to weaken the blood brain barrier (527). It would be pertinent to measure markers of BBB integrity in my mouse model in future work.

6.3.4 Conclusion

Taking the results from all three chapters into account, there is some evidence that maternal obesity has an effect on certain aspects of inflammation such as microglial activation in male offspring, and maternal obesity coupled with further postnatal obesity may worsen inflammation, however it seems that there are sex-specific and age-dependant factors involved in both cytokine and activated microglial expression. Changes in inflammation appear to be associated to changes in HPA and anxiety/memory in 15 week-old male, but not female offspring, and at 52 weeks of age cytokine changes were not generally associated with changes to HPA or anxiety/memory in either sex, but changes in microglia may be associated to HPA function. Further investigation of inflammatory response to stress or LPS-treatment, inflammation associated genes (such as BBB) and also using other methods of inflammation expression analysis, such as IHC, at 15 and 52 weeks of age would be the best next step to help conclude whether neuroinflammation is associated to the changes in anxiety and HPA seen (at 52 weeks of age in particular) in this thesis.

6.4 Limitations and future work

The data from this thesis provide a novel contribution to understanding of the effects of maternal and postnatal obesity on the HPA axis. However, there are areas within this topic that warrant further research to fully explore and explain the changes in behaviour/cognition, HPA and inflammation, that were beyond the scope of this thesis.

Firstly, due to the inconsistency between anxiety behaviour in the males between OF and EPM tests, it would be beneficial to perform further anxiety tests (such as social interaction or light-dark exploration tests) to confirm the changes I saw in my study. In addition, as only non-spatial recognition memory was assessed it would also be beneficial to measure other types of memory such as spatial memory which can be measured, for example, by Morris water maze testing. It is also unclear whether stress phenotypes may change throughout the weaning phase. USVs in C57Bl/6 mice in particular peak at postnatal day 3 (534) so it would be interesting to see whether USVs are reduced at this age. Testing at earlier ages was unable to be performed due to HF-fed mothers requiring 7 days with pups without human interference to reduce potential cannibalism and to allow mother-pup bonding, as these offspring also undergo behavioural testing in adult life. In the male offspring particularly, it would be interesting to understand whether aspects of the HPA axis are already altered in fetal life. Future research of HPA and inflammatory markers could be measured during gestation and also in P7 pups to match anxiety phenotypes we see in the males at this age.

The analysis of HPA axis function could be further explored in a few ways. ACTH levels at 15 weeks of age could be measured to give a fuller overview of total HPA output in this obese mouse model. In order to understand the changes in HPA hormones observed at 52 weeks of age it would be beneficial to assess pituitary (CRFR) and adrenal receptor (MC2R) expression as well as MRAP for adrenal sensitivity. Both pituitary and adrenal glands were taken from mice sacrificed from Cohort C, and MC2R and MRAP expression from my mouse cohort is currently being analysed. This can be done via qPCR and IHC, as a subset of pituitary and adrenal organs were collected and stored appropriately for histology work. Performing this work will allow further clarification as to where in the HPA axis changes are taking place due to obesity. Alternative techniques could also be used in addition to mRNA levels such as measuring protein expression via western blotting. As there may also be brain region-specific effects on FKBP51 levels due to inflammation as measured by LPS-treated mice, it would be beneficial to assess this with larger sample numbers.

In order to strengthen the findings in Chapter 5, there was some uncertainty about the validity of 3 of the neuroinflammatory genes assessed via qPCR (IL-1β, IL-6 and TNF-α), possibly due to sub-

optimal primers. Further assessment of inflammatory status would also be beneficial such as assessment of BBB integrity. In addition, due to the 30 week brain tissue in my study not being collected appropriately for IHC work, it would be beneficial to use the subset of brains from my Cohort C that were collected by perfusion and stored correctly for histology (slow frozen in OCT) to assess activated microglial expression by IHC in order to align with results from qPCR analysis. In addition, previous research has shown that basal differences in brain inflammation are hard to detect via qPCR due to their low levels (52, 277). To truly assess whether maternal obesity may prime obese offspring for exacerbated inflammatory response, subjection to a stressor (such as restraint) or LPS-treatment should be performed and the same inflammatory and HPA markers reassessed. It would also be beneficial to measure markers of HPA function and inflammation in other areas of the brain such as the cortex and amygdala which have been shown to contribute to anxiety and neuropsychiatric disorders.

The mouse model of obesity via HF-feeding that was used has been well-validated and previously used to assess metabolic function successfully. Initially I had planned to assess a third age of 30 weeks in order to match outcomes to preliminary neuroinflammation results observed in mouse Cohort A, and also allow for easier assessment of the transition period from young to older adulthood, however this proved to be over-ambitious given the workload of measuring behaviour in a large cohort. Due to problems in mouse Cohort C with maternal cannibalism, maternal weight gain and difficulties with successful pregnancies, there would have been an overlap between testing ages (as the age gaps between first and last offspring used in the study were approximately 5 months).

The problems faced with staggered breeding times also meant that some subsets of animals required behavioural testing in the same week that other subsets of mice were due to be sacrificed. Due to this overlap, sacrifice had to take place in the afternoon hours (13.00-16.00 hours) which meant that mRNA expression analysis results from these mice may not have been entirely representative of behavioural testing (which was generally performed 9.00-12.00 hours) due to diurnal HPA regulation. Although banked tissue from Cohort B were used due to the same age (15 weeks), obese mouse model and sampling time (13.00-16.00 hours) as Cohort C, this cohort had low sample sizes for qPCR analysis, particularly in the females.

In general, studies assessing behaviour do not perform tests repeatedly on mice, which may have influenced outcomes in my study. However, due to the large period of time between the behavioural tests, it is likely that assessment will not be impaired, and any potential habituation effect will be obviated by comparing the different experimental groups.

As previously explained in Chapter 2, a HF diet consisting of 45% calories from saturated fat was used to induce obesity as compared to a control diet of 7% calories from fat. Although the overall aim of the HF diet was to induce obesity, there were also differences in other energy forms such as carbohydrate content which was much lower in the HF diet at 35% as compared to 75% in the C diet (protein content only different by 2.5% between diets). This additional change in diet composition must be taken into account when assessing the mechanisms of how obesity is able to alter HPA function, behaviour and inflammation. Besides the diet composition, the time window of behavioural testing, the type and concentration of dietary fat seems to be crucial for the manifestation of cognitive and behavioural impairment in existing literature. This must be taken into account when comparing results.

During behavioural assessments, locomotor activity at 52 weeks was generally reduced in the postnatal HF groups. It could be argued that analysis of anxiety-related thigmotaxis behaviour may be skewed due to obesity-induced inactivity. In order to account for this reduction in activity the relative percentage of distance travelled in the inner vs outer areas was calculated, and in addition percentage time resting in the inner vs outer areas was also calculated as an extra measure to ensure that regardless of the locomotor activity reduction, these mice being tested were simply not resting in the inner area for longer which was confirmed in my study.

6.5 Conclusion

Critically, this research highlights the significance of appropriate maternal nutrition on long-term health of offspring, particularly in males who were most affected by maternal obesity, but also the impact of postnatal obesity which was detrimental in both sexes. Sex and age-specific effects are an underlying feature of this thesis, with female offspring being relatively protected from the negative effects of maternal obesity, and older age exacerbating anxiety phenotypes and HPA dysregulation. Despite negative effects of both maternal and postnatal obesity separately, few significant interactions between maternal and postnatal obesity were found. However, the HFHF group was regularly the most significantly different group from the CC group, particularly in males, indicating that programming effects of maternal diet may be subtle. Interestingly, there were also some opposing (instead of additive) effects of maternal and postnatal obesity in males, particularly in response to stress, which may show differential long-term changes to the HPA axis. The data from this thesis adds to the limited knowledge in this research area, highlighting the necessity to understand the complex nature of maternal environment to cause differential sex effects. Understanding the potential roles of maternal and further postnatal obesity on long-term pathophysiology of the HPA axis will lead to a greater understanding of the causes of its related neuropsychiatric changes and help to improve health in future generations.

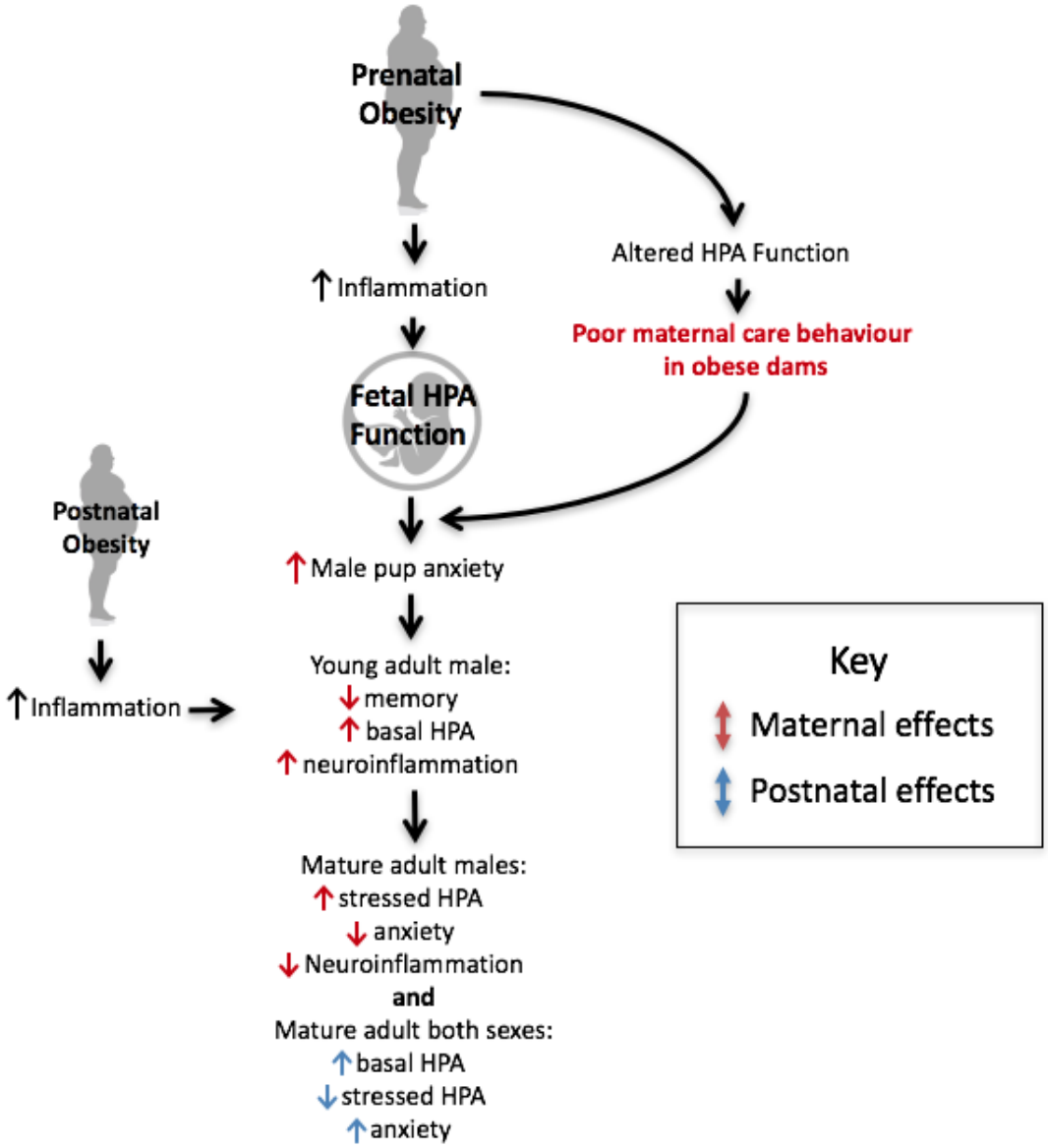

Figure 6.3 – **Thesis overview.** A summary of the flow of logic with main outcomes of maternal (red) and postnatal (blue) obesity on maternal behaviour and offspring at all ages.

Appendix A Mouse model diet

A.1 Control diet feed formulation

Rat and Mouse No. 1 Maintenance

Calculated Analysis

NUTRIENTS		Total	Supp (9)
Proximate Analysis			
Moisture (1)	%	10.00	
Crude Oil	%	2.71	
Crude Protein	%	14.38	
Crude Fibre	%	4.65	
Ash	%	6.00	
Nitrogen Free Extract	%	61.73	
Digestibility Co-Efficients (7)			
Digestible Crude Oil	%	2.47	
Digestible Crude Protein	%	12.92	
Carbohydrates, Fibre and Non Starch Polysaccharides (NSP)			
Total Dietary Fibre	%	17.05	
Pectin	%	1.52	
Hemicellulose	%	10.17	
Cellulose	%	4.32	
Lignin	%	1.68	
Starch	%	44.97	
Sugar	%	4.05	
Energy (5)			
Gross Energy	MJ/kg	14.74	
Digestible Energy (15)	MJ/kg	11.90	
Metabolisable Energy (15)	MJ/kg	10.74	
Atwater Fuel Energy (AFE)(8)	MJ/kg	13.75	
AFE from Oil	%	7.42	
AFE from Protein	%	17.49	
AFE from Carbohydrate	%	75.09	
Fatty Acids			
Saturated Fatty Acids			
C12:0 Lauric	%	0.02	
C14:0 Myristic	%	0.14	
C16:0 Palmitic	%	0.31	
C18:0 Stearic	%	0.04	
Monounsaturated Fatty Acids			
C14:1 Myristoleic	%	0.02	
C16:1 Palmitoleic	%	0.09	
C18:1 Oleic	%	0.77	
Polyunsaturated Fatty Acids			
C18:2(ω6) Linoleic	%	0.69	
C18:3(ω3) Linolenic	%	0.06	
C20:4(ω6) Arachidonic	%	0.13	
C22:5(ω3) Clupanodonic	%		
Amino Acids			
Arginine	%	0.91	
Lysine (6)	%	0.66	0.07
Methionine	%	0.22	0.04
Cystine	%	0.24	
Tryptophan	%	0.18	
Histidine	%	0.35	
Threonine	%	0.49	
Isoleucine	%	0.54	
Leucine	%	0.98	
Phenylalanine	%	0.66	
Valine	%	0.69	
Tyrosine	%	0.49	
Taurine	%		
Glycine	%	1.11	
Aspartic Acid	%	0.67	

NUTRIENTS		Total	Supp (9)
Glutamic Acid	%	3.17	
Proline	%	1.20	
Serine	%	0.56	
Hydroxyproline	%		
Hydroxylysine	%		
Alanine	%	0.16	
Macro Minerals			
Calcium	%	0.73	0.63
Total Phosphorus	%	0.52	0.04
Phytate Phosphorus	%	0.24	
Available Phosphorus	%	0.28	0.04
Sodium	%	0.25	0.19
Chloride	%	0.38	0.32
Potassium	%	0.67	
Magnesium	%	0.23	
Micro Minerals			
Iron	mg/kg	159.30	82.50
Copper	mg/kg	11.50	1.94
Manganese	mg/kg	72.44	19.22
Zinc	mg/kg	35.75	
Cobalt	µg/kg	634.10	550.00
Iodine	µg/kg	1202.69	1085.00
Selenium	µg/kg	298.99	100.00
Fluorine	mg/kg	10.49	
Vitamins			
β-Carotene (2)	mg/kg	0.16	
Retinol (2)	µg/kg	2566.38	2400.00
Vitamin A (2)	iu/kg	8554.27	8000.00
Cholecalciferol (3)	µg/kg	15.54	15.00
Vitamin D (3)	iu/kg	621.70	600.00
α-Tocopherol (4)	mg/kg	76.45	56.82
Vitamin E (4)	iu/kg	84.10	62.50
Vitamin B_1 (Thiamine)	mg/kg	8.58	1.96
Vitamin B_2 (Riboflavin)	mg/kg	4.33	2.94
Vitamin B_6 (Pyridoxine)	mg/kg	4.81	0.98
Vitamin B_{12} (Cyanocobalamine)	µg/kg	7.49	6.00
Vitamin C (Ascorbic Acid)	mg/kg	2.59	
Vitamin K (Menadione)	mg/kg	10.17	9.36
Folic Acid (Vitamin B_9)	mg/kg	0.79	
Nicotinic Acid (Vitamin PP) (6)	mg/kg	61.32	2.45
Pantothenic Acid (Vitamin $B_{3/5}$)	mg/kg	20.17	5.80
Choline (Vitamin $B_{4/7}$)	mg/kg	1080.14	366.60
Inositol	mg/kg	2369.59	
Biotin (Vitamin H) (6)	µg/kg	277.13	

Notes

1. All values are calculated using a moisture basis of 10%. Typical moisture levels will range between 9.5 - 11.5%.
2. a. Vitamin A includes Retinol and the Retinol equivalents of β-carotene
   b. Retinol includes the Retinol equivalents of β-Carotene.
   c. 0.48 µg Retinol = 1 µg β-carotene = 1.6 iu Vitamin A activity
   d. 1 µg Retinol = 3.33* iu Vitamin A activity
   e. 1 iu Vitamin A = 0.3 µg Retinol = 0.6 µg β-carotene
   f. The standard analysis for Vitamin A does not detect β-carotene
3. 1µg Cholecalciferol (D_3) = 40.0 iu Vitamin D
4. 1 mg all-*rac*-α-tocopherol = 1.1 iu Vitamin E activity
   1 mg all-*rac*-α-tocopherol acetate = 1.0 iu Vitamin E activity
5. 1 MJ = 239.23 Kcalories = 239.23 Calories = 239,230 calories
6. These nutrients coming from natural raw materials such as cereals may have low availabilities due to the interactions with other compounds.
7. Based on in-vitro digestibility analysis.
8. AF Energy = Atwater Fuel Energy = ((CO%/100)*9000)+((CP%/100)*4000)+((NFE%/100)*4000)/239.23
9. Supplemented nutrients from manufactured and mined sources.
15. Calculated.

A.2 High-fat diet feed formulation

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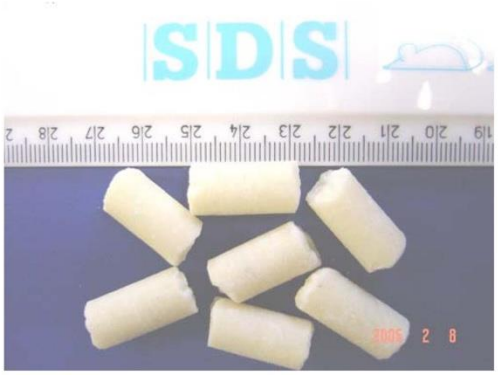
Data Sheet: 824053 - '45% AFE FAT'



Diet Code: **824053**

Diet Name:

RM AFE45%FAT 20%CP 35%CHO (P)

Description:

Purified laboratory rodent diet.

'High Fat' diet for use with 829050 or other 'Rodent AFE' diets in this series.

Useful for studying obesity, diabetes and other fat/energy induced diseases.

BASIC INFORMATION

DIET	824053 - '45% AFE Fat'
INGREDIENT	g% (w/w)
Casein	26.533
Choline Bitartrate	0.296
L-Cystine	0.399
Lard	17.895
Rice Starch	28.344
Cellulose	6.171
Soya Oil	4.319
Sucrose	10.490
Mineral Mix	4.319
Vitamin Mix	1.234
Total	100.000

SPECIFICATION	% (w/w)	kcal/g	% kcal
Crude Fat	22.6	2.03	45
Crude Protein	23.0	0.92	20
Crude Fibre	4.6	/	/
Ash	4.2	/	/
Carbohydrate	39.8	1.59	35
Total AFE		4.54	100

OTHER INFORMATION

Storage:
This is a perishable material. Please store in a cool dry place. If possible store refrigerated or even freeze to reduce nutrient oxidation.

Shelf Life:
3 months from date of manufacture.
6 months if stored refrigerated.
9 months if stored frozen.

Feeding Directions:
Feed ad-libitum. Clean drinking water should be available at all times.

Product Form, Packaging & Net Weight:
Standard: 10 mm diameter pellets. Packed in sealed plastic buckets. **3 kg net weight.**

Nutrient Information:
AFE = ATWATER FUEL ENERGY = Decimal fractions of Fat, Protein, Carbohydrate multiplied by 9, 4 & 4 respectively to give kcal AFE / g of diet.
1 MJ = 239.23 Kcal
Nutrient figures on a fresh weight basis unless otherwise stated

Contact SDS for further details.
Tel: +44 (0) 1376 511 260
Fax: +44 (0) 1376 511 247
e-mail: info@sdsdiets.com

824053 COM 050209

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DIET FORMULATION AND SPECIFICATION DATA

BASIC DIET INFORMATION:

Code:	824018
Name:	RM AFE 45% FAT SY(P)
Date:	04/02/2011

CALCULATED ANALYSIS:

		FRESH	10% H2O
TOTAL	%	100.00	100.00
MOISTURE	%	4.11	10.00
CRUDE OIL	%	22.61	21.22
CRUDE PROTEIN	%	22.97	21.56
CRUDE FIBRE	%	4.50	4.22
ASH	%	4.42	4.15
NFE	%	40.38	37.90
PECTIN	%	0.00	0.00
HEMICELLULOSE	%	0.12	0.11
CELLULOSE	%	5.92	5.56
LIGNIN	%	0.00	0.00
STARCH	%	15.85	14.88
SUGAR	%	22.66	21.27
GROSS ENERGY	MJ/kg	20.30	19.05
DIGESTIBLE ENERGY	MJ/kg	18.61	17.47
METABOLISABLE ENERGY	MJ/kg	17.19	16.13
AF ENERGY	kcal/kg	4568.36	4287.75
C14 1 MYRISTOLEIC	%	0.02	0.02
C16 1 PALMITOLEIC	%	0.03	0.03
C18 1 W9 OLEIC	%	6.46	6.06
C18 2 W6 LINOLEIC	%	3.76	3.53
C18 3 W3 LINOLENIC	%	0.39	0.37
C20 4 W6 ARICHIDONIC	%	0.01	0.01
C22 5 W3 CLUPANODONIC	%	0.00	0.00
C12:0 LAURIC	%	0.03	0.03
C14:0 MYRISTIC	%	0.31	0.29
C16:0 PALMITIC	%	4.30	4.04
C18:0 STEARIC	%	1.92	1.80
ARGININE	%	0.73	0.69
LYSINE	%	1.46	1.37
S LYS	%	0.00	0.00
METHIONINE	%	0.57	0.53
S METH	%	0.00	0.00
CYSTINE	%	0.45	0.42
S CYST	%	0.39	0.37

		FRESH	10% H2O
CL	%	0.28	0.26
S CL	%	0.19	0.18
K	%	0.45	0.42
S K	%	0.44	0.41
MG	%	0.08	0.08
S MG	%	0.06	0.06
FE	mg/kg	58.86	55.24
S FE	mg/kg	52.92	49.67
CU	mg/kg	8.76	8.22
S CU	mg/kg	7.43	6.97
MN	mg/kg	13.29	12.47
S MN	mg/kg	11.96	11.23
ZN	mg/kg	68.90	64.67
S ZN	mg/kg	37.09	34.81
CO	µg/kg	0.00	0.00
S CO	µg/kg	0.00	0.00
I	µg/kg	254.59	238.95
S I	µg/kg	254.59	238.95
SE	µg/kg	197.20	185.09
S SE	µg/kg	197.20	185.09
F	mg/kg	1.24	1.16
VIT A	iu/kg	4931.57	4628.65
S VIT A	iu/kg	4931.57	4628.65
VIT D3	iu/kg	7863.40	7380.39
S VIT D3	iu/kg	1232.89	1157.16
VIT E	iu/kg	100.64	94.46
S VIT E	iu/kg	96.39	90.47
VIT B1 THI	mg/kg	7.36	6.91
S VIT B1	mg/kg	7.25	6.80
VIT B2 RIB	mg/kg	6.37	5.98
S VIT B2	mg/kg	5.92	5.56
VIT B6 PYR	mg/kg	8.65	8.12
S VIT B6	mg/kg	8.54	8.02
VIT B12 CY	µg/kg	30.82	28.93

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

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TRYPTOPHAN	%	0.20	0.19
S TRYPT	%	0.00	0.00
HISTIDINE	%	0.52	0.49
THREONINE	%	0.80	0.75
S THREO	%	0.00	0.00
ISOLEUCINE	%	1.16	1.09
LEUCINE	%	1.75	1.64
PHENYLALAN	%	0.96	0.90
VALINE	%	1.39	1.30
TYROSINE	%	0.96	0.90
TAURINE	%	0.00	0.00
GLYCINE	%	0.91	0.85
ASPARTIC A	%	1.30	1.22
GLUTAMIC A	%	3.76	3.53
PROLINE	%	1.57	1.47
SERINE	%	0.87	0.82
HYD PROLIN	%	0.00	0.00
HYD LYSINE	%	0.00	0.00
ALANINE	%	0.74	0.69
CA	%	0.63	0.59
S CA	%	0.62	0.58
TOTAL P	%	0.37	0.35
S PHOS	%	0.29	0.27
PHYTATE P	%	0.00	0.00
AVAIL P	%	0.27	0.25
NA	%	0.35	0.33
S NA	%	0.12	0.11

S VIT B12	µg/kg	30.82	28.93
VIT C ASCO	mg/kg	0.00	0.00
S VIT C	mg/kg	0.00	0.00
VIT K MENE	mg/kg	0.95	0.89
S VIT K	mg/kg	0.95	0.89
FOLIC ACID	mg/kg	2.37	2.22
S FOLIC	mg/kg	2.34	2.20
NICOTINIC	mg/kg	36.62	34.37
S NICOTIN	mg/kg	36.25	34.02
PANTOTHENI	mg/kg	18.46	17.33
S PANTOTH	mg/kg	17.69	16.60
CHOLINE	mg/kg	1330.10	1248.40
S CHOLINE	mg/kg	1216.13	1141.43
INOSITOL	mg/kg	0.00	0.00
S INOSITOL	mg/kg	0.00	0.00
BIOTIN	µg/kg	246.58	231.43
S BIOTIN	µg/kg	246.58	231.43

S = Supplemented nutrients from manufactured and mined sources. The TOTAL nutrient level including theoretical natural contribution for the diet pre-processing is found immediately above the SUPP nutrient.

INGREDIENTS:

NAME	% INCLUSION
CASEIN	26.51
SUCROSE	20.34
RICE STARCH	18.43
LARD	18.00
CELLULOSE	6.16
SOYA OIL	4.32
MINERAL MIX	4.32
VITAMIN MIX	1.23
L-CYSTINE	0.40
CHOLINE BITARTRATE	0.30

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

1. Caspi A, Moffitt TE, Newman DL, Silva PA. Behavioral observations at age 3 years predict adult psychiatric disorders. Longitudinal evidence from a birth cohort. Arch Gen Psychiatry. 1996;53(11):1033-9.
2. Wittchen HU, Jacobi F, Rehm J, Gustavsson A, Svensson M, Jonsson B, et al. The size and burden of mental disorders and other disorders of the brain in Europe 2010. Eur Neuropsychopharmacol. 2011;21(9):655-79.
3. Kessler RC, Berglund P, Demler O, Jin R, Merikangas KR, Walters EE. Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. Arch Gen Psychiatry. 2005;62(6):593-602.
4. CQC. The state of care in mental health services 2014 to 2017. 2017.
5. Kessler RC, Wang PS. The descriptive epidemiology of commonly occurring mental disorders in the United States. Annu Rev Public Health. 2008;29:115-29.
6. Cooper R. Diagnostic and Statistical Manual of Mental Disorders (DSM). Knowledge Organization. 2017;44(8):668-76.
7. Conrad CD. A critical review of chronic stress effects on spatial learning and memory. Prog Neuropsychopharmacol Biol Psychiatry. 2010;34(5):742-55.
8. Roy-Byrne PP, Davidson KW, Kessler RC, Asmundson GJ, Goodwin RD, Kubzansky L, et al. Anxiety disorders and comorbid medical illness. Gen Hosp Psychiatry. 2008;30(3):208-25.
9. Sareen J, Jacobi F, Cox BJ, Belik SL, Clara I, Stein MB. Disability and poor quality of life associated with comorbid anxiety disorders and physical conditions. Arch Intern Med. 2006;166(19):2109-16.
10. Musazzi L, Marrocco J. The Many Faces of Stress: Implications for Neuropsychiatric Disorders. Neural Plast. 2016;2016:8389737.
11. Ahmed S, Venigalla H, Mekala HM, Dar S, Hassan M, Ayub S. Traumatic Brain Injury and Neuropsychiatric Complications. Indian J Psychol Med. 2017;39(2):114-21.
12. Zhu X, Need AC, Petrovski S, Goldstein DB. One gene, many neuropsychiatric disorders: lessons from Mendelian diseases. Nat Neurosci. 2014;17(6):773-81.
13. Munjal S, Ferrando SJ, Freyberg Z. Neuropsychiatric Aspects of Infectious Diseases: An Update. Crit Care Clin. 2017;33(3):681-712.
14. Sheidow AJ, McCart M, Zajac K, Davis M. Prevalence and impact of substance use among emerging adults with serious mental health conditions. Psychiatr Rehabil J. 2012;35(3):235-43.
15. Kinderman P, Schwannauer M, Pontin E, Tai S. Psychological processes mediate the impact of familial risk, social circumstances and life events on mental health. PloS one. 2013;8(10):e76564.
16. Gross C, Hen R. The developmental origins of anxiety. Nat Rev Neurosci. 2004;5(7):545-52.
17. Estes ML, McAllister AK. Maternal immune activation: Implications for neuropsychiatric disorders. Science. 2016;353(6301):772-7.
18. Veena SR, Gale CR, Krishnaveni GV, Kehoe SH, Srinivasan K, Fall CH. Association between maternal nutritional status in pregnancy and offspring cognitive function during childhood and adolescence; a systematic review. BMC Pregnancy Childbirth. 2016;16:220.
19. Barker DJ, Osmond C, Law CM. The intrauterine and early postnatal origins of cardiovascular disease and chronic bronchitis. Journal of epidemiology and community health. 1989;43(3):237-40.
20. Barker DJ, Winter PD, Osmond C, Margetts B, Simmonds SJ. Weight in infancy and death from ischaemic heart disease. Lancet. 1989;2(8663):577-80.
21. Dennison EM, Arden NK, Keen RW, Syddall H, Day IN, Spector TD, et al. Birthweight, vitamin D receptor genotype and the programming of osteoporosis. Paediatric and perinatal epidemiology. 2001;15(3):211-9.

22. Curhan GC, Willett WC, Rimm EB, Spiegelman D, Ascherio AL, Stampfer MJ. Birth weight and adult hypertension, diabetes mellitus, and obesity in US men. Circulation. 1996;94(12):3246-50.
23. Colman I, Ploubidis GB, Wadsworth ME, Jones PB, Croudace TJ. A longitudinal typology of symptoms of depression and anxiety over the life course. Biological psychiatry. 2007;62(11):1265-71.
24. Van den Bergh BR, Mulder EJ, Mennes M, Glover V. Antenatal maternal anxiety and stress and the neurobehavioural development of the fetus and child: links and possible mechanisms. A review. Neuroscience and biobehavioral reviews. 2005;29(2):237-58.
25. Welberg LA, Seckl JR. Prenatal stress, glucocorticoids and the programming of the brain. Journal of neuroendocrinology. 2001;13(2):113-28.
26. Woods LL, Ingelfinger JR, Nyengaard JR, Rasch R. Maternal protein restriction suppresses the newborn renin-angiotensin system and programs adult hypertension in rats. Pediatric research. 2001;49(4):460-7.
27. Sasaki A, de Vega W, Sivanathan S, St-Cyr S, McGowan PO. Maternal high-fat diet alters anxiety behavior and glucocorticoid signaling in adolescent offspring. Neuroscience. 2014;272:92-101.
28. Kuzawa CW. Fetal origins of developmental plasticity: are fetal cues reliable predictors of future nutritional environments? American journal of human biology. 2005;17(1):5-21.
29. Monaghan P. Early growth conditions, phenotypic development and environmental change. Philos Trans R Soc Lond B Biol Sci. 2008;363(1497):1635-45.
30. Hales CN, Barker DJ. The thrifty phenotype hypothesis. Br Med Bull. 2001;60:5-20.
31. Gregorio BM, Souza-Mello V, Carvalho JJ, Mandarim-de-Lacerda CA, Aguila MB. Maternal high-fat intake predisposes nonalcoholic fatty liver disease in C57BL/6 offspring. Am J Obstet Gynecol. 2010;203(5):495 e1-8.
32. Bagby SP. Maternal nutrition, low nephron number, and hypertension in later life: pathways of nutritional programming. J Nutr. 2007;137(4):1066-72.
33. Tang WY, Ho SM. Epigenetic reprogramming and imprinting in origins of disease. Rev Endocr Metab Disord. 2007;8(2):173-82.
34. Roseboom T, de Rooij S, Painter R. The Dutch famine and its long-term consequences for adult health. Early Hum Dev. 2006;82(8):485-91.
35. Ravelli GP, Stein ZA, Susser MW. Obesity in young men after famine exposure in utero and early infancy. N Engl J Med. 1976;295(7):349-53.
36. Susser ES BA, Gorman JM. Prenatal exposures in schizophrenia. Washington, DC: American Psychiatric Press. 1999.
37. Taylor PD, Poston L. Developmental programming of obesity in mammals. Exp Physiol. 2007;92(2):287-98.
38. World Health Organization. Obesity and overweight. Fact sheet. 2016 [cited 2018 December 3]. Available from: http://www.who.int/mediacentre/factsheets/fs311/en/.
39. World Health Organization. Global status report on noncommunicable diseases. Geneva, Switzerland: World Health Organization. p. volumes.
40. NHS Digital. Statistics on Obesity, Physical Activity and Diet 2018 [updated 2018; cited 2018 December 3]. Available from: https://files.digital.nhs.uk/publication/0/0/obes-phys-acti-diet-eng-2018-rep.pdf.
41. WHO. WHO Fact Files: Ten facts on obesity. Geneva: WHO. 2010.
42. Hossain P, Kawar B, El Nahas M. Obesity and diabetes in the developing world--a growing challenge. The New England journal of medicine. 2007;356(3):213-5.
43. Guh DP, Zhang W, Bansback N, Amarsi Z, Birmingham CL, Anis AH. The incidence of co-morbidities related to obesity and overweight: a systematic review and meta-analysis. BMC public health. 2009;9:88.
44. Di Angelantonio E, Bhupathiraju Sh N, Wormser D, Gao P, Kaptoge S, Berrington de Gonzalez A, et al. Body-mass index and all-cause mortality: individual-participant-data meta-analysis of 239 prospective studies in four continents. Lancet. 2016;388(10046):776-86.

45. Misiak B, Leszek J, Kiejna A. Metabolic syndrome, mild cognitive impairment and Alzheimer's disease--the emerging role of systemic low-grade inflammation and adiposity. Brain research bulletin. 2012;89(3-4):144-9.
46. Luppino FS, de Wit LM, Bouvy PF, Stijnen T, Cuijpers P, Penninx BW, et al. Overweight, obesity, and depression: a systematic review and meta-analysis of longitudinal studies. Arch Gen Psychiatry. 2010;67(3):220-9.
47. Gariepy G, Nitka D, Schmitz N. The association between obesity and anxiety disorders in the population: a systematic review and meta-analysis. International journal of obesity. 2010;34(3):407-19.
48. Cheke LG, Bonnici HM, Clayton NS, Simons JS. Obesity and insulin resistance are associated with reduced activity in core memory regions of the brain. Neuropsychologia. 2017;96:137-49.
49. Hasler G, Pine DS, Gamma A, Milos G, Ajdacic V, Eich D, et al. The associations between psychopathology and being overweight: a 20-year prospective study. Psychol Med. 2004;34(6):1047-57.
50. Zemdegs J, Quesseveur G, Jarriault D, Penicaud L, Fioramonti X, Guiard BP. High-fat diet-induced metabolic disorders impairs 5-HT function and anxiety-like behavior in mice. Br J Pharmacol. 2016;173(13):2095-110.
51. Sivanathan S, Thavartnam K, Arif S, Elegino T, McGowan PO. Chronic high fat feeding increases anxiety-like behaviour and reduces transcript abundance of glucocorticoid signalling genes in the hippocampus of female rats. Behavioural brain research. 2015;286:265-70.
52. Andre C, Dinel AL, Ferreira G, Laye S, Castanon N. Diet-induced obesity progressively alters cognition, anxiety-like behavior and lipopolysaccharide-induced depressive-like behavior: focus on brain indoleamine 2,3-dioxygenase activation. Brain, behavior, and immunity. 2014;41:10-21.
53. Cordner ZA, Tamashiro KL. Effects of high-fat diet exposure on learning & memory. Physiology & behavior. 2015.
54. Sharma S, Fulton S. Diet-induced obesity promotes depressive-like behaviour that is associated with neural adaptations in brain reward circuitry. International journal of obesity. 2013;37(3):382-9.
55. Kurhe Y, Mahesh R, Gupta D. Effect of a selective cyclooxygenase type 2 inhibitor celecoxib on depression associated with obesity in mice: an approach using behavioral tests. Neurochem Res. 2014;39(7):1395-402.
56. Bruce-Keller AJ, Salbaum JM, Luo M, Blanchard Et, Taylor CM, Welsh DA, et al. Obese-type gut microbiota induce neurobehavioral changes in the absence of obesity. Biological psychiatry. 2015;77(7):607-15.
57. Poston L, Harthoorn LF, Van Der Beek EM, Contributors to the IEW. Obesity in pregnancy: implications for the mother and lifelong health of the child. A consensus statement. Pediatric research. 2011;69(2):175-80.
58. Heslehurst N, Rankin J, Wilkinson JR, Summerbell CD. A nationally representative study of maternal obesity in England, UK: trends in incidence and demographic inequalities in 619 323 births, 1989-2007. International journal of obesity. 2010;34(3):420-8.
59. Lindberg SM, Adams AK, Prince RJ. Early predictors of obesity and cardiovascular risk among American Indian children. Matern Child Health J. 2012;16(9):1879-86.
60. Barisione M, Carlini F, Gradaschi R, Camerini G, Adami GF. Body weight at developmental age in siblings born to mothers before and after surgically induced weight loss. Surg Obes Relat Dis. 2012;8(4):387-91.
61. Murabayashi N, Sugiyama T, Zhang L, Kamimoto Y, Umekawa T, Ma N, et al. Maternal high-fat diets cause insulin resistance through inflammatory changes in fetal adipose tissue. European journal of obstetrics, gynecology, and reproductive biology. 2013;169(1):39-44.
62. Drake AJ, Reynolds RM. Impact of maternal obesity on offspring obesity and cardiometabolic disease risk. Reproduction. 2010;140(3):387-98.

63. Dunn GA, Bale TL. Maternal high-fat diet promotes body length increases and insulin insensitivity in second-generation mice. Endocrinology. 2009;150(11):4999-5009.
64. Odaka Y, Nakano M, Tanaka T, Kaburagi T, Yoshino H, Sato-Mito N, et al. The influence of a high-fat dietary environment in the fetal period on postnatal metabolic and immune function. Obesity. 2010;18(9):1688-94.
65. Boney CM, Verma A, Tucker R, Vohr BR. Metabolic syndrome in childhood: association with birth weight, maternal obesity, and gestational diabetes mellitus. Pediatrics. 2005;115(3):e290-6.
66. Mingrone G, Manco M, Mora ME, Guidone C, Iaconelli A, Gniuli D, et al. Influence of maternal obesity on insulin sensitivity and secretion in offspring. Diabetes Care. 2008;31(9):1872-6.
67. Tsadok MA, Friedlander Y, Paltiel O, Manor O, Meiner V, Hochner H, et al. Obesity and blood pressure in 17-year-old offspring of mothers with gestational diabetes: insights from the Jerusalem Perinatal Study. Exp Diabetes Res. 2011;2011:906154.
68. Hochner H, Friedlander Y, Calderon-Margalit R, Meiner V, Sagy Y, Avgil-Tsadok M, et al. Associations of maternal prepregnancy body mass index and gestational weight gain with adult offspring cardiometabolic risk factors: the Jerusalem Perinatal Family Follow-up Study. Circulation. 2012;125(11):1381-9.
69. Reynolds RM, Allan KM, Raja EA, Bhattacharya S, McNeill G, Hannaford PC, et al. Maternal obesity during pregnancy and premature mortality from cardiovascular event in adult offspring: follow-up of 1 323 275 person years. Bmj. 2013;347:f4539.
70. Simmen FA, Simmen RC. The maternal womb: a novel target for cancer prevention in the era of the obesity pandemic? Eur J Cancer Prev. 2011;20(6):539-48.
71. Ahlgren M, Wohlfahrt J, Olsen LW, Sorensen TI, Melbye M. Birth weight and risk of cancer. Cancer. 2007;110(2):412-9.
72. Krakowiak P, Walker CK, Bremer AA, Baker AS, Ozonoff S, Hansen RL, et al. Maternal metabolic conditions and risk for autism and other neurodevelopmental disorders. Pediatrics. 2012;129(5):e1121-8.
73. Rodriguez A, Miettunen J, Henriksen TB, Olsen J, Obel C, Taanila A, et al. Maternal adiposity prior to pregnancy is associated with ADHD symptoms in offspring: evidence from three prospective pregnancy cohorts. International journal of obesity. 2008;32(3):550-7.
74. Rivera HM, Christiansen KJ, Sullivan EL. The role of maternal obesity in the risk of neuropsychiatric disorders. Front Neurosci-Switz. 2015;9.
75. Balsevich G, Baumann V, Uribe A, Chen A, Schmidt MV. Prenatal Exposure to Maternal Obesity Alters Anxiety and Stress Coping Behaviors in Aged Mice. Neuroendocrinology. 2016;103(3-4):354-68.
76. Sasaki A, de Vega WC, St-Cyr S, Pan P, McGowan PO. Perinatal high fat diet alters glucocorticoid signaling and anxiety behavior in adulthood. Neuroscience. 2013;240:1-12.
77. Peleg-Raibstein D, Luca E, Wolfrum C. Maternal high-fat diet in mice programs emotional behavior in adulthood. Behavioural brain research. 2012;233(2):398-404.
78. Kanasaki K, Koya D. Biology of obesity: lessons from animal models of obesity. J Biomed Biotechnol. 2011;2011:197636.
79. Winzell MS, Ahren B. The high-fat diet-fed mouse: a model for studying mechanisms and treatment of impaired glucose tolerance and type 2 diabetes. Diabetes. 2004;53 Suppl 3:S215-9.
80. Fraulob JC, Ogg-Diamantino R, Fernandes-Santos C, Aguila MB, Mandarim-de-Lacerda CA. A Mouse Model of Metabolic Syndrome: Insulin Resistance, Fatty Liver and Non-Alcoholic Fatty Pancreas Disease (NAFPD) in C57BL/6 Mice Fed a High Fat Diet. J Clin Biochem Nutr. 2010;46(3):212-23.
81. Zhang J, Wang C, Terroni PL, Cagampang FR, Hanson M, Byrne CD. High-unsaturated-fat, high-protein, and low-carbohydrate diet during pregnancy and lactation modulates hepatic lipid metabolism in female adult offspring. American journal of physiology Regulatory, integrative and comparative physiology. 2005;288(1):R112-8.

82. Torrens C, Ethirajan P, Bruce KD, Cagampang FR, Siow RC, Hanson MA, et al. Interaction between maternal and offspring diet to impair vascular function and oxidative balance in high fat fed male mice. PloS one. 2012;7(12):e50671.
83. Elahi MM, Cagampang FR, Mukhtar D, Anthony FW, Ohri SK, Hanson MA. Long-term maternal high-fat feeding from weaning through pregnancy and lactation predisposes offspring to hypertension, raised plasma lipids and fatty liver in mice. The British journal of nutrition. 2009;102(4):514-9.
84. Sellayah D, Sek K, Anthony FW, Watkins AJ, Osmond C, Fleming TP, et al. Appetite regulatory mechanisms and food intake in mice are sensitive to mismatch in diets between pregnancy and postnatal periods. Brain Res. 2008;1237:146-52.
85. Bruce KD, Cagampang FR, Argenton M, Zhang J, Ethirajan PL, Burdge GC, et al. Maternal high-fat feeding primes steatohepatitis in adult mice offspring, involving mitochondrial dysfunction and altered lipogenesis gene expression. Hepatology. 2009;50(6):1796-808.
86. Bailey KR CJ. Anxiety-Related Behaviors in Mice. In: JJ B, editor. Methods of Behavior Analysis in Neuroscience. 2nd edition ed. Boca Raton (FL): CRC Press/Taylor & Francis; 2009.
87. Dudchenko PA. An overview of the tasks used to test working memory in rodents. Neurosci Biobehav Rev. 2004;28(7):699-709.
88. Faa G, Manchia M, Pintus R, Gerosa C, Marcialis MA, Fanos V. Fetal programming of neuropsychiatric disorders. Birth Defects Res C Embryo Today. 2016;108(3):207-23.
89. Mason LH, Harp JP, Han DY. Pb neurotoxicity: neuropsychological effects of lead toxicity. Biomed Res Int. 2014;2014:840547.
90. Kaur Multani PaS, Nitin and Kaur, Ravneet and Sharma, Praveen and Gupta, Ramesh C. Chapter 59 - Biomarkers for drugs of abuse and neuropsychiatric disorders: Models and mechanisms. Biomarkers in Toxicology. Boston: Academic Press; 2014.
91. Dempster EL, Pidsley R, Schalkwyk LC, Owens S, Georgiades A, Kane F, et al. Disease-associated epigenetic changes in monozygotic twins discordant for schizophrenia and bipolar disorder. Hum Mol Genet. 2011;20(24):4786-96.
92. Singhal P, Naswa S, Marfatia YS. Pregnancy and sexually transmitted viral infections. Indian J Sex Transm Dis AIDS. 2009;30(2):71-8.
93. Tselis A, Booss J. Behavioral consequences of infections of the central nervous system: with emphasis on viral infections. J Am Acad Psychiatry Law. 2003;31(3):289-98.
94. Hatanaka Y, Kabuta T, Wada K. Disturbance in Maternal Environment Leads to Abnormal Synaptic Instability during Neuronal Circuitry Development. Front Neurosci. 2017;11:35.
95. Glynn LM, Sandman CA. Prenatal Origins of Neurological Development: A Critical Period for Fetus and Mother. Current Directions in Psychological Science. 2011;20(6):384-9.
96. Piedrahita JA, Oetama B, Bennett GD, van Waes J, Kamen BA, Richardson J, et al. Mice lacking the folic acid-binding protein Folbp1 are defective in early embryonic development. Nat Genet. 1999;23(2):228-32.
97. Garabedian BH, Fraser FC. A familial association between twinning and upper-neural tube defects. Am J Hum Genet. 1994;55(5):1050-3.
98. Copp AJ, Greene ND, Murdoch JN. The genetic basis of mammalian neurulation. Nat Rev Genet. 2003;4(10):784-93.
99. Detrait ER, George TM, Etchevers HC, Gilbert JR, Vekemans M, Speer MC. Human neural tube defects: developmental biology, epidemiology, and genetics. Neurotoxicol Teratol. 2005;27(3):515-24.
100. Zohn IE, Sarkar AA. Modeling neural tube defects in the mouse. Curr Top Dev Biol. 2008;84:1-35.
101. Florio M, Huttner WB. Neural progenitors, neurogenesis and the evolution of the neocortex. Development. 2014;141(11):2182-94.
102. Sur M, Rubenstein JL. Patterning and plasticity of the cerebral cortex. Science. 2005;310(5749):805-10.
103. Bystron I, Blakemore C, Rakic P. Development of the human cerebral cortex: Boulder Committee revisited. Nat Rev Neurosci. 2008;9(2):110-22.

104. Pilz D, Stoodley N, Golden JA. Neuronal migration, cerebral cortical development, and cerebral cortical anomalies. J Neuropathol Exp Neurol. 2002;61(1):1-11.
105. Burbridge S, Stewart I, Placzek M. Development of the Neuroendocrine Hypothalamus. Compr Physiol. 2016;6(2):623-43.
106. Xie Y, Dorsky RI. Development of the hypothalamus: conservation, modification and innovation. Development. 2017;144(9):1588-99.
107. Shimogori T, Lee DA, Miranda-Angulo A, Yang Y, Wang H, Jiang L, et al. A genomic atlas of mouse hypothalamic development. Nat Neurosci. 2010;13(6):767-75.
108. Bouret SG. Early life origins of obesity: role of hypothalamic programming. J Pediatr Gastroenterol Nutr. 2009;48 Suppl 1:S31-8.
109. Plagemann A, Davidowa H, Harder T, Dudenhausen JW. Developmental programming of the hypothalamus: a matter of insulin. A comment on: Horvath, T. L., Bruning, J. C.: Developmental programming of the hypothalamus: a matter of fat. Nat. Med. (2006) 12: 52-53. Neuro Endocrinol Lett. 2006;27(1-2):70-2.
110. Markakis EA. Development of the neuroendocrine hypothalamus. Front Neuroendocrinol. 2002;23(3):257-91.
111. Altman J, Bayer SA. The development of the rat hypothalamus. Adv Anat Embryol Cell Biol. 1986;100:1-178.
112. Mai JK, Lensing-Hohn S, Ende AA, Sofroniew MV. Developmental organization of neurophysin neurons in the human brain. J Comp Neurol. 1997;385(3):477-89.
113. Koutcherov Y, Mai JK, Ashwell KW, Paxinos G. Organization of human hypothalamus in fetal development. J Comp Neurol. 2002;446(4):301-24.
114. McClellan KM, Parker KL, Tobet S. Development of the ventromedial nucleus of the hypothalamus. Front Neuroendocrinol. 2006;27(2):193-209.
115. Bouret SG, Draper SJ, Simerly RB. Formation of projection pathways from the arcuate nucleus of the hypothalamus to hypothalamic regions implicated in the neural control of feeding behavior in mice. The Journal of neuroscience. 2004;24(11):2797-805.
116. Bouret SG. Nutritional programming of hypothalamic development: critical periods and windows of opportunity. Int J Obes Suppl. 2012;2(Suppl 2):S19-24.
117. Grayson BE, Levasseur PR, Williams SM, Smith MS, Marks DL, Grove KL. Changes in melanocortin expression and inflammatory pathways in fetal offspring of nonhuman primates fed a high-fat diet. Endocrinology. 2010;151(4):1622-32.
118. Biran J, Tahor M, Wircer E, Levkowitz G. Role of developmental factors in hypothalamic function. Front Neuroanat. 2015;9:47.
119. Yu YC, Bultje RS, Wang X, Shi SH. Specific synapses develop preferentially among sister excitatory neurons in the neocortex. Nature. 2009;458(7237):501-4.
120. Brunne B, Zhao S, Derouiche A, Herz J, May P, Frotscher M, et al. Origin, maturation, and astroglial transformation of secondary radial glial cells in the developing dentate gyrus. Glia. 2010;58(13):1553-69.
121. Lagali PS, Corcoran CP, Picketts DJ. Hippocampus development and function: role of epigenetic factors and implications for cognitive disease. Clin Genet. 2010;78(4):321-33.
122. Tole S, Christian C, Grove EA. Early specification and autonomous development of cortical fields in the mouse hippocampus. Development. 1997;124(24):4959-70.
123. Angevine JB, Jr. Time of neuron origin in the hippocampal region. An autoradiographic study in the mouse. Exp Neurol Suppl. 1965:Suppl 2:1-70.
124. Pleasure SJ, Anderson S, Hevner R, Bagri A, Marin O, Lowenstein DH, et al. Cell migration from the ganglionic eminences is required for the development of hippocampal GABAergic interneurons. Neuron. 2000;28(3):727-40.
125. Bayer SA, Altman J. Hippocampal development in the rat: cytogenesis and morphogenesis examined with autoradiography and low-level X-irradiation. J Comp Neurol. 1974;158(1):55-79.
126. Altman J, Bayer SA. Migration and distribution of two populations of hippocampal granule cell precursors during the perinatal and postnatal periods. J Comp Neurol. 1990;301(3):365-81.

127. Arnold SE, Trojanowski JQ. Human fetal hippocampal development: I. Cytoarchitecture, myeloarchitecture, and neuronal morphologic features. J Comp Neurol. 1996;367(2):274-92.
128. Hoeijmakers L, Lucassen PJ, Korosi A. The interplay of early-life stress, nutrition, and immune activation programs adult hippocampal structure and function. Front Mol Neurosci. 2014;7:103.
129. Smith SM, Vale WW. The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. Dialogues in clinical neuroscience. 2006;8(4):383-95.
130. Silverman MN, Sternberg EM. Glucocorticoid regulation of inflammation and its functional correlates: from HPA axis to glucocorticoid receptor dysfunction. Ann N Y Acad Sci. 2012;1261:55-63.
131. Bose M, Olivan B, Laferrere B. Stress and obesity: the role of the hypothalamic-pituitary-adrenal axis in metabolic disease. Current opinion in endocrinology, diabetes, and obesity. 2009;16(5):340-6.
132. Aisa B, Tordera R, Lasheras B, Del Rio J, Ramirez MJ. Cognitive impairment associated to HPA axis hyperactivity after maternal separation in rats. Psychoneuroendocrinology. 2007;32(3):256-66.
133. Faravelli C, Lo Sauro C, Godini L, Lelli L, Benni L, Pietrini F, et al. Childhood stressful events, HPA axis and anxiety disorders. World journal of psychiatry. 2012;2(1):13-25.
134. Nader N, Chrousos GP, Kino T. Interactions of the circadian CLOCK system and the HPA axis. Trends in endocrinology and metabolism: TEM. 2010;21(5):277-86.
135. Kirschbaum C, Pirke KM, Hellhammer DH. The 'Trier Social Stress Test'--a tool for investigating psychobiological stress responses in a laboratory setting. Neuropsychobiology. 1993;28(1-2):76-81.
136. Wei Q, Hebda-Bauer EK, Pletsch A, Luo J, Hoversten MT, Osetek AJ, et al. Overexpressing the glucocorticoid receptor in forebrain causes an aging-like neuroendocrine phenotype and mild cognitive dysfunction. The Journal of neuroscience. 2007;27(33):8836-44.
137. Ditkoff EC, Fruzzetti F, Chang L, Stancyzk FZ, Lobo RA. The impact of estrogen on adrenal androgen sensitivity and secretion in polycystic ovary syndrome. J Clin Endocrinol Metab. 1995;80(2):603-7.
138. Vamvakopoulos NC, Chrousos GP. Evidence of direct estrogenic regulation of human corticotropin-releasing hormone gene expression. Potential implications for the sexual dimophism of the stress response and immune/inflammatory reaction. The Journal of clinical investigation. 1993;92(4):1896-902.
139. Young EA. The role of gonadal steroids in hypothalamic-pituitary-adrenal axis regulation. Crit Rev Neurobiol. 1995;9(4):371-81.
140. Goel N, Workman JL, Lee TT, Innala L, Viau V. Sex differences in the HPA axis. Compr Physiol. 2014;4(3):1121-55.
141. Oakley RH, Cidlowski JA. The biology of the glucocorticoid receptor: new signaling mechanisms in health and disease. The Journal of allergy and clinical immunology. 2013;132(5):1033-44.
142. Reul JM, de Kloet ER. Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology. 1985;117(6):2505-11.
143. Amsterdam A, Tajima K, Sasson R. Cell-specific regulation of apoptosis by glucocorticoids: implication to their anti-inflammatory action. Biochem Pharmacol. 2002;64(5-6):843-50.
144. Jacobson L, Sapolsky R. The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. Endocr Rev. 1991;12(2):118-34.
145. De Kloet ER, Vreugdenhil E, Oitzl MS, Joels M. Brain corticosteroid receptor balance in health and disease. Endocr Rev. 1998;19(3):269-301.
146. Laryea G, Schutz G, Muglia LJ. Disrupting hypothalamic glucocorticoid receptors causes HPA axis hyperactivity and excess adiposity. Molecular endocrinology. 2013;27(10):1655-65.

147. Solomon MB, Loftspring M, de Kloet AD, Ghosal S, Jankord R, Flak JN, et al. Neuroendocrine Function After Hypothalamic Depletion of Glucocorticoid Receptors in Male and Female Mice. Endocrinology. 2015;156(8):2843-53.
148. Gass P, Reichardt HM, Strekalova T, Henn F, Tronche F. Mice with targeted mutations of glucocorticoid and mineralocorticoid receptors: models for depression and anxiety? Physiology & behavior. 2001;73(5):811-25.
149. Gass P, Kretz O, Wolfer DP, Berger S, Tronche F, Reichardt HM, et al. Genetic disruption of mineralocorticoid receptor leads to impaired neurogenesis and granule cell degeneration in the hippocampus of adult mice. EMBO Rep. 2000;1(5):447-51.
150. Rozeboom AM, Akil H, Seasholtz AF. Mineralocorticoid receptor overexpression in forebrain decreases anxiety-like behavior and alters the stress response in mice. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(11):4688-93.
151. Lai M, Horsburgh K, Bae SE, Carter RN, Stenvers DJ, Fowler JH, et al. Forebrain mineralocorticoid receptor overexpression enhances memory, reduces anxiety and attenuates neuronal loss in cerebral ischaemia. Eur J Neurosci. 2007;25(6):1832-42.
152. Phillipov G, Palermo M, Shackleton CH. Apparent cortisone reductase deficiency: a unique form of hypercortisolism. J Clin Endocrinol Metab. 1996;81(11):3855-60.
153. Edgar RS, Green EW, Zhao Y, van Ooijen G, Olmedo M, Qin X, et al. Peroxiredoxins are conserved markers of circadian rhythms. Nature. 2012;485(7399):459-64.
154. Chung S, Son GH, Kim K. Circadian rhythm of adrenal glucocorticoid: its regulation and clinical implications. Biochim Biophys Acta. 2011;1812(5):581-91.
155. Ivars K, Nelson N, Theodorsson A, Theodorsson E, Strom JO, Morelius E. Development of salivary cortisol circadian rhythm in preterm infants. PloS one. 2017;12(8):e0182685.
156. Wang M. The role of glucocorticoid action in the pathophysiology of the Metabolic Syndrome. Nutr Metab (Lond). 2005;2(1):3.
157. Joseph JJ, Golden SH. Cortisol dysregulation: the bidirectional link between stress, depression, and type 2 diabetes mellitus. Ann N Y Acad Sci. 2017;1391(1):20-34.
158. Rubinow DR, Post RM, Savard R, Gold PW. Cortisol hypersecretion and cognitive impairment in depression. Arch Gen Psychiatry. 1984;41(3):279-83.
159. Carroll T, Raff H, Findling JW. Late-night salivary cortisol measurement in the diagnosis of Cushing's syndrome. Nat Clin Pract Endocrinol Metab. 2008;4(6):344-50.
160. Crown A, Lightman S. Management of patients with glucocorticoid deficiency. Nat Clin Pract Endocrinol Metab. 2005;1(2):62-3.
161. Rosmond R, Holm G, Bjorntorp P. Food-induced cortisol secretion in relation to anthropometric, metabolic and haemodynamic variables in men. Int J Obes Relat Metab Disord. 2000;24(4):416-22.
162. Jung CM, Khalsa SB, Scheer FA, Cajochen C, Lockley SW, Czeisler CA, et al. Acute effects of bright light exposure on cortisol levels. J Biol Rhythms. 2010;25(3):208-16.
163. Raul JS, Cirimele V, Ludes B, Kintz P. Detection of physiological concentrations of cortisol and cortisone in human hair. Clin Biochem. 2004;37(12):1105-11.
164. Wust S, Wolf J, Hellhammer DH, Federenko I, Schommer N, Kirschbaum C. The cortisol awakening response - normal values and confounds. Noise Health. 2000;2(7):79-88.
165. Stetler C, Miller GE. Blunted cortisol response to awakening in mild to moderate depression: regulatory influences of sleep patterns and social contacts. J Abnorm Psychol. 2005;114(4):697-705.
166. Edwards S, Hucklebridge F, Clow A, Evans P. Components of the diurnal cortisol cycle in relation to upper respiratory symptoms and perceived stress. Psychosom Med. 2003;65(2):320-7.
167. Herman JP, Figueiredo H, Mueller NK, Ulrich-Lai Y, Ostrander MM, Choi DC, et al. Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. Front Neuroendocrinol. 2003;24(3):151-80.
168. Fendler K, Karmos G, Telegdy G. The effect of hippocampal lesion on pituitary-adrenal function. Acta Physiol Acad Sci Hung. 1961;20:293-7.

169. Herman JP. Regulation of adrenocorticosteroid receptor mRNA expression in the central nervous system. Cell Mol Neurobiol. 1993;13(4):349-72.
170. Furay AR, Bruestle AE, Herman JP. The role of the forebrain glucocorticoid receptor in acute and chronic stress. Endocrinology. 2008;149(11):5482-90.
171. Smythe JW, Murphy D, Timothy C, Costall B. Hippocampal mineralocorticoid, but not glucocorticoid, receptors modulate anxiety-like behavior in rats. Pharmacology, biochemistry, and behavior. 1997;56(3):507-13.
172. Fanselow MS, Dong HW. Are the dorsal and ventral hippocampus functionally distinct structures? Neuron. 2010;65(1):7-19.
173. Cullinan WE, Herman JP, Watson SJ. Ventral subicular interaction with the hypothalamic paraventricular nucleus: evidence for a relay in the bed nucleus of the stria terminalis. J Comp Neurol. 1993;332(1):1-20.
174. Herman JP, Dolgas CM, Carlson SL. Ventral subiculum regulates hypothalamo-pituitary-adrenocortical and behavioural responses to cognitive stressors. Neuroscience. 1998;86(2):449-59.
175. Sapolsky RM, Krey LC, McEwen BS. The neuroendocrinology of stress and aging: the glucocorticoid cascade hypothesis. Endocr Rev. 1986;7(3):284-301.
176. Chen Y, Brunson KL, Adelmann G, Bender RA, Frotscher M, Baram TZ. Hippocampal corticotropin releasing hormone: pre- and postsynaptic location and release by stress. Neuroscience. 2004;126(3):533-40.
177. Row BW, Dohanich GP. Post-training administration of corticotropin-releasing hormone (CRH) enhances retention of a spatial memory through a noradrenergic mechanism in male rats. Neurobiol Learn Mem. 2008;89(4):370-8.
178. Ma YL, Chen KY, Wei CL, Lee EH. Corticotropin-releasing factor enhances brain-derived neurotrophic factor gene expression to facilitate memory retention in rats. Chin J Physiol. 1999;42(2):73-81.
179. Blank T, Nijholt I, Eckart K, Spiess J. Priming of long-term potentiation in mouse hippocampus by corticotropin-releasing factor and acute stress: implications for hippocampus-dependent learning. The Journal of neuroscience. 2002;22(9):3788-94.
180. Wang XD, Rammes G, Kraev I, Wolf M, Liebl C, Scharf SH, et al. Forebrain CRF(1) modulates early-life stress-programmed cognitive deficits. The Journal of neuroscience. 2011;31(38):13625-34.
181. Heinrichs SC, Stenzel-Poore MP, Gold LH, Battenberg E, Bloom FE, Koob GF, et al. Learning impairment in transgenic mice with central overexpression of corticotropin-releasing factor. Neuroscience. 1996;74(2):303-11.
182. Ivy AS, Rex CS, Chen Y, Dube C, Maras PM, Grigoriadis DE, et al. Hippocampal dysfunction and cognitive impairments provoked by chronic early-life stress involve excessive activation of CRH receptors. The Journal of neuroscience. 2010;30(39):13005-15.
183. Wang XD, Chen Y, Wolf M, Wagner KV, Liebl C, Scharf SH, et al. Forebrain CRHR1 deficiency attenuates chronic stress-induced cognitive deficits and dendritic remodeling. Neurobiol Dis. 2011;42(3):300-10.
184. Chen Y, Bender RA, Brunson KL, Pomper JK, Grigoriadis DE, Wurst W, et al. Modulation of dendritic differentiation by corticotropin-releasing factor in the developing hippocampus. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(44):15782-7.
185. Rose J, Schwartz J, Green J, Kerr D. Development of the corticotropin-releasing factor adrenocorticotropic hormone/beta-endorphin system in the mammalian fetus. Fetal and Neonatal Physiology WB Saunders: Philadelphia, USA. 1998:2431-42.
186. McNutt NS, Jones AL. Observations on the ultrastructure of cytodifferentiation in the human fetal adrenal cortex. Lab Invest. 1970;22(6):513-27.
187. Campbell AL, Murphy BE. The maternal-fetal cortisol gradient during pregnancy and at delivery. J Clin Endocrinol Metab. 1977;45(3):435-40.
188. Mesiano S, Jaffe RB. Developmental and functional biology of the primate fetal adrenal cortex. Endocr Rev. 1997;18(3):378-403.

189. Liggins GC. The role of the hypothalamic-pituitary-adrenal axis in preparing the fetus for birth. Am J Obstet Gynecol. 2000;182(2):475-7.
190. Sandman CA, Wadhwa PD, Chicz-DeMet A, Dunkel-Schetter C, Porto M. Maternal stress, HPA activity, and fetal/infant outcome. Ann N Y Acad Sci. 1997;814:266-75.
191. Kapoor A, Dunn E, Kostaki A, Andrews MH, Matthews SG. Fetal programming of hypothalamo-pituitary-adrenal function: prenatal stress and glucocorticoids. J Physiol. 2006;572(Pt 1):31-44.
192. Sapolsky RM, Meaney MJ. Maturation of the adrenocortical stress response: neuroendocrine control mechanisms and the stress hyporesponsive period. Brain Res. 1986;396(1):64-76.
193. Diaz R, Brown RW, Seckl JR. Distinct ontogeny of glucocorticoid and mineralocorticoid receptor and 11beta-hydroxysteroid dehydrogenase types I and II mRNAs in the fetal rat brain suggest a complex control of glucocorticoid actions. The Journal of neuroscience. 1998;18(7):2570-80.
194. Costa A, Rocci MP, Arisio R, Benedetto C, Fabris C, Bertino E, et al. Glucocorticoid receptors immunoreactivity in tissue of human embryos. J Endocrinol Invest. 1996;19(2):92-8.
195. Condon J, Gosden C, Gardener D, Nickson P, Hewison M, Howie AJ, et al. Expression of type 2 11beta-hydroxysteroid dehydrogenase and corticosteroid hormone receptors in early human fetal life. J Clin Endocrinol Metab. 1998;83(12):4490-7.
196. Noorlander CW, De Graan PN, Middeldorp J, Van Beers JJ, Visser GH. Ontogeny of hippocampal corticosteroid receptors: effects of antenatal glucocorticoids in human and mouse. J Comp Neurol. 2006;499(6):924-32.
197. Graeff FG. [Anxiety, panic and the hypothalamic-pituitary-adrenal axis]. Revista brasileira de psiquiatria. 2007;29 Suppl 1:S3-6.
198. Kallen VL, Tulen JHM, Utens EMWJ, Treffers PDA, De Jong FH, Ferdinand RE. Associations between HPA axis functioning and level of anxiety in children and adolescents with an anxiety disorder. Depression and anxiety. 2008;25(2):131-41.
199. Vreeburg SA, Zitman FG, van Pelt J, Derijk RH, Verhagen JC, van Dyck R, et al. Salivary cortisol levels in persons with and without different anxiety disorders. Psychosom Med. 2010;72(4):340-7.
200. Greaves-Lord K, Ferdinand RF, Oldehinkel AJ, Sondeijker FE, Ormel J, Verhulst FC. Higher cortisol awakening response in young adolescents with persistent anxiety problems. Acta psychiatrica Scandinavica. 2007;116(2):137-44.
201. Kallen VL, Tulen JH, Utens EM, Treffers PD, De Jong FH, Ferdinand RF. Associations between HPA axis functioning and level of anxiety in children and adolescents with an anxiety disorder. Depression and anxiety. 2008;25(2):131-41.
202. Sotnikov S, Wittmann A, Bunck M, Bauer S, Deussing J, Schmidt M, et al. Blunted HPA axis reactivity reveals glucocorticoid system dysbalance in a mouse model of high anxiety-related behavior. Psychoneuroendocrinology. 2014;48:41-51.
203. Soravia LM, Heinrichs M, Aerni A, Maroni C, Schelling G, Ehlert U, et al. Glucocorticoids reduce phobic fear in humans. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(14):5585-90.
204. Abelson JL, Curtis GC. Hypothalamic-pituitary-adrenal axis activity in panic disorder. 24-hour secretion of corticotropin and cortisol. Arch Gen Psychiatry. 1996;53(4):323-31.
205. Erhardt A, Ising M, Unschuld PG, Kern N, Lucae S, Putz B, et al. Regulation of the hypothalamic-pituitary-adrenocortical system in patients with panic disorder. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. 2006;31(11):2515-22.
206. Lenze EJ, Mantella RC, Shi P, Goate AM, Nowotny P, Butters MA, et al. Elevated cortisol in older adults with generalized anxiety disorder is reduced by treatment: a placebo-controlled evaluation of escitalopram. Am J Geriatr Psychiatry. 2011;19(5):482-90.
207. Condren RM, O'Neill A, Ryan MC, Barrett P, Thakore JH. HPA axis response to a psychological stressor in generalised social phobia. Psychoneuroendocrinology. 2002;27(6):693-703.

208. Roelofs K, van Peer J, Berretty E, Jong P, Spinhoven P, Elzinga BM. Hypothalamus-pituitary-adrenal axis hyperresponsiveness is associated with increased social avoidance behavior in social phobia. Biological psychiatry. 2009;65(4):336-43.
209. Sapolsky RM. Do glucocorticoid concentrations rise with age in the rat? Neurobiology of Aging. 1992;13(1):171-4.
210. Martin SA, Jameson CH, Allan SM, Lawrence CB. Maternal high-fat diet worsens memory deficits in the triple-transgenic (3xTgAD) mouse model of Alzheimer's disease. PloS one. 2014;9(6):e99226.
211. Page KC, Jones EK, Anday EK. Maternal and postweaning high-fat diets disturb hippocampal gene expression, learning, and memory function. American journal of physiology Regulatory, integrative and comparative physiology. 2014;306(8):R527-37.
212. Moreira PS, Almeida PR, Leite-Almeida H, Sousa N, Costa P. Impact of Chronic Stress Protocols in Learning and Memory in Rodents: Systematic Review and Meta-Analysis. PloS one. 2016;11(9):e0163245.
213. Conrad CD, Galea LA, Kuroda Y, McEwen BS. Chronic stress impairs rat spatial memory on the Y maze, and this effect is blocked by tianeptine pretreatment. Behav Neurosci. 1996;110(6):1321-34.
214. Luine V, Villegas M, Martinez C, McEwen BS. Repeated stress causes reversible impairments of spatial memory performance. Brain Res. 1994;639(1):167-70.
215. Bodnoff SR, Humphreys AG, Lehman JC, Diamond DM, Rose GM, Meaney MJ. Enduring effects of chronic corticosterone treatment on spatial learning, synaptic plasticity, and hippocampal neuropathology in young and mid-aged rats. J Neurosci. 1995;15(1 Pt 1):61-9.
216. Elizalde N, Gil-Bea FJ, Ramirez MJ, Aisa B, Lasheras B, Del Rio J, et al. Long-lasting behavioral effects and recognition memory deficit induced by chronic mild stress in mice: effect of antidepressant treatment. Psychopharmacology (Berl). 2008;199(1):1-14.
217. Solas M, Aisa B, Tordera RM, Mugueta MC, Ramirez MJ. Stress contributes to the development of central insulin resistance during aging: implications for Alzheimer's disease. Biochim Biophys Acta. 2013;1832(12):2332-9.
218. Luine VN, Spencer RL, McEwen BS. Effects of chronic corticosterone ingestion on spatial memory performance and hippocampal serotonergic function. Brain Res. 1993;616(1-2):65-70.
219. de Quervain DJ, Roozendaal B, McGaugh JL. Stress and glucocorticoids impair retrieval of long-term spatial memory. Nature. 1998;394(6695):787-90.
220. Kirschbaum C, Wolf OT, May M, Wippich W, Hellhammer DH. Stress- and treatment-induced elevations of cortisol levels associated with impaired declarative memory in healthy adults. Life sciences. 1996;58(17):1475-83.
221. Newcomer JW, Craft S, Hershey T, Askins K, Bardgett ME. Glucocorticoid-induced impairment in declarative memory performance in adult humans. The Journal of neuroscience. 1994;14(4):2047-53.
222. Lupien SJ, Gaudreau S, Tchiteya BM, Maheu F, Sharma S, Nair NP, et al. Stress-induced declarative memory impairment in healthy elderly subjects: relationship to cortisol reactivity. J Clin Endocrinol Metab. 1997;82(7):2070-5.
223. Brunson KL, Eghbal-Ahmadi M, Bender R, Chen Y, Baram TZ. Long-term, progressive hippocampal cell loss and dysfunction induced by early-life administration of corticotropin-releasing hormone reproduce the effects of early-life stress. Proceedings of the National Academy of Sciences of the United States of America. 2001;98(15):8856-61.
224. Wingenfeld K, Wolf OT. HPA axis alterations in mental disorders: impact on memory and its relevance for therapeutic interventions. CNS neuroscience & therapeutics. 2011;17(6):714-22.
225. Sterner EY, Kalynchuk LE. Behavioral and neurobiological consequences of prolonged glucocorticoid exposure in rats: relevance to depression. Prog Neuropsychopharmacol Biol Psychiatry. 2010;34(5):777-90.
226. Conrad CD. What is the functional significance of chronic stress-induced CA3 dendritic retraction within the hippocampus? Behav Cogn Neurosci Rev. 2006;5(1):41-60.

227. Herman JP, Adams D, Prewitt C. Regulatory changes in neuroendocrine stress-integrative circuitry produced by a variable stress paradigm. Neuroendocrinology. 1995;61(2):180-90.
228. Paskitti ME, McCreary BJ, Herman JP. Stress regulation of adrenocorticosteroid receptor gene transcription and mRNA expression in rat hippocampus: time-course analysis. Brain Res Mol Brain Res. 2000;80(2):142-52.
229. Finsterwald C, Alberini CM. Stress and glucocorticoid receptor-dependent mechanisms in long-term memory: from adaptive responses to psychopathologies. Neurobiol Learn Mem. 2014;112:17-29.
230. Kanatsou S, Fearey BC, Kuil LE, Lucassen PJ, Harris AP, Seckl JR, et al. Overexpression of Mineralocorticoid Receptors Partially Prevents Chronic Stress-Induced Reductions in Hippocampal Memory and Structural Plasticity. PloS one. 2015;10(11):e0142012.
231. Burton C, Cottrell E, Edwards J. Addison's disease: identification and management in primary care. Br J Gen Pract. 2015;65(638):488-90.
232. Eller-Vainicher C, Morelli V, Salcuni AS, Torlontano M, Coletti F, Iorio L, et al. Post-surgical hypocortisolism after removal of an adrenal incidentaloma: is it predictable by an accurate endocrinological work-up before surgery? Eur J Endocrinol. 2010;162(1):91-9.
233. Tokuyama K, Himms-Hagen J. Adrenalectomy prevents obesity in glutamate-treated mice. Am J Physiol. 1989;257(2 Pt 1):E139-44.
234. Rebuffe-Scrive M, Krotkiewski M, Elfverson J, Bjorntorp P. Muscle and adipose tissue morphology and metabolism in Cushing's syndrome. J Clin Endocrinol Metab. 1988;67(6):1122-8.
235. Tataranni PA, Larson DE, Snitker S, Young JB, Flatt JP, Ravussin E. Effects of glucocorticoids on energy metabolism and food intake in humans. Am J Physiol. 1996;271(2 Pt 1):E317-25.
236. George SA, Khan S, Briggs H, Abelson JL. CRH-stimulated cortisol release and food intake in healthy, non-obese adults. Psychoneuroendocrinology. 2010;35(4):607-12.
237. De Vriendt T, Moreno LA, De Henauw S. Chronic stress and obesity in adolescents: scientific evidence and methodological issues for epidemiological research. Nutrition, metabolism, and cardiovascular diseases : NMCD. 2009;19(7):511-9.
238. Incollingo Rodriguez AC, Epel ES, White ML, Standen EC, Seckl JR, Tomiyama AJ. Hypothalamic-pituitary-adrenal axis dysregulation and cortisol activity in obesity: A systematic review. Psychoneuroendocrinology. 2015;62:301-18.
239. Marin P, Darin N, Amemiya T, Andersson B, Jern S, Bjorntorp P. Cortisol secretion in relation to body fat distribution in obese premenopausal women. Metabolism: clinical and experimental. 1992;41(8):882-6.
240. Rosmond R, Dallman MF, Bjorntorp P. Stress-related cortisol secretion in men: relationships with abdominal obesity and endocrine, metabolic and hemodynamic abnormalities. The Journal of clinical endocrinology and metabolism. 1998;83(6):1853-9.
241. Epel ES, McEwen B, Seeman T, Matthews K, Castellazzo G, Brownell KD, et al. Stress and body shape: stress-induced cortisol secretion is consistently greater among women with central fat. Psychosomatic medicine. 2000;62(5):623-32.
242. Levin BE, Richard D, Michel C, Servatius R. Differential stress responsivity in diet-induced obese and resistant rats. American journal of physiology Regulatory, integrative and comparative physiology. 2000;279(4):R1357-64.
243. Bjorntorp P, Rosmond R. Obesity and cortisol. Nutrition. 2000;16(10):924-36.
244. Stewart PM, Boulton A, Kumar S, Clark PM, Shackleton CH. Cortisol metabolism in human obesity: impaired cortisone-->cortisol conversion in subjects with central adiposity. J Clin Endocrinol Metab. 1999;84(3):1022-7.
245. Rask E, Olsson T, Soderberg S, Andrew R, Livingstone DE, Johnson O, et al. Tissue-specific dysregulation of cortisol metabolism in human obesity. J Clin Endocrinol Metab. 2001;86(3):1418-21.
246. Vicennati V, Pasquali R. Abnormalities of the hypothalamic-pituitary-adrenal axis in nondepressed women with abdominal obesity and relations with insulin resistance: evidence for a central and a peripheral alteration. J Clin Endocrinol Metab. 2000;85(11):4093-8.

247. Champaneri S, Xu X, Carnethon MR, Bertoni AG, Seeman T, DeSantis AS, et al. Diurnal salivary cortisol is associated with body mass index and waist circumference: the Multiethnic Study of Atherosclerosis. Obesity. 2013;21(1):E56-63.
248. Stalder T, Steudte S, Alexander N, Miller R, Gao W, Dettenborn L, et al. Cortisol in hair, body mass index and stress-related measures. Biological psychology. 2012;90(3):218-23.
249. Abraham SB, Rubino D, Sinaii N, Ramsey S, Nieman LK. Cortisol, obesity, and the metabolic syndrome: a cross-sectional study of obese subjects and review of the literature. Obesity. 2013;21(1):E105-17.
250. Rosmond R, Bjorntorp P. The hypothalamic-pituitary-adrenal axis activity as a predictor of cardiovascular disease, type 2 diabetes and stroke. J Intern Med. 2000;247(2):188-97.
251. Ursache A, Wedin W, Tirsi A, Convit A. Preliminary evidence for obesity and elevations in fasting insulin mediating associations between cortisol awakening response and hippocampal volumes and frontal atrophy. Psychoneuroendocrinology. 2012;37(8):1270-6.
252. Duclos M, Marquez Pereira P, Barat P, Gatta B, Roger P. Increased cortisol bioavailability, abdominal obesity, and the metabolic syndrome in obese women. Obes Res. 2005;13(7):1157-66.
253. Wallerius S, Rosmond R, Ljung T, Holm G, Bjorntorp P. Rise in morning saliva cortisol is associated with abdominal obesity in men: a preliminary report. J Endocrinol Invest. 2003;26(7):616-9.
254. Therrien F, Drapeau V, Lalonde J, Lupien SJ, Beaulieu S, Tremblay A, et al. Awakening cortisol response in lean, obese, and reduced obese individuals: effect of gender and fat distribution. Obesity. 2007;15(2):377-85.
255. Kumari M, Chandola T, Brunner E, Kivimaki M. A nonlinear relationship of generalized and central obesity with diurnal cortisol secretion in the Whitehall II study. J Clin Endocrinol Metab. 2010;95(9):4415-23.
256. Auvinen HE, Romijn JA, Biermasz NR, Havekes LM, Smit JW, Rensen PC, et al. Effects of high fat diet on the Basal activity of the hypothalamus-pituitary-adrenal axis in mice: a systematic review. Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme. 2011;43(13):899-906.
257. Rask E, Walker BR, Soderberg S, Livingstone DE, Eliasson M, Johnson O, et al. Tissue-specific changes in peripheral cortisol metabolism in obese women: increased adipose 11beta-hydroxysteroid dehydrogenase type 1 activity. The Journal of clinical endocrinology and metabolism. 2002;87(7):3330-6.
258. Livingstone DE, Jones GC, Smith K, Jamieson PM, Andrew R, Kenyon CJ, et al. Understanding the role of glucocorticoids in obesity: tissue-specific alterations of corticosterone metabolism in obese Zucker rats. Endocrinology. 2000;141(2):560-3.
259. Abildgaard A, Lund S, Hougaard KS. Chronic high-fat diet increases acute neuroendocrine stress response independently of prenatal dexamethasone treatment in male rats. Acta Neuropsychiatr. 2014;26(1):8-18.
260. Tannenbaum BM, Brindley DN, Tannenbaum GS, Dallman MF, McArthur MD, Meaney MJ. High-fat feeding alters both basal and stress-induced hypothalamic-pituitary-adrenal activity in the rat. Am J Physiol. 1997;273(6 Pt 1):E1168-77.
261. Kamara K, Eskay R, Castonguay T. High-fat diets and stress responsivity. Physiology & behavior. 1998;64(1):1-6.
262. Bellisario V, Panetta P, Balsevich G, Baumann V, Noble J, Raggi C, et al. Maternal high-fat diet acts as a stressor increasing maternal glucocorticoids' signaling to the fetus and disrupting maternal behavior and brain activation in C57BL/6J mice. Psychoneuroendocrinology. 2015;60:138-50.
263. Jessop DS, Dallman MF, Fleming D, Lightman SL. Resistance to glucocorticoid feedback in obesity. J Clin Endocrinol Metab. 2001;86(9):4109-14.
264. Heyward FD, Walton RG, Carle MS, Coleman MA, Garvey WT, Sweatt JD. Adult mice maintained on a high-fat diet exhibit object location memory deficits and reduced hippocampal SIRT1 gene expression. Neurobiol Learn Mem. 2012;98(1):25-32.

265. McNeilly AD, Stewart CA, Sutherland C, Balfour DJ. High fat feeding is associated with stimulation of the hypothalamic-pituitary-adrenal axis and reduced anxiety in the rat. Psychoneuroendocrinology. 2015;52:272-80.
266. Underwood EL, Thompson LT. A High-Fat Diet Causes Impairment in Hippocampal Memory and Sex-Dependent Alterations in Peripheral Metabolism. Neural Plast. 2016;2016:7385314.
267. Ronsaville DS, Municchi G, Laney C, Cizza G, Meyer SE, Haim A, et al. Maternal and environmental factors influence the hypothalamic-pituitary-adrenal axis response to corticotropin-releasing hormone infusion in offspring of mothers with or without mood disorders. Development and psychopathology. 2006;18(1):173-94.
268. O'Connor TG, Ben-Shlomo Y, Heron J, Golding J, Adams D, Glover V. Prenatal anxiety predicts individual differences in cortisol in pre-adolescent children. Biological psychiatry. 2005;58(3):211-7.
269. Van den Bergh BR, Van Calster B, Smits T, Van Huffel S, Lagae L. Antenatal maternal anxiety is related to HPA-axis dysregulation and self-reported depressive symptoms in adolescence: a prospective study on the fetal origins of depressed mood. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. 2008;33(3):536-45.
270. Brunton PJ. Effects of maternal exposure to social stress during pregnancy: consequences for mother and offspring. Reproduction. 2013;146(5):R175-89.
271. Mina TH, Lahti M, Drake AJ, Forbes S, Denison FC, Raikkonen K, et al. Maternal lipids in pregnancy are associated with increased offspring cortisol reactivity in childhood. Psychoneuroendocrinology. 2017;83:79-83.
272. Mina TH, Lahti M, Drake AJ, Raikkonen K, Minnis H, Denison FC, et al. Prenatal exposure to very severe maternal obesity is associated with adverse neuropsychiatric outcomes in children. Psychol Med. 2017;47(2):353-62.
273. Long NM, Nathanielsz PW, Ford SP. The impact of maternal overnutrition and obesity on hypothalamic-pituitary-adrenal axis response of offspring to stress. Domestic animal endocrinology. 2012;42(4):195-202.
274. Trottier G, Koski KG, Brun T, Toufexis DJ, Richard D, Walker CD. Increased fat intake during lactation modifies hypothalamic-pituitary-adrenal responsiveness in developing rat pups: a possible role for leptin. Endocrinology. 1998;139(9):3704-11.
275. Chin EH, Schmidt KL, Martel KM, Wong CK, Hamden JE, Gibson WT, et al. A maternal high-fat, high-sucrose diet has sex-specific effects on fetal glucocorticoids with little consequence for offspring metabolism and voluntary locomotor activity in mice. PloS one. 2017;12(3):e0174030.
276. Niculescu MD, Lupu DS. High fat diet-induced maternal obesity alters fetal hippocampal development. Int J Dev Neurosci. 2009;27(7):627-33.
277. Bilbo SD, Tsang V. Enduring consequences of maternal obesity for brain inflammation and behavior of offspring. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2010;24(6):2104-15.
278. White CL, Pistell PJ, Purpera MN, Gupta S, Fernandez-Kim SO, Hise TL, et al. Effects of high fat diet on Morris maze performance, oxidative stress, and inflammation in rats: contributions of maternal diet. Neurobiol Dis. 2009;35(1):3-13.
279. Winther G, Elfving B, Muller HK, Lund S, Wegener G. Maternal High-fat Diet Programs Offspring Emotional Behavior in Adulthood. Neuroscience. 2018;388:87-101.
280. Steinberg L. Reciprocal relation between parent child distance and pubertal maturation. Developmental Psychology. 1988;24(1):122-8.
281. Lissau I, Sorensen TI. Parental neglect during childhood and increased risk of obesity in young adulthood. Lancet. 1994;343(8893):324-7.
282. Russek LG, Schwartz GE. Feelings of parental caring predict health status in midlife: a 35-year follow-up of the Harvard Mastery of Stress Study. J Behav Med. 1997;20(1):1-13.

283. Kawai M, Minabe Y, Takagai S, Ogai M, Matsumoto H, Mori N, et al. Poor maternal care and high maternal body mass index in pregnancy as a risk factor for schizophrenia in offspring. Acta Psychiatr Scand. 2004;110(4):257-63.
284. Zhao G, Ford ES, Dhingra S, Li C, Strine TW, Mokdad AH. Depression and anxiety among US adults: associations with body mass index. International journal of obesity. 2009;33(2):257-66.
285. Okubo H, Miyake Y, Sasaki S, Tanaka K, Murakami K, Hirota Y, et al. Dietary patterns during pregnancy and the risk of postpartum depression in Japan: the Osaka Maternal and Child Health Study. The British journal of nutrition. 2011;105(8):1251-7.
286. Molyneaux E, Poston L, Ashurst-Williams S, Howard LM. Obesity and mental disorders during pregnancy and postpartum: a systematic review and meta-analysis. Obstetrics and gynecology. 2014;123(4):857-67.
287. Aubuchon-Endsley NL, Bublitz MH, Stroud LR. Pre-pregnancy obesity and maternal circadian cortisol regulation: Moderation by gestational weight gain. Biological psychology. 2014;102:38-43.
288. Liu D, Diorio J, Tannenbaum B, Caldji C, Francis D, Freedman A, et al. Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. Science. 1997;277(5332):1659-62.
289. Connor KL, Vickers MH, Beltrand J, Meaney MJ, Sloboda DM. Nature, nurture or nutrition? Impact of maternal nutrition on maternal care, offspring development and reproductive function. J Physiol. 2012;590(9):2167-80.
290. Baptissart M, Lamb HE, To K, Bradish C, Tehrani J, Reif D, et al. Neonatal mice exposed to a high-fat diet in utero influence the behaviour of their nursing dam. Proceedings of the Royal Society B: Biological Sciences. 2018;285(1891).
291. Rankin JA. Biological mediators of acute inflammation. AACN clinical issues. 2004;15(1):3-17.
292. Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: when the immune system subjugates the brain. Nature reviews Neuroscience. 2008;9(1):46-56.
293. Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. Nature. 2008;454(7203):445-54.
294. Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002;420(6917):860-7.
295. Osiecki H. The role of chronic inflammation in cardiovascular disease and its regulation by nutrients. Alternative medicine review : a journal of clinical therapeutic. 2004;9(1):32-53.
296. Pekny M, Nilsson M. Astrocyte activation and reactive gliosis. Glia. 2005;50(4):427-34.
297. Besedovsky HO, del Rey A. Immune-neuro-endocrine interactions: facts and hypotheses. Endocr Rev. 1996;17(1):64-102.
298. Glezer I, Rivest S. Glucocorticoids: protectors of the brain during innate immune responses. Neuroscientist. 2004;10(6):538-52.
299. Dunn AJ. Cytokine activation of the HPA axis. Ann N Y Acad Sci. 2000;917:608-17.
300. Beck IM, Vanden Berghe W, Vermeulen L, Yamamoto KR, Haegeman G, De Bosscher K. Crosstalk in inflammation: the interplay of glucocorticoid receptor-based mechanisms and kinases and phosphatases. Endocr Rev. 2009;30(7):830-82.
301. Forsythe LK, Wallace JM, Livingstone MB. Obesity and inflammation: the effects of weight loss. Nutr Res Rev. 2008;21(2):117-33.
302. Greenberg AS, Obin MS. Obesity and the role of adipose tissue in inflammation and metabolism. Am J Clin Nutr. 2006;83(2):461S-5S.
303. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science. 1993;259(5091):87-91.
304. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. The Journal of clinical investigation. 1995;95(5):2409-15.

305. Gnacinska M, Malgorzewicz S, Lysiak-Szydlowska W, Sworczak K. The serum profile of adipokines in overweight patients with metabolic syndrome. Endokrynologia Polska. 2010;61(1):36-41.
306. Hotamisligil GS. Inflammation and metabolic disorders. Nature. 2006;444(7121):860-7.
307. Cai D, Yuan M, Frantz DF, Melendez PA, Hansen L, Lee J, et al. Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. Nature medicine. 2005;11(2):183-90.
308. Ehses JA, Perren A, Eppler E, Ribaux P, Pospisilik JA, Maor-Cahn R, et al. Increased number of islet-associated macrophages in type 2 diabetes. Diabetes. 2007;56(9):2356-70.
309. Pillon NJ, Arane K, Bilan PJ, Chiu TT, Klip A. Muscle cells challenged with saturated fatty acids mount an autonomous inflammatory response that activates macrophages. Cell communication and signaling : CCS. 2012;10(1):30.
310. De Souza CT, Araujo EP, Bordin S, Ashimine R, Zollner RL, Boschero AC, et al. Consumption of a fat-rich diet activates a proinflammatory response and induces insulin resistance in the hypothalamus. Endocrinology. 2005;146(10):4192-9.
311. Lykouras L, Michopoulos J. Anxiety disorders and obesity. Psychiatrike = Psychiatriki. 2011;22(4):307-13.
312. Thaler JP, Yi CX, Schur EA, Guyenet SJ, Hwang BH, Dietrich MO, et al. Obesity is associated with hypothalamic injury in rodents and humans. The Journal of clinical investigation. 2012;122(1):153-62.
313. Thaler JP, Schwartz MW. Minireview: Inflammation and obesity pathogenesis: the hypothalamus heats up. Endocrinology. 2010;151(9):4109-15.
314. Kanoski SE, Zhang Y, Zheng W, Davidson TL. The effects of a high-energy diet on hippocampal function and blood-brain barrier integrity in the rat. Journal of Alzheimer's disease : JAD. 2010;21(1):207-19.
315. Jurdak N, Lichtenstein AH, Kanarek RB. Diet-induced obesity and spatial cognition in young male rats. Nutr Neurosci. 2008;11(2):48-54.
316. Erion JR, Wosiski-Kuhn M, Dey A, Hao S, Davis CL, Pollock NK, et al. Obesity elicits interleukin 1-mediated deficits in hippocampal synaptic plasticity. The Journal of neuroscience. 2014;34(7):2618-31.
317. Christian LM, Porter K. Longitudinal changes in serum proinflammatory markers across pregnancy and postpartum: effects of maternal body mass index. Cytokine. 2014;70(2):134-40.
318. Urakubo A, Jarskog LF, Lieberman JA, Gilmore JH. Prenatal exposure to maternal infection alters cytokine expression in the placenta, amniotic fluid, and fetal brain. Schizophr Res. 2001;47(1):27-36.
319. Patterson PH. Maternal infection and immune involvement in autism. Trends in molecular medicine. 2011;17(7):389-94.
320. Khandaker GM, Zimbron J, Lewis G, Jones PB. Prenatal maternal infection, neurodevelopment and adult schizophrenia: a systematic review of population-based studies. Psychol Med. 2013;43(2):239-57.
321. Leibowitz KL, Moore RH, Ahima RS, Stunkard AJ, Stallings VA, Berkowitz RI, et al. Maternal obesity associated with inflammation in their children. World journal of pediatrics : WJP. 2012;8(1):76-9.
322. Yan X, Huang Y, Wang H, Du M, Hess BW, Ford SP, et al. Maternal obesity induces sustained inflammation in both fetal and offspring large intestine of sheep. Inflammatory bowel diseases. 2011;17(7):1513-22.
323. Dudele A, Hougaard KS, Kjolby M, Hokland M, Winther G, Elfving B, et al. Chronic maternal inflammation or high-fat-feeding programs offspring obesity in a sex-dependent manner. International journal of obesity. 2017;41(9):1420-6.
324. Kang SS, Kurti A, Fair DA, Fryer JD. Dietary intervention rescues maternal obesity induced behavior deficits and neuroinflammation in offspring. Journal of neuroinflammation. 2014;11:156.

325. Ginsberg Y, Khatib N, Weiner Z, Beloosesky R. Maternal Inflammation, Fetal Brain Implications and Suggested Neuroprotection: A Summary of 10 Years of Research in Animal Models. Rambam Maimonides Med J. 2017;8(2).
326. Wang X, Rousset CI, Hagberg H, Mallard C. Lipopolysaccharide-induced inflammation and perinatal brain injury. Semin Fetal Neonatal Med. 2006;11(5):343-53.
327. Huleihel M, Golan H, Hallak M. Intrauterine infection/inflammation during pregnancy and offspring brain damages: possible mechanisms involved. Reprod Biol Endocrinol. 2004;2:17.
328. Cai Z, Lin S, Pang Y, Rhodes PG. Brain injury induced by intracerebral injection of interleukin-1beta and tumor necrosis factor-alpha in the neonatal rat. Pediatr Res. 2004;56(3):377-84.
329. Jurewicz A, Matysiak M, Tybor K, Kilianek L, Raine CS, Selmaj K. Tumour necrosis factor-induced death of adult human oligodendrocytes is mediated by apoptosis inducing factor. Brain. 2005;128(Pt 11):2675-88.
330. Pang Y, Cai Z, Rhodes PG. Effect of tumor necrosis factor-alpha on developing optic nerve oligodendrocytes in culture. J Neurosci Res. 2005;80(2):226-34.
331. Wang X, Hagberg H, Zhu C, Jacobsson B, Mallard C. Effects of intrauterine inflammation on the developing mouse brain. Brain Res. 2007;1144:180-5.
332. Smith SE, Li J, Garbett K, Mirnics K, Patterson PH. Maternal immune activation alters fetal brain development through interleukin-6. The Journal of neuroscience. 2007;27(40):10695-702.
333. Fowden AL, Ward JW, Wooding FP, Forhead AJ, Constancia M. Programming placental nutrient transport capacity. J Physiol. 2006;572(Pt 1):5-15.
334. Fowden AL, Forhead AJ. Endocrine mechanisms of intrauterine programming. Reproduction. 2004;127(5):515-26.
335. Fowden AL, Sferruzzi-Perri AN, Coan PM, Constancia M, Burton GJ. Placental efficiency and adaptation: endocrine regulation. J Physiol. 2009;587(Pt 14):3459-72.
336. Baur R. Morphometry of the placental exchange area. Adv Anat Embryol Cell Biol. 1977;53(1):3-65.
337. Aye IL, Lager S, Ramirez VI, Gaccioli F, Dudley DJ, Jansson T, et al. Increasing maternal body mass index is associated with systemic inflammation in the mother and the activation of distinct placental inflammatory pathways. Biol Reprod. 2014;90(6):129.
338. Fernandez-Twinn DS, Gascoin G, Musial B, Carr S, Duque-Guimaraes D, Blackmore HL, et al. Exercise rescues obese mothers' insulin sensitivity, placental hypoxia and male offspring insulin sensitivity. Scientific reports. 2017;7:44650.
339. Challier JC, Basu S, Bintein T, Minium J, Hotmire K, Catalano PM, et al. Obesity in pregnancy stimulates macrophage accumulation and inflammation in the placenta. Placenta. 2008;29(3):274-81.
340. Zaretsky MV, Alexander JM, Byrd W, Bawdon RE. Transfer of inflammatory cytokines across the placenta. Obstetrics and gynecology. 2004;103(3):546-50.
341. Dosch NC, Guslits EF, Weber MB, Murray SE, Ha B, Coe CL, et al. Maternal Obesity Affects Inflammatory and Iron Indices in Umbilical Cord Blood. J Pediatr. 2016;172:20-8.
342. Stewart FM, Freeman DJ, Ramsay JE, Greer IA, Caslake M, Ferrell WR. Longitudinal assessment of maternal endothelial function and markers of inflammation and placental function throughout pregnancy in lean and obese mothers. J Clin Endocrinol Metab. 2007;92(3):969-75.
343. Wedekind L, Belkacemi L. Altered cytokine network in gestational diabetes mellitus affects maternal insulin and placental-fetal development. J Diabetes Complications. 2016;30(7):1393-400.
344. Kim DW, Young SL, Grattan DR, Jasoni CL. Obesity during pregnancy disrupts placental morphology, cell proliferation, and inflammation in a sex-specific manner across gestation in the mouse. Biol Reprod. 2014;90(6):130.

345. Raison CL, Miller AH. When not enough is too much: the role of insufficient glucocorticoid signaling in the pathophysiology of stress-related disorders. The American journal of psychiatry. 2003;160(9):1554-65.
346. Miller AH, Pariante CM, Pearce BD. Effects of cytokines on glucocorticoid receptor expression and function. Glucocorticoid resistance and relevance to depression. Adv Exp Med Biol. 1999;461:107-16.
347. Wang X, Wu H, Miller AH. Interleukin 1alpha (IL-1alpha) induced activation of p38 mitogen-activated protein kinase inhibits glucocorticoid receptor function. Mol Psychiatry. 2004;9(1):65-75.
348. Pace TW, Hu F, Miller AH. Cytokine-effects on glucocorticoid receptor function: relevance to glucocorticoid resistance and the pathophysiology and treatment of major depression. Brain, behavior, and immunity. 2007;21(1):9-19.
349. Davies TH, Ning YM, Sanchez ER. A new first step in activation of steroid receptors: hormone-induced switching of FKBP51 and FKBP52 immunophilins. J Biol Chem. 2002;277(7):4597-600.
350. Wagner KV, Marinescu D, Hartmann J, Wang XD, Labermaier C, Scharf SH, et al. Differences in FKBP51 regulation following chronic social defeat stress correlate with individual stress sensitivity: influence of paroxetine treatment. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. 2012;37(13):2797-808.
351. Zannas AS, Wiechmann T, Gassen NC, Binder EB. Gene-Stress-Epigenetic Regulation of FKBP5: Clinical and Translational Implications. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. 2016;41(1):261-74.
352. Scharf SH, Liebl C, Binder EB, Schmidt MV, Muller MB. Expression and regulation of the Fkbp5 gene in the adult mouse brain. PloS one. 2011;6(2):e16883.
353. Gallo LI, Ghini AA, Piwien Pilipuk G, Galigniana MD. Differential recruitment of tetratricorpeptide repeat domain immunophilins to the mineralocorticoid receptor influences both heat-shock protein 90-dependent retrotransport and hormone-dependent transcriptional activity. Biochemistry. 2007;46(49):14044-57.
354. Galigniana MD, Erlejman AG, Monte M, Gomez-Sanchez C, Piwien-Pilipuk G. The hsp90-FKBP52 complex links the mineralocorticoid receptor to motor proteins and persists bound to the receptor in early nuclear events. Mol Cell Biol. 2010;30(5):1285-98.
355. Deppe CE, Heering PJ, Viengchareun S, Grabensee B, Farman N, Lombes M. Cyclosporine a and FK506 inhibit transcriptional activity of the human mineralocorticoid receptor: a cell-based model to investigate partial aldosterone resistance in kidney transplantation. Endocrinology. 2002;143(5):1932-41.
356. Latouche C, Sainte-Marie Y, Steenman M, Castro Chaves P, Naray-Fejes-Toth A, Fejes-Toth G, et al. Molecular signature of mineralocorticoid receptor signaling in cardiomyocytes: from cultured cells to mouse heart. Endocrinology. 2010;151(9):4467-76.
357. Stechschulte LA, Sanchez ER. FKBP51-a selective modulator of glucocorticoid and androgen sensitivity. Current opinion in pharmacology. 2011;11(4):332-7.
358. Denny WB, Valentine DL, Reynolds PD, Smith DF, Scammell JG. Squirrel monkey immunophilin FKBP51 is a potent inhibitor of glucocorticoid receptor binding. Endocrinology. 2000;141(11):4107-13.
359. Hartmann J, Wagner KV, Gaali S, Kirschner A, Kozany C, Ruhter G, et al. Pharmacological Inhibition of the Psychiatric Risk Factor FKBP51 Has Anxiolytic Properties. The Journal of neuroscience. 2015;35(24):9007-16.
360. Attwood BK, Bourgognon JM, Patel S, Mucha M, Schiavon E, Skrzypiec AE, et al. Neuropsin cleaves EphB2 in the amygdala to control anxiety. Nature. 2011;473(7347):372-5.
361. Hoeijmakers L, Harbich D, Schmid B, Lucassen PJ, Wagner KV, Schmidt MV, et al. Depletion of FKBP51 in female mice shapes HPA axis activity. PloS one. 2014;9(4):e95796.
362. Escoll P, Ranz I, Munoz-Anton N, van-den-Rym A, Alvarez-Mon M, Martinez-Alonso C, et al. Sustained interleukin-1beta exposure modulates multiple steps in glucocorticoid receptor signaling, promoting split-resistance to the transactivation of prominent anti-inflammatory genes by glucocorticoids. Mediators Inflamm. 2015;2015:347965.

363. Wohleb ES, Hanke ML, Corona AW, Powell ND, Stiner LM, Bailey MT, et al. beta-Adrenergic receptor antagonism prevents anxiety-like behavior and microglial reactivity induced by repeated social defeat. The Journal of neuroscience. 2011;31(17):6277-88.
364. Pariante CM, Pearce BD, Pisell TL, Sanchez CI, Po C, Su C, et al. The proinflammatory cytokine, interleukin-1alpha, reduces glucocorticoid receptor translocation and function. Endocrinology. 1999;140(9):4359-66.
365. Kam JC, Szefler SJ, Surs W, Sher ER, Leung DY. Combination IL-2 and IL-4 reduces glucocorticoid receptor-binding affinity and T cell response to glucocorticoids. Journal of immunology. 1993;151(7):3460-6.
366. Spahn JD, Szefler SJ, Surs W, Doherty DE, Nimmagadda SR, Leung DY. A novel action of IL-13: induction of diminished monocyte glucocorticoid receptor-binding affinity. Journal of immunology. 1996;157(6):2654-9.
367. Henry CJ, Huang Y, Wynne AM, Godbout JP. Peripheral lipopolysaccharide (LPS) challenge promotes microglial hyperactivity in aged mice that is associated with exaggerated induction of both pro-inflammatory IL-1beta and anti-inflammatory IL-10 cytokines. Brain, behavior, and immunity. 2009;23(3):309-17.
368. Godbout JP, Chen J, Abraham J, Richwine AF, Berg BM, Kelley KW, et al. Exaggerated neuroinflammation and sickness behavior in aged mice following activation of the peripheral innate immune system. FASEB journal. 2005;19(10):1329-31.
369. Kessler MS, Bosch OJ, Bunck M, Landgraf R, Neumann ID. Maternal care differs in mice bred for high vs. low trait anxiety: impact of brain vasopressin and cross-fostering. Social neuroscience. 2011;6(2):156-68.
370. Champagne FA, Curley JP, Keverne EB, Bateson PP. Natural variations in postpartum maternal care in inbred and outbred mice. Physiology & behavior. 2007;91(2-3):325-34.
371. Scattoni ML, Crawley J, Ricceri L. Ultrasonic vocalizations: a tool for behavioural phenotyping of mouse models of neurodevelopmental disorders. Neurosci Biobehav Rev. 2009;33(4):508-15.
372. Hofer MA, Shair HN, Brunelli SA. Ultrasonic vocalizations in rat and mouse pups. Current protocols in neuroscience / editorial board, Jacqueline N Crawley [et al]. 2002;Chapter 8:Unit 8 14.
373. D'Amato FR, Scalera E, Sarli C, Moles A. Pups call, mothers rush: does maternal responsiveness affect the amount of ultrasonic vocalizations in mouse pups? Behavior genetics. 2005;35(1):103-12.
374. Bailey KR, Crawley JN. Anxiety-Related Behaviors in Mice. In: Buccafusco JJ, editor. Methods of Behavior Analysis in Neuroscience. Frontiers in Neuroscience. 2nd ed. Boca Raton (FL)2009.
375. Rasmussen S, Miller MM, Filipski SB, Tolwani RJ. Cage change influences serum corticosterone and anxiety-like behaviors in the mouse. Journal of the American Association for Laboratory Animal Science : JAALAS. 2011;50(4):479-83.
376. Pritchard LM, Van Kempen TA, Zimmerberg B. Behavioral effects of repeated handling differ in rats reared in social isolation and environmental enrichment. Neuroscience letters. 2013;536:47-51.
377. Crawley JN. What's wrong with my mouse? : behavioral phenotyping of transgenic and knockout mice. New York: Wiley-Liss; 2000. xiii, 329 p. p.
378. Walf AA, Frye CA. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nature protocols. 2007;2(2):322-8.
379. Swiergiel AH, Dunn AJ. Effects of interleukin-1beta and lipopolysaccharide on behavior of mice in the elevated plus-maze and open field tests. Pharmacology, biochemistry, and behavior. 2007;86(4):651-9.
380. Leger M, Quiedeville A, Bouet V, Haelewyn B, Boulouard M, Schumann-Bard P, et al. Object recognition test in mice. Nature protocols. 2013;8(12):2531-7.
381. Barker GR, Warburton EC. When is the hippocampus involved in recognition memory? The Journal of neuroscience. 2011;31(29):10721-31.

382. Zimprich A, Garrett L, Deussing JM, Wotjak CT, Fuchs H, Gailus-Durner V, et al. A robust and reliable non-invasive test for stress responsivity in mice. Frontiers in behavioral neuroscience. 2014;8:125.
383. O'Neil A, Itsiopoulos C, Skouteris H, Opie RS, McPhie S, Hill B, et al. Preventing mental health problems in offspring by targeting dietary intake of pregnant women. BMC Med. 2014;12:208.
384. Capra L, Tezza G, Mazzei F, Boner AL. The origins of health and disease: the influence of maternal diseases and lifestyle during gestation. Ital J Pediatr. 2013;39:7.
385. de Rooij SR, Wouters H, Yonker JE, Painter RC, Roseboom TJ. Prenatal undernutrition and cognitive function in late adulthood. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(39):16881-6.
386. O'Reilly JR, Reynolds RM. The risk of maternal obesity to the long-term health of the offspring. Clin Endocrinol (Oxf). 2013;78(1):9-16.
387. Rodriguez A. Maternal pre-pregnancy obesity and risk for inattention and negative emotionality in children. J Child Psychol Psychiatry. 2010;51(2):134-43.
388. Hinkle SN, Schieve LA, Stein AD, Swan DW, Ramakrishnan U, Sharma AJ. Associations between maternal prepregnancy body mass index and child neurodevelopment at 2 years of age. International journal of obesity. 2012;36(10):1312-9.
389. Nohr EA, Vaeth M, Baker JL, Sorensen T, Olsen J, Rasmussen KM. Combined associations of prepregnancy body mass index and gestational weight gain with the outcome of pregnancy. Am J Clin Nutr. 2008;87(6):1750-9.
390. Colman I, Ataullahjan A, Naicker K, Van Lieshout RJ. Birth weight, stress, and symptoms of depression in adolescence: evidence of fetal programming in a national Canadian cohort. Can J Psychiatry. 2012;57(7):422-8.
391. Weaver IC, Meaney MJ, Szyf M. Maternal care effects on the hippocampal transcriptome and anxiety-mediated behaviors in the offspring that are reversible in adulthood. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(9):3480-5.
392. Verbeek T, Bockting CL, van Pampus MG, Ormel J, Meijer JL, Hartman CA, et al. Postpartum depression predicts offspring mental health problems in adolescence independently of parental lifetime psychopathology. J Affect Disord. 2012;136(3):948-54.
393. Buss C, Davis EP, Hobel CJ, Sandman CA. Maternal pregnancy-specific anxiety is associated with child executive function at 6-9 years age. Stress. 2011;14(6):665-76.
394. Hariri N, Thibault L. High-fat diet-induced obesity in animal models. Nutr Res Rev. 2010;23(2):270-99.
395. Graf AE, Lallier SW, Waidyaratne G, Thompson MD, Tipple TE, Hester ME, et al. Maternal high fat diet exposure is associated with increased hepcidin levels, decreased myelination, and neurobehavioral changes in male offspring. Brain, behavior, and immunity. 2016;58:369-78.
396. Cheke LG, Simons JS, Clayton NS. Higher body mass index is associated with episodic memory deficits in young adults. Q J Exp Psychol (Hove). 2016;69(11):2305-16.
397. Heyward FD, Gilliam D, Coleman MA, Gavin CF, Wang J, Kaas G, et al. Obesity Weighs down Memory through a Mechanism Involving the Neuroepigenetic Dysregulation of Sirt1. The Journal of neuroscience. 2016;36(4):1324-35.
398. Kesby JP, Kim JJ, Scadeng M, Woods G, Kado DM, Olefsky JM, et al. Spatial Cognition in Adult and Aged Mice Exposed to High-Fat Diet. PloS one. 2015;10(10):e0140034.
399. Gluckman PD, Hanson MA. Living with the past: evolution, development, and patterns of disease. Science. 2004;305(5691):1733-6.
400. Li SW, Yu HR, Sheen JM, Tiao MM, Tain YL, Lin IC, et al. A maternal high-fat diet during pregnancy and lactation, in addition to a postnatal high-fat diet, leads to metabolic syndrome with spatial learning and memory deficits: beneficial effects of resveratrol. Oncotarget. 2017;8(67):111998-2013.

401. Mendes-da-Silva C, Giriko CA, Mennitti LV, Hosoume LF, Souto Tdos S, Silva AV. Maternal high-fat diet during pregnancy or lactation changes the somatic and neurological development of the offspring. Arq Neuropsiquiatr. 2014;72(2):136-44.
402. Purcell RH, Sun B, Pass LL, Power ML, Moran TH, Tamashiro KL. Maternal stress and high-fat diet effect on maternal behavior, milk composition, and pup ingestive behavior. Physiology & behavior. 2011;104(3):474-9.
403. Tamashiro KL, Terrillion CE, Hyun J, Koenig JI, Moran TH. Prenatal stress or high-fat diet increases susceptibility to diet-induced obesity in rat offspring. Diabetes. 2009;58(5):1116-25.
404. Ribaroff GA, Wastnedge E, Drake AJ, Sharpe RM, Chambers TJG. Animal models of maternal high fat diet exposure and effects on metabolism in offspring: a meta-regression analysis. Obes Rev. 2017;18(6):673-86.
405. Shankar K, Harrell A, Liu X, Gilchrist JM, Ronis MJ, Badger TM. Maternal obesity at conception programs obesity in the offspring. American journal of physiology Regulatory, integrative and comparative physiology. 2008;294(2):R528-38.
406. Bayol SA, Farrington SJ, Stickland NC. A maternal 'junk food' diet in pregnancy and lactation promotes an exacerbated taste for 'junk food' and a greater propensity for obesity in rat offspring. The British journal of nutrition. 2007;98(4):843-51.
407. Parente LB, Aguila MB, Mandarim-de-Lacerda CA. Deleterious effects of high-fat diet on perinatal and postweaning periods in adult rat offspring. Clin Nutr. 2008;27(4):623-34.
408. Bellisario V, Panetta P, Balsevich G, Baumann V, Noble J, Raggi C, et al. High-fat diet during pregnancy acts as a stressor increasing maternal glucocorticoids' signaling to the fetus and disrupting maternal behavior in a mouse model. Psychoneuroendocrinology. 2015;61:10.
409. Ehret G. Infant rodent ultrasounds -- a gate to the understanding of sound communication. Behavior genetics. 2005;35(1):19-29.
410. Blumberg MS, Sokoloff G. Do infant rats cry? Psychological Review. 2001;108(1):83-95.
411. Shoji H, Takao K, Hattori S, Miyakawa T. Age-related changes in behavior in C57BL/6J mice from young adulthood to middle age. Mol Brain. 2016;9:11.
412. Bordner KA, Kitchen RR, Carlyle B, George ED, Mahajan MC, Mane SM, et al. Parallel declines in cognition, motivation, and locomotion in aging mice: association with immune gene upregulation in the medial prefrontal cortex. Exp Gerontol. 2011;46(8):643-59.
413. Elias PK, Elias MF, Eleftheriou BE. Emotionality, exploratory behavior, and locomotion in aging inbred strains of mice. Gerontologia. 1975;21(1):46-55.
414. Fernandes C, Grayton H, Poston L, Samuelsson AM, Taylor PD, Collier DA, et al. Prenatal exposure to maternal obesity leads to hyperactivity in offspring. Mol Psychiatry. 2012;17(12):1159-60.
415. Chen Q, Sjolander A, Langstrom N, Rodriguez A, Serlachius E, D'Onofrio BM, et al. Maternal pre-pregnancy body mass index and offspring attention deficit hyperactivity disorder: a population-based cohort study using a sibling-comparison design. Int J Epidemiol. 2014;43(1):83-90.
416. Visser SN, Danielson ML, Bitsko RH, Holbrook JR, Kogan MD, Ghandour RM, et al. Trends in the parent-report of health care provider-diagnosed and medicated attention-deficit/hyperactivity disorder: United States, 2003-2011. J Am Acad Child Adolesc Psychiatry. 2014;53(1):34-46 e2.
417. Ennaceur A. Tests of unconditioned anxiety - pitfalls and disappointments. Physiology & behavior. 2014;135:55-71.
418. Costall B, Jones BJ, Kelly ME, Naylor RJ, Tomkins DM. Exploration of mice in a black and white test box: validation as a model of anxiety. Pharmacology, biochemistry, and behavior. 1989;32(3):777-85.
419. Carli M, Prontera C, Samanin R. Effect of 5-HT1A agonists on stress-induced deficit in open field locomotor activity of rats: evidence that this model identifies anxiolytic-like activity. Neuropharmacology. 1989;28(5):471-6.
420. Lamprea MR, Cardenas FP, Setem J, Morato S. Thigmotactic responses in an open-field. Braz J Med Biol Res. 2008;41(2):135-40.

421. Rincel M, Lepinay AL, Delage P, Fioramonti J, Theodorou VS, Laye S, et al. Maternal high-fat diet prevents developmental programming by early-life stress. Transl Psychiatry. 2016;6(11):e966.
422. Murray AJ, Knight NS, Cochlin LE, McAleese S, Deacon RM, Rawlins JN, et al. Deterioration of physical performance and cognitive function in rats with short-term high-fat feeding. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2009;23(12):4353-60.
423. Garcia AM, Cardenas FP, Morato S. Effect of different illumination levels on rat behavior in the elevated plus-maze. Physiology & behavior. 2005;85(3):265-70.
424. Pardon MC, Gould GG, Garcia A, Phillips L, Cook MC, Miller SA, et al. Stress reactivity of the brain noradrenergic system in three rat strains differing in their neuroendocrine and behavioral responses to stress: implications for susceptibility to stress-related neuropsychiatric disorders. Neuroscience. 2002;115(1):229-42.
425. Sarrazin N, Di Blasi F, Roullot-Lacarriere V, Rouge-Pont F, Le Roux A, Costet P, et al. Transcriptional effects of glucocorticoid receptors in the dentate gyrus increase anxiety-related behaviors. PloS one. 2009;4(11):e7704.
426. Estanislau C, Morato S. Behavior ontogeny in the elevated plus-maze: prenatal stress effects. Int J Dev Neurosci. 2006;24(4):255-62.
427. Hwang LL, Wang CH, Li TL, Chang SD, Lin LC, Chen CP, et al. Sex differences in high-fat diet-induced obesity, metabolic alterations and learning, and synaptic plasticity deficits in mice. Obesity. 2010;18(3):463-9.
428. Piato AL, Detanico BC, Jesus JF, Lhullier FL, Nunes DS, Elisabetsky E. Effects of Marapuama in the chronic mild stress model: further indication of antidepressant properties. J Ethnopharmacol. 2008;118(2):300-4.
429. Yalcin I, Aksu F, Belzung C. Effects of desipramine and tramadol in a chronic mild stress model in mice are altered by yohimbine but not by pindolol. European journal of pharmacology. 2005;514(2-3):165-74.
430. Wang D, An SC, Zhang X. Prevention of chronic stress-induced depression-like behavior by inducible nitric oxide inhibitor. Neurosci Lett. 2008;433(1):59-64.
431. Barros HM, Tannhauser SL, Tannhauser MA, Tannhauser M. The effects of GABAergic drugs on grooming behaviour in the open field. Pharmacol Toxicol. 1994;74(6):339-44.
432. Enginar N, Hatipoglu I, Firtina M. Evaluation of the acute effects of amitriptyline and fluoxetine on anxiety using grooming analysis algorithm in rats. Pharmacology, biochemistry, and behavior. 2008;89(3):450-5.
433. de Andrade IS, Zemdegs JC, de Souza AP, Watanabe RL, Telles MM, Nascimento CM, et al. Diet-induced obesity impairs hypothalamic glucose sensing but not glucose hypothalamic extracellular levels, as measured by microdialysis. Nutr Diabetes. 2015;5:e162.
434. Friend DM, Devarakonda K, O'Neal TJ, Skirzewski M, Papazoglou I, Kaplan AR, et al. Basal Ganglia Dysfunction Contributes to Physical Inactivity in Obesity. Cell metabolism. 2017;25(2):312-21.
435. Hassan AM, Mancano G, Kashofer K, Frohlich EE, Matak A, Mayerhofer R, et al. High-fat diet induces depression-like behaviour in mice associated with changes in microbiome, neuropeptide Y, and brain metabolome. Nutr Neurosci. 2018:1-17.
436. Aslani S, Vieira N, Marques F, Costa PS, Sousa N, Palha JA. The effect of high-fat diet on rat's mood, feeding behavior and response to stress. Transl Psychiatry. 2015;5:e684.
437. Albani SH, Andrawis MM, Abella RJ, Fulghum JT, Vafamand N, Dumas TC. Behavior in the elevated plus maze is differentially affected by testing conditions in rats under and over three weeks of age. Frontiers in behavioral neuroscience. 2015;9:31.
438. Rodgers RJ, Dalvi A. Anxiety, defence and the elevated plus-maze. Neurosci Biobehav Rev. 1997;21(6):801-10.
439. File SE, Zangrossi H, Jr., Viana M, Graeff FG. Trial 2 in the elevated plus-maze: a different form of fear? Psychopharmacology (Berl). 1993;111(4):491-4.

440. Elhaik Goldman S, Goez D, Last D, Naor S, Liraz Zaltsman S, Sharvit-Ginon I, et al. High-fat diet protects the blood-brain barrier in an Alzheimer's disease mouse model. Aging Cell. 2018:e12818.
441. Darcet F, Mendez-David I, Tritschler L, Gardier AM, Guilloux JP, David DJ. Learning and memory impairments in a neuroendocrine mouse model of anxiety/depression. Frontiers in behavioral neuroscience. 2014;8:136.
442. Traschutz A, Kummer MP, Schwartz S, Heneka MT. Variability and temporal dynamics of novel object recognition in aging male C57BL/6 mice. Behav Processes. 2017.
443. Jurdak N, Kanarek RB. Sucrose-induced obesity impairs novel object recognition learning in young rats. Physiology & behavior. 2009;96(1):1-5.
444. Camer D, Yu Y, Szabo A, Fernandez F, Dinh CHL, Huang XF. Bardoxolone methyl prevents high-fat diet-induced alterations in prefrontal cortex signalling molecules involved in recognition memory. Prog Neuropsychopharmacol Biol Psychiatry. 2015;59:68-75.
445. Lavin DN, Joesting JJ, Chiu GS, Moon ML, Meng J, Dilger RN, et al. Fasting induces an anti-inflammatory effect on the neuroimmune system which a high-fat diet prevents. Obesity. 2011;19(8):1586-94.
446. Tucker KR, Godbey SJ, Thiebaud N, Fadool DA. Olfactory ability and object memory in three mouse models of varying body weight, metabolic hormones, and adiposity. Physiology & behavior. 2012;107(3):424-32.
447. Knight EM, Martins IV, Gumusgoz S, Allan SM, Lawrence CB. High-fat diet-induced memory impairment in triple-transgenic Alzheimer's disease (3xTgAD) mice is independent of changes in amyloid and tau pathology. Neurobiol Aging. 2014;35(8):1821-32.
448. Can OD, Ulupinar E, Ozkay UD, Yegin B, Ozturk Y. The effect of simvastatin treatment on behavioral parameters, cognitive performance, and hippocampal morphology in rats fed a standard or a high-fat diet. Behav Pharmacol. 2012;23(5-6):582-92.
449. Zhu C, Han TL, Zhao Y, Zhou X, Mao X, Qi H, et al. A mouse model of pre-pregnancy maternal obesity combined with offspring exposure to a high-fat diet resulted in cognitive impairment in male offspring. Exp Cell Res. 2018;368(2):159-66.
450. Verma R, Balhara YP, Gupta CS. Gender differences in stress response: Role of developmental and biological determinants. Ind Psychiatry J. 2011;20(1):4-10.
451. Jacobson L. Hypothalamic-pituitary-adrenocortical axis: neuropsychiatric aspects. Compr Physiol. 2014;4(2):715-38.
452. Pasquali R, Vicennati V, Cacciari M, Pagotto U. The hypothalamic-pituitary-adrenal axis activity in obesity and the metabolic syndrome. Ann N Y Acad Sci. 2006;1083:111-28.
453. Herman JP, McKlveen JM, Ghosal S, Kopp B, Wulsin A, Makinson R, et al. Regulation of the Hypothalamic-Pituitary-Adrenocortical Stress Response. Compr Physiol. 2016;6(2):603-21.
454. Auvinen HE, Romijn JA, Biermasz NR, Pijl H, Havekes LM, Smit JW, et al. The effects of high fat diet on the basal activity of the hypothalamus-pituitary-adrenal axis in mice. J Endocrinol. 2012;214(2):191-7.
455. Pasquali R, Cantobelli S, Casimirri F, Capelli M, Bortoluzzi L, Flamia R, et al. The hypothalamic-pituitary-adrenal axis in obese women with different patterns of body fat distribution. J Clin Endocrinol Metab. 1993;77(2):341-6.
456. Swierczynska MM, Mateska I, Peitzsch M, Bornstein SR, Chavakis T, Eisenhofer G, et al. Changes in morphology and function of adrenal cortex in mice fed a high-fat diet. International journal of obesity. 2015;39(2):321-30.
457. Dhabhar FS, McEwen BS. Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: a potential role for leukocyte trafficking. Brain, behavior, and immunity. 1997;11(4):286-306.
458. Gadek-Michalska A, Spyrka J, Rachwalska P, Tadeusz J, Bugajski J. Influence of chronic stress on brain corticosteroid receptors and HPA axis activity. Pharmacological reports : PR. 2013;65(5):1163-75.
459. Kalyani M, Hasselfeld K, Janik JM, Callahan P, Shi H. Effects of High-Fat Diet on Stress Response in Male and Female Wildtype and Prolactin Knockout Mice. PloS one. 2016;11(11):e0166416.

460. Van Pett K, Viau V, Bittencourt JC, Chan RK, Li HY, Arias C, et al. Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. J Comp Neurol. 2000;428(2):191-212.
461. Gomez-Sanchez E, Gomez-Sanchez CE. The multifaceted mineralocorticoid receptor. Compr Physiol. 2014;4(3):965-94.
462. De Kloet ER. Hormones and the stressed brain. Ann N Y Acad Sci. 2004;1018:1-15.
463. Cole MA, Kalman BA, Pace TW, Topczewski F, Lowrey MJ, Spencer RL. Selective blockade of the mineralocorticoid receptor impairs hypothalamic-pituitary-adrenal axis expression of habituation. Journal of neuroendocrinology. 2000;12(10):1034-42.
464. Mattsson C, Lai M, Noble J, McKinney E, Yau JL, Seckl JR, et al. Obese Zucker rats have reduced mineralocorticoid receptor and 11beta-hydroxysteroid dehydrogenase type 1 expression in hippocampus-implications for dysregulation of the hypothalamic-pituitary-adrenal axis in obesity. Endocrinology. 2003;144(7):2997-3003.
465. Zhe D, Fang H, Yuxiu S. Expressions of hippocampal mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) in the single-prolonged stress-rats. Acta Histochem Cytochem. 2008;41(4):89-95.
466. Westberry JM, Sadosky PW, Hubler TR, Gross KL, Scammell JG. Glucocorticoid resistance in squirrel monkeys results from a combination of a transcriptionally incompetent glucocorticoid receptor and overexpression of the glucocorticoid receptor co-chaperone FKBP51. J Steroid Biochem Mol Biol. 2006;100(1-3):34-41.
467. Woodruff PG, Boushey HA, Dolganov GM, Barker CS, Yang YH, Donnelly S, et al. Genome-wide profiling identifies epithelial cell genes associated with asthma and with treatment response to corticosteroids. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(40):15858-63.
468. Vermeer H, Hendriks-Stegeman BI, van der Burg B, van Buul-Offers SC, Jansen M. Glucocorticoid-induced increase in lymphocytic FKBP51 messenger ribonucleic acid expression: a potential marker for glucocorticoid sensitivity, potency, and bioavailability. J Clin Endocrinol Metab. 2003;88(1):277-84.
469. O'Leary JC, 3rd, Dharia S, Blair LJ, Brady S, Johnson AG, Peters M, et al. A new anti-depressive strategy for the elderly: ablation of FKBP5/FKBP51. PloS one. 2011;6(9):e24840.
470. Legendre A, Papakonstantinou E, Roy MC, Richard D, Harris RB. Differences in response to corticotropin-releasing factor after short- and long-term consumption of a high-fat diet. American journal of physiology Regulatory, integrative and comparative physiology. 2007;293(3):R1076-85.
471. Metherell LA, Chapple JP, Cooray S, David A, Becker C, Ruschendorf F, et al. Mutations in MRAP, encoding a new interacting partner of the ACTH receptor, cause familial glucocorticoid deficiency type 2. Nat Genet. 2005;37(2):166-70.
472. Ramachandrappa S, Gorrigan RJ, Clark AJ, Chan LF. The melanocortin receptors and their accessory proteins. Front Endocrinol (Lausanne). 2013;4:9.
473. Jackson DS, Ramachandrappa S, Clark AJ, Chan LF. Melanocortin receptor accessory proteins in adrenal disease and obesity. Front Neurosci. 2015;9:213.
474. Dakin RS, Walker BR, Seckl JR, Hadoke PW, Drake AJ. Estrogens protect male mice from obesity complications and influence glucocorticoid metabolism. International journal of obesity. 2015;39(10):1539-47.
475. Richard D, Rivest R, Naimi N, Timofeeva E, Rivest S. Expression of corticotropin-releasing factor and its receptors in the brain of lean and obese Zucker rats. Endocrinology. 1996;137(11):4786-95.
476. Bethea CL, Reddy AP, Flowers M, Shapiro RA, Colman RJ, Abbott DH, et al. High fat diet decreases beneficial effects of estrogen on serotonin-related gene expression in marmosets. Prog Neuropsychopharmacol Biol Psychiatry. 2015;58:71-80.
477. Wei J, Yuen EY, Liu W, Li X, Zhong P, Karatsoreos IN, et al. Estrogen protects against the detrimental effects of repeated stress on glutamatergic transmission and cognition. Mol Psychiatry. 2014;19(5):588-98.

478. Grundwald NJ, Brunton PJ. Prenatal stress programs neuroendocrine stress responses and affective behaviors in second generation rats in a sex-dependent manner. Psychoneuroendocrinology. 2015;62:204-16.
479. Karbaschi R, Zardooz H, Khodagholi F, Dargahi L, Salimi M, Rashidi F. Maternal high-fat diet intensifies the metabolic response to stress in male rat offspring. Nutr Metab (Lond). 2017;14:20.
480. Monteiro R, Azevedo I. Chronic inflammation in obesity and the metabolic syndrome. Mediators of inflammation. 2010;2010.
481. Castanon N, Luheshi G, Laye S. Role of neuroinflammation in the emotional and cognitive alterations displayed by animal models of obesity. Front Neurosci. 2015;9:229.
482. Guillemot-Legris O, Muccioli GG. Obesity-Induced Neuroinflammation: Beyond the Hypothalamus. Trends Neurosci. 2017;40(4):237-53.
483. Silverman MN, Pearce BD, Biron CA, Miller AH. Immune modulation of the hypothalamic-pituitary-adrenal (HPA) axis during viral infection. Viral Immunol. 2005;18(1):41-78.
484. Besedovsky HO, del Rey A, Klusman I, Furukawa H, Monge Arditi G, Kabiersch A. Cytokines as modulators of the hypothalamus-pituitary-adrenal axis. J Steroid Biochem Mol Biol. 1991;40(4-6):613-8.
485. Kneeland RE, Fatemi SH. Viral infection, inflammation and schizophrenia. Progress in neuro-psychopharmacology & biological psychiatry. 2013;42:35-48.
486. Short SJ, Lubach GR, Karasin AI, Olsen CW, Styner M, Knickmeyer RC, et al. Maternal influenza infection during pregnancy impacts postnatal brain development in the rhesus monkey. Biological psychiatry. 2010;67(10):965-73.
487. Valdearcos M, Robblee MM, Benjamin DI, Nomura DK, Xu AW, Koliwad SK. Microglia dictate the impact of saturated fat consumption on hypothalamic inflammation and neuronal function. Cell Rep. 2014;9(6):2124-38.
488. de Kloet AD, Pioquinto DJ, Nguyen D, Wang L, Smith JA, Hiller H, et al. Obesity induces neuroinflammation mediated by altered expression of the renin-angiotensin system in mouse forebrain nuclei. Physiology & behavior. 2014;136:31-8.
489. Tucsek Z, Toth P, Sosnowska D, Gautam T, Mitschelen M, Koller A, et al. Obesity in aging exacerbates blood-brain barrier disruption, neuroinflammation, and oxidative stress in the mouse hippocampus: effects on expression of genes involved in beta-amyloid generation and Alzheimer's disease. J Gerontol A Biol Sci Med Sci. 2014;69(10):1212-26.
490. Kim DW, Glendining KA, Grattan DR, Jasoni CL. Maternal Obesity in the Mouse Compromises the Blood-Brain Barrier in the Arcuate Nucleus of Offspring. Endocrinology. 2016;157(6):2229-42.
491. Franceschi C, Campisi J. Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. J Gerontol A Biol Sci Med Sci. 2014;69 Suppl 1:S4-9.
492. Lee CK, Weindruch R, Prolla TA. Gene-expression profile of the ageing brain in mice. Nat Genet. 2000;25(3):294-7.
493. Ulvestad E, Williams K, Bjerkvig R, Tiekotter K, Antel J, Matre R. Human microglial cells have phenotypic and functional characteristics in common with both macrophages and dendritic antigen-presenting cells. J Leukoc Biol. 1994;56(6):732-40.
494. Ajmone-Cat MA, Nicolini A, Minghetti L. Prolonged exposure of microglia to lipopolysaccharide modifies the intracellular signaling pathways and selectively promotes prostaglandin E2 synthesis. J Neurochem. 2003;87(5):1193-203.
495. Fantuzzi G. Adipose tissue, adipokines, and inflammation. J Allergy Clin Immunol. 2005;115(5):911-9; quiz 20.
496. Das UN. Is obesity an inflammatory condition? Nutrition. 2001;17(11):953-66.
497. Jeon BT, Jeong EA, Shin HJ, Lee Y, Lee DH, Kim HJ, et al. Resveratrol attenuates obesity-associated peripheral and central inflammation and improves memory deficit in mice fed a high-fat diet. Diabetes. 2012;61(6):1444-54.
498. Sobesky JL, Barrientos RM, De May HS, Thompson BM, Weber MD, Watkins LR, et al. High-fat diet consumption disrupts memory and primes elevations in hippocampal IL-1beta, an

effect that can be prevented with dietary reversal or IL-1 receptor antagonism. Brain, behavior, and immunity. 2014;42:22-32.
499. Buckman LB, Thompson MM, Moreno HN, Ellacott KL. Regional astrogliosis in the mouse hypothalamus in response to obesity. J Comp Neurol. 2013;521(6):1322-33.
500. Ziko I, De Luca S, Dinan T, Barwood JM, Sominsky L, Cai G, et al. Neonatal overfeeding alters hypothalamic microglial profiles and central responses to immune challenge long-term. Brain, behavior, and immunity. 2014;41:32-43.
501. Yi CX, Tschop MH, Woods SC, Hofmann SM. High-fat-diet exposure induces IgG accumulation in hypothalamic microglia. Dis Model Mech. 2012;5(5):686-90.
502. Ferguson D, Sapolsky R. Mineralocorticoid receptor overexpression differentially modulates specific phases of spatial and nonspatial memory. The Journal of neuroscience. 2007;27(30):8046-52.
503. Chen Y, Fenoglio KA, Dube CM, Grigoriadis DE, Baram TZ. Cellular and molecular mechanisms of hippocampal activation by acute stress are age-dependent. Mol Psychiatry. 2006;11(11):992-1002.
504. ter Heegde F, De Rijk RH, Vinkers CH. The brain mineralocorticoid receptor and stress resilience. Psychoneuroendocrinology. 2015;52:92-110.
505. Kellendonk C, Gass P, Kretz O, Schutz G, Tronche F. Corticosteroid receptors in the brain: gene targeting studies. Brain Res Bull. 2002;57(1):73-83.
506. Raber J. Detrimental effects of chronic hypothalamic-pituitary-adrenal axis activation. From obesity to memory deficits. Mol Neurobiol. 1998;18(1):1-22.
507. Arborelius L, Owens MJ, Plotsky PM, Nemeroff CB. The role of corticotropin-releasing factor in depression and anxiety disorders. J Endocrinol. 1999;160(1):1-12.
508. Schreiber W, Lauer CJ, Krumrey K, Holsboer F, Krieg JC. Dysregulation of the hypothalamic-pituitary-adrenocortical system in panic disorder. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. 1996;15(1):7-15.
509. Carola V, D'Olimpio F, Brunamonti E, Mangia F, Renzi P. Evaluation of the elevated plus-maze and open-field tests for the assessment of anxiety-related behaviour in inbred mice. Behavioural brain research. 2002;134(1-2):49-57.
510. Zhang R, Asai M, Mahoney CE, Joachim M, Shen Y, Gunner G, et al. Loss of hypothalamic corticotropin-releasing hormone markedly reduces anxiety behaviors in mice. Mol Psychiatry. 2017;22(5):733-44.
511. O'Leary JC, 3rd, Zhang B, Koren J, 3rd, Blair L, Dickey CA. The role of FKBP5 in mood disorders: action of FKBP5 on steroid hormone receptors leads to questions about its evolutionary importance. CNS Neurol Disord Drug Targets. 2013;12(8):1157-62.
512. Fries GR, Gassen NC, Rein T. The FKBP51 Glucocorticoid Receptor Co-Chaperone: Regulation, Function, and Implications in Health and Disease. Int J Mol Sci. 2017;18(12).
513. Calvo N, Volosin M. Glucocorticoid and mineralocorticoid receptors are involved in the facilitation of anxiety-like response induced by restraint. Neuroendocrinology. 2001;73(4):261-71.
514. Chrousos GP. The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. N Engl J Med. 1995;332(20):1351-62.
515. McCann SM, Lyson K, Karanth S, Gimeno M, Belova N, Kamat A, et al. Mechanism of action of cytokines to induce the pattern of pituitary hormone secretion in infection. Ann N Y Acad Sci. 1995;771:386-95.
516. Monje ML, Toda H, Palmer TD. Inflammatory blockade restores adult hippocampal neurogenesis. Science. 2003;302(5651):1760-5.
517. Munoz-Durango N, Vecchiola A, Gonzalez-Gomez LM, Simon F, Riedel CA, Fardella CE, et al. Modulation of Immunity and Inflammation by the Mineralocorticoid Receptor and Aldosterone. Biomed Res Int. 2015;2015:652738.
518. Tuckermann JP, Kleiman A, McPherson KG, Reichardt HM. Molecular mechanisms of glucocorticoids in the control of inflammation and lymphocyte apoptosis. Crit Rev Clin Lab Sci. 2005;42(1):71-104.

519. Wynne BM, Kronk TA, Al-Khalili O, Mallick R, Ko B, Eaton DC, et al. Interleukin-6 Transactivation of the Mineralocorticoid Receptor. The FASEB Journal. 2016;30(1_supplement):1223.3-.3.
520. Abbott AN, Welsh KJ, Hwang SA, Ploszaj P, Choudhury T, Boyd S, et al. IL-6 mediates 11betaHSD type 2 to effect progression of the mycobacterial cord factor trehalose 6,6'-dimycolate-induced granulomatous response. Neuroimmunomodulation. 2011;18(4):212-25.
521. Kossintseva I, Wong S, Johnstone E, Guilbert L, Olson DM, Mitchell BF. Proinflammatory cytokines inhibit human placental 11beta-hydroxysteroid dehydrogenase type 2 activity through Ca2+ and cAMP pathways. Am J Physiol Endocrinol Metab. 2006;290(2):E282-8.
522. Yu HM, Wang Q, Sun WB. Silencing of FKBP51 alleviates the mechanical pain threshold, inhibits DRG inflammatory factors and pain mediators through the NF-kappaB signaling pathway. Gene. 2017;627:169-75.
523. Yang L, Wang M, Guo YY, Sun T, Li YJ, Yang Q, et al. Systemic inflammation induces anxiety disorder through CXCL12/CXCR4 pathway. Brain, behavior, and immunity. 2016;56:352-62.
524. Kritas SK, Saggini A, Cerulli G, Caraffa A, Antinolfi P, Pantalone A, et al. Corticotropin-releasing hormone, microglia and mental disorders. Int J Immunopathol Pharmacol. 2014;27(2):163-7.
525. Tanaka J, Fujita H, Matsuda S, Toku K, Sakanaka M, Maeda N. Glucocorticoid- and mineralocorticoid receptors in microglial cells: the two receptors mediate differential effects of corticosteroids. Glia. 1997;20(1):23-37.
526. Donzis EJ, Tronson NC. Modulation of learning and memory by cytokines: signaling mechanisms and long term consequences. Neurobiol Learn Mem. 2014;115:68-77.
527. Mooradian AD. Effect of aging on the blood-brain barrier. Neurobiol Aging. 1988;9(1):31-9.
528. Baufeld C, Osterloh A, Prokop S, Miller KR, Heppner FL. High-fat diet-induced brain region-specific phenotypic spectrum of CNS resident microglia. Acta Neuropathol. 2016;132(3):361-75.
529. Schwanhausser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, et al. Global quantification of mammalian gene expression control. Nature. 2011;473(7347):337-42.
530. Straub RH, Cutolo M. Glucocorticoids and chronic inflammation. Rheumatology (Oxford). 2016;55(suppl 2):ii6-ii14.
531. Remus JL, Dantzer R. Inflammation Models of Depression in Rodents: Relevance to Psychotropic Drug Discovery. Int J Neuropsychopharmacol. 2016;19(9).
532. Liu YZ, Wang YX, Jiang CL. Inflammation: The Common Pathway of Stress-Related Diseases. Front Hum Neurosci. 2017;11:316.
533. Sankowski R, Mader S, Valdes-Ferrer SI. Systemic inflammation and the brain: novel roles of genetic, molecular, and environmental cues as drivers of neurodegeneration. Front Cell Neurosci. 2015;9:28.
534. Roubertoux PL, Martin B, Le Roy I, Beau J, Marchaland C, Perez-Diaz F, et al. Vocalizations in newborn mice: genetic analysis. Behavior genetics. 1996;26(4):427-37.