Effect of High-Fat Diet-Induced Obesity on Maternal Mouse Behaviour Prepartum and Postpartum

by

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Dedicated to my mother and my late father,

for their endless love, support and encouragement.

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ABSTRACT

Background: Obesity is prevalent in women of childbearing age. Approximately 18.5 to 38.5% of women in North America are considered to be obese at the time they become pregnant. Obesity in pregnancy increases the risk of developing maternal mental behavioural disorders, which include anxiety and depression. These disorders may be influenced by changes in lactogen levels and signalling during pregnancy and at time of delivery. Brain-derived neurotrophic factor (BDNF) is one possible biomarker of mental health problems. Low serum BDNF levels during pregnancy are associated with depression during the peripartum period. Here, a high-fat diet (HFD)-induced mouse model of obesity was used to study the relationship between HFD and maternal behaviour outcome. It was ***hypothesized*** that HFD-induced obesity in pregnancy increases risk of impaired maternal behaviour, an anxiety-like phenotype, compromised working memory and anhedonia that is associated with a reduction in total brain BDNF levels in CD-1 mice prepartum and postpartum. It was further ***hypothesized*** that increasing placental lactogen availability will reduce risk of impaired maternal behaviour.

Approach: Four-week old wild-type CD-1[WT] and CD-1[171hGH/CS] mice, which contain a transgene that includes genes coding for human placental lactogen, were fed a HFD (fat=60 kcal%; carbohydrate=20 kcal%; protein=20 kcal%) or regular chow diet (RCD; fat=14 kcal%; carbohydrate=60 kcal%; protein=26 kcal%) throughout the study. Mice were bred after five weeks on the diet. Maternal behaviour was assessed in all mice by testing nest building prepartum, followed by nursing and pup-retrieval behaviour postpartum. Anxiety-like behaviour was assessed prepartum and postpartum via an elevated-plus maze (EPM) test. Working memory was assessed

using a novel object recognition (NOR) test postpartum. Anhedonia was assessed pre-weaning and post-weaning in the postpartum period using a sucrose preference test. BDNF RNA levels were measured by quantitative real-time reverse transcriptase-polymerase chain reaction and total brain protein levels via BDNF-specific enzyme linked immunosorbent assay and, through collaboration, by protein immunoblotting.

Results: HFD-induced obesity impaired nest building and pup-retrieval behaviour in CD-1[WT] mice but had no significant negative effect on these behaviours in CD-1[171hGH/CS] mice. HFD-induced obesity impaired postpartum anxiety-like behaviour, working memory, and was associated with anhedonia in dams pre-weaning in the CD-1[WT] mice. Conversely, HFD-induced obesity did not impair anxiety-like behaviour and working memory in CD-1[171hGH/CS] mice. An increase in brain BDNF levels in non-pregnant and pregnant CD-1[WT] mice on HFD was observed in the postpartum period. However, in the CD-1[171hGH/CS] mice, higher BDNF levels were seen prepartum in non-pregnant and pregnant mice on a RCD, but only in the pregnant mice postpartum.

Conclusions: A negative effect of HFD-induced obesity on maternal behaviour, postpartum anxiety, working memory and anhedonia was observed in CD-1[WT] but not CD-1[171hGH/CS] mice. Furthermore, the negative effects observed on maternal behaviour in CD-1[WT] mice, and specifically pup retrieval, is associated with increased total brain BDNF levels in the postpartum period. This increase was not seen in the CD-1[171hGH/CS] mice where pup-retrieval was unaffected by HFD. This raises the possibility that the lack of response in the CD-1[171hGH/CS]

mice to the negative effects induced by HFD in the in the CD-1[WT] mice might be mitigated by a direct or indirect effect of the products of the transgene, including on brain BDNF levels.

ABBREVIATIONS

β	beta
°C	degree Celsius
%	percentage
a.m.	ante meridiem
ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
BMI	body mass index
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
cm	centimetre
CO_2	carbon dioxide
CS	chorionic somatomammotropin
DI	discrimination index
DNA	deoxyribonucleic acid
DOC	sodium deoxycholate
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPM	elevated plus maze
GD	gestation day
g	gram

GH	growth hormone
GTT	glucose tolerance test
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hGH	human growth hormone
HFD	high-fat diet
hPL	human placental lactogen
i.p.	intraperitoneal injection
kcal	kilocalorie
kDa	kilodalton
kg	kilogram
L	litre
mBDNF	mature brain-derived neurotrophic factor
µL	microliter
µg	microgram
µM	micromolar
mM	millimolar
ml	millilitre
mRNA	messenger ribonucleic acid
n	sample size
NaCl	sodium chloride
ng	nanogram
nm	nanometer
NOR	novel object recognition

NP-40	nonylphenoxypolyethoxyethanol (tergitol)
OD	optical density
PBS	Dulbucco's Phosphate Buffered Saline
PD	postpartum day
pH	potential hydrogen
PL	placental lactogen
p.m.	post meridiem
Ponceau Red	Ponceau S, Acid Red 112 with the formula 3-hydroxy-4-(2-sulfo-4-[4-sulfophenylazo]phenylazo)-2,7-naphthalenedisulfonic acid sodium salt
PPD	peripartum depression
proBDNF	precursor brain-derived neurotrophic factor
RCD	regular chow diet
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
sdH_2O	sterile distilled water
SDS	sodium dodecyl sulfate
S-HRP	streptavidin horseradish peroxidase
TG	transgenic
TrkB	tropomyosin-related kinase receptor type B
WT	wild-type

Please note that all abbreviations will be re-introduced in each chapter as needed.

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CHAPTER 1

Introduction

1.1 The global health problem: obesity

Obesity is a global health problem and is considered to be an epidemic [1]. This medical condition affects more than 30% of people worldwide and is associated with increased risk of complex, chronic health issues such as metabolic disease, hypertension and heart failure [1-3]. Obesity is normally a direct result of excessive caloric intake, specifically high-fat foods and decreased physical activity and energy expenditure [2]. In humans, obesity is often measured in terms of body mass index (BMI; weight in kilograms (kg)/ height in metres squared) of 30 kg/m^2 or higher or a waist-hip ratio ≥102 cm for men and ≥88 for women [2,4]. The prevalence of obesity is greater in women, than in men [5]. Obesity or overweight-status is becoming increasingly prevalent in women of child-bearing age (between the ages of 20-39) [6]. Approximately 18.5 to 38.5% of women in North America are obese at the time they become pregnant [7]. This is associated with an increased risk for additional health problems, like gestational diabetes, which can be characterized by abnormally high insulin resistance [8]. Other consequences of pre-pregnancy obesity include preeclampsia, fetal mortality and preterm birth [7,9]. However, one of the leading problems of pre-pregnancy obesity or overweight status are maternal mental health disorders [9], which include depression (specifically peripartum depression (PPD)) that can have negative affects both before and after parturition [7]. Pre-pregnancy obesity has also been associated with higher anxiety symptoms during pregnancy and in the postpartum period [10]. Previous research has shown that maternal mental health disorders are one of the major causes of death in pregnant women and within the first year postpartum [11].

In pregnancy, one example of a physiological stressor is ingestion of a high-fat diet [12], which can result in impaired placental function and harmful metabolic changes in children [13]. Maternal obesity can have long-term health complications for the offspring, as explained by the "developmental overnutrition hypothesis" [13]. This hypothesis suggests that increased glucose, fatty acids and amino acid levels at the maternal-fetal interface leads to irreversible changes in fetal neuroendocrine function and metabolism, increasing the risk of obesity in adult life [13]. Furthermore, children born to or raised by mothers with obesity and depression have an increased risk of impaired cognitive function, antisocial behaviour, and depression later in life [14]. In addition, high-fat diet has been linked to an increased risk of developing anxiety and depression [15]. Furthermore, it also associated with a reduction in hippocampal volume, which has the ability to affect both memory and cognitive function of the mother [15].

1.2 The relationship between obesity and placental hormones in pregnancy

During pregnancy, the human body undergoes physiological changes to support the fetus and prepare for birth [16]. These changes in maternal physiology are primarily mediated by the placenta, an organ responsible for delivering nutrients and removing waste from the fetus, as well as secreting hormones and growth factors to facilitate the pregnancy [16]. The human placenta releases hormones belonging to the growth hormone (GH) family [16], which play important roles in pregnancy and contribute to lactation and maternal behaviour outcomes [17]. This includes lactogens such as placental lactogen (PL) and prolactin [18]. Human (h) PL, also known as chorionic somatomammotropin (CS) hormone, is released predominantly by villous synctiotrophoblast and has the potential to influence metabolic changes such as insulin resistance, mobilization of free fatty acids as well as availability of amino acids and glucose for the fetus in pregnancy [16,19]. Prolactin is secreted from the anterior pituitary lactotrophic cells as well as the

decidua during pregnancy [17,20], and is important for lactation [21]. Both prolactin and PL levels increase throughout pregnancy, but PL levels are 50-fold higher than prolactin at parturition [22]. In the maternal circulation, plasma prolactin levels range from 6 ng/ml in early pregnancy to 210 ng/ml close to parturition in humans [23]. On the other hand, PL levels increase throughout pregnancy, with a serum concentration of 5000-7000 ng/ml close to end of pregnancy [24].

Obesity is one factor that can affect lactogen levels in pregnancy. In pregnant women with obesity or overweight status, a 40% reduction in serum human (h) PL protein levels was detected in the 28th week of pregnancy and reductions in term placental hPL RNA and protein levels were still detectable at term [8,25]. Specifically, the hPL levels in women with obesity or overweight status corresponded to the levels observed at 21 weeks of pregnancy in lean women [25]. A recent study also noted a 44% decrease in hPL ribonucleic acid (RNA) levels in placental tissue at term, which was associated with clinical depression [11]. Although not known, this presents the potential of hPL being a possible biomarker for the onset of mood disorders in the peripartum period.

1.3 Role of lactogenic signalling in maternal behaviours

Maternal behaviour is a broad term used to describe a range of behaviours expressed by the mother, in order to care and protect her newborn(s) during pregnancy and after birth. In humans, maternal behaviour includes preparing for the arrival of the baby, feeding/nursing and ensuring the safety of the baby [26]. These behaviours are often difficult to study in humans due to the ethical and privacy concerns, including but not only harm to either the health of the mother or baby [27]. Hence, animal models have been largely used to study mother-infant relationships [27]. Studies in rodents have proven valuable in providing insights into the relationship between the mother and baby in humans [27].

In rodents, maternal behaviour encompasses several elements, which includes nest building, gathering or retrieval of the pups, nursing behaviour, pup grooming and protecting the pups from dangerous stimuli [21]. These behaviours can be comparable to those in humans. Nest building is essential for shelter, reproduction and thermoregulation and, in female mice, is often influenced by hormones and pregnancy-status [28]. Pregnant female mice build large brood nests with multiple entrances, which are considered to be "maternal nests" [29]. Nest building behaviour can be assessed in many ways, of which the most common is to provide mice with a cotton sheet (nestlet) overnight [28]. The nest quality is assessed the following morning based on an existing rating scale [28].

Retrieval behaviour is a characteristic of dams (female mice that recently gave birth), as they will retrieve any pup that is missing from the nest [29]. Hence, maternal behaviour can be assessed using a pup retrieval test, in which pups are removed and placed away from the nest, and the time taken by the dam to retrieve each or all of her pups is recorded [30].

Nursing or feeding of the newborn pups is an essential aspect of maternal behaviour [31]. In fact, during the first 3 weeks after giving birth, dams spend greater than 90% of their time nursing [29]. Dams can nurse using different postures, including arched-back, blanket, and supine nursing, in which all positions require direct contact with the pups [31]. Dams also spend time grooming the pups by anogenital licking or body licking [31]. Thus, time spent nursing can be assessed as a measure of maternal behaviour.

These maternal behaviours are thought to be primed by lactogenic signalling through the prolactin receptor in the rodent brain in pregnancy [21,32-38]. Prolactin receptors are transmembrane receptors, belonging to the cytokine receptor family [17]. Prolactin and PL both carry out their actions by binding to the prolactin receptor, but in humans, PL has ten times greater affinity to the prolactin receptor than prolactin itself in pregnancy [33] and is present at greater

concentrations in the maternal circulation [24]. Both prolactin and PL are 22 kDa in size [39,40] and it is assumed that prolactin enters the cerebrospinal fluid through a carrier-mediated process, and accesses the brain at the choroid plexus [34]. The high levels of hPL in the maternal circulation presumably translates into greater levels crossing the blood brain barrier (also referred to as the neurovascular unit). Hence, hPL is detected in the cerebral spinal fluid, likely given its size and similarity to hGH through a process of simple diffusion [41]. In mice, ablation of the prolactin receptor prevents lactogenic signalling and has been shown to impair nursing and pup retrieval behaviour in mice [33]. In contrast, a mutation in the prolactin gene did not disrupt maternal behaviour [42]. The fact that maternal behaviour remains intact even in the absence of prolactin implicates the role of other ligands that can bind to and signal via prolactin receptors [43]. Hence, it is possible that lactogens, such as PL can compensate for deficits associated with maternal behaviours or alternatively may normally play a more important role at least prepartum.

Prolactin is crucial for the development of the mammary gland for production of milk in pregnancy and secretion of milk after birth [21]. In rodents, during pregnancy, prolactin is secreted in nocturnal and diurnal surges until mid-pregnancy, when mouse PL secretion dominates [21]. In mice, two of the known PLs in pregnancy include prolactin-related PL-I (Prl3d1) and PL-II (Prl3b1) [44,45]. PL-I and PL-II are produced by trophoblast giant cells of the placenta [45]. PL-I increases from the start of pregnancy until about mid-gestation, when it rapidly declines, and is replaced by PL-II which increases until parturition [45]. Close to parturition, prolactin levels also increase once again in rodents, as it is important for initiation of maternal behaviour postpartum [35]. The suckling stimulus by the pups after birth initiates milk letdown [22]. Prolactin action is mediated by the prolactin receptor in the brain [17], specifically, suckling can activate neuronal pathway to brain regions that are involved in maternal behaviour [21]. The medial preoptic area of the hypothalamus is one major region where prolactin action through the prolactin receptor is crucial

for expression of normal maternal behaviour postpartum, including nursing behaviour [32]. Deletion of the prolactin receptor in the medial preoptic area results in dams abandoning their pups and reduced nursing behaviour postpartum [32]. In addition, a reduction in prolactin levels in early pregnancy can increase maternal anxiety and impair maternal behaviour, as measured by pup retrieval, in the postpartum period [36].

1.4 Effect of high-fat diet-induced obesity on maternal behaviours

Hight-fat diet (HFD) consumption in pregnancy has been linked with damaging effects on maternal behaviour in mice, such as an increase in cannibalistic behaviour and maternal mortality in the perinatal period of wild-type C57BL6/J mice [46]. A HFD can also modify gene expression, growth of the placenta, transport of nutrients to the fetus and cause fetal overgrowth in C57BL/6J mice [47]. A 15% survival rate was observed in newborn pups of female C57BL/6 mice exposed to HFD for approximately 12 weeks [48]. A recent study found that a HFD of approximately 11 weeks increased disorganized behaviour in dams postpartum [49]. Specifically, these dams spent more time engaging in self-directed behaviour (such as grooming and feeding) and less time engaging in pup-directed behaviours [49]. In addition, during a pup retrieval test, dams fed HFD had a higher latency to sniff the first pup, when compared to dams on regular-chow diet (RCD) [49]. Similarly, dams exposed to chronic HFD had a higher latency to retrieve pups back to the nest [48].

HFD-induced obesity has negative consequences on breastfeeding and mammary gland function in humans [50]. Women with a higher BMI experience earlier termination of breastfeeding in the postpartum period, due to lactation failure or insufficient milk production [51]. In fact, the response of prolactin to suckling is more critical for production of milk, than for lactogenesis [52]. Similarly, recent studies in mice have shown that a diet high in fat may impair

mammary development and lactogenesis, involving a decrease or absence of milk production [50]. Female C57BL/6 mice exposed to chronic HFD experienced a 33% decrease in milk production and mammopoiesis [48]. Taken together, these observations suggest that HFD-induced obesity may impair nursing behaviour outcome in postpartum female mice. These studies, however, did not look at the effect of a HFD on prepartum maternal mouse behaviour including the effect of a HFD on nest building in pregnancy.

1.5 Relationship between HFD-induced obesity and anxiety

Anxiety is defined as the body's natural response to stress, and is often characterized by excessive worrying and fear, fatigue and discomfort in the chest [53]. Obesity or overweight status has also been correlated with anxiety disorders in humans [54]. This could be due to the detrimental effects of obesity on health and life quality, or discrimination and stigma associated with obesity [54]. Anxiety and depression are associated with obese women with a BMI of 30, but in men with a BMI of 40 higher [55]. Hence, women with obesity appear to be more susceptible to anxiety disorders than men. Pregnancy is a critical time when women may experience increased anxiety, especially if they are under a lot of additional stress [56]. This increase in anxiety has been linked to premature birth and low birth weight of the baby, which can increase the risk of cognitive dysfunction and poor social development later in life [57]. Anxiety can also have a negative effect on the mother's ability to bond with the newborn baby [58]. However, due to the heterogenous nature of obesity and anxiety, the association between them [10,53], and similarly whether this would exacerbate or influence any anxiety associated with pregnancy remains unclear.

Rodent models have been used to study anxiety-like behaviour, which are behaviours that reflect an anxiety state in humans [59]. Both anxiety and fear elicit similar behavioural reactions that include increased attentiveness, decreased activity, freezing, and decreased feeding [59]. The

amygdala is a region of the brain known to process fear and stress-like behaviours, whereas, the bed nucleus of the stria terminalis processes anxiety-like behaviours [60]. Many behavioural tests can be used to assess anxiety-like behaviours in rodents, but the most common tests measure approach-avoidant behaviours [61]. This includes the elevated plus maze (EPM) test, which appeals to the innate preference of rodents to prefer dark, enclosed spaces and distaste towards elevated, open and bright places [61]. The EPM is a plus-shaped maze elevated a few feet from the ground and is composed of two open arms (absence of walls) and two closed arms (presence of walls) [62]. Increased time spent on the closed arms indicates increased anxiety-like behaviour [62].

HFD-induced rodent models of obesity have been established to study the relationship between HFD and anxiety-like behaviours. A HFD of 3 weeks has been shown to increase anxiety-like behaviour in C57BL/6J male mice [63]. However, pregnant C57BL/6J showed no difference in anxiety-like behaviour during mid-pregnancy after being fed a HFD or RCD for approximately 11 weeks [49]. To date, there is a paucity of studies investigating the effect of HFD-induced obesity or overeating status on anxiety-like behaviour in pregnancy. Specifically, there are no studies in the postpartum period in mice, and, thus, this warrants further investigation.

1.6 Effect of HFD-induced obesity on memory

Obesity has been associated with impaired cognition in individuals either early in life or mid-life [64]. This can affect memory, attention, and decision-making abilities [65]. It can also increase the risk of developing dementia in late adulthood [66]. Individuals with obesity exhibit deficits in executive functions, which consists of working memory, decision-making, and problem solving [67,68]. Working memory is defined as the ability of the brain to retain/store information temporarily that is essential for cognitive tasks such as learning, reasoning and understanding

language [69]. Working memory is involved in short-term memory and helps to store and process incoming information [69]. A sex-related difference has been noted in memory function [70]. A recent study found that women with obesity score lower on working memory tasks, in comparison to men with a similar BMI [70]. Likewise, females with higher body fat percentage score lower on executive function tests than males [71,72]. Moreover, impaired working memory has been associated with increased ingestion of fat-rich foods and decreased consumption of healthier foods, such as fruits and vegetables [73]. This can exacerbate the risk of developing obesity. Functional magnetic resonance imaging studies have implicated a possible role of impaired hippocampal function in poor working memory outcome in older adults [74].

The hippocampus is located in the temporal lobe of the brain and plays a role in learning and memory (Figure 1.6) [75]. The hippocampus receives cortical inputs from the perirhinal, parahippocampal and entorhinal cortices (Figure 1.6) [75,76]. The perirhinal cortex helps with illustrating complex objects, as well as with object recognition and storing information about objects [75]. The parahippocampal cortex plays a role in processing visuospatial information [75]. The entorhinal cortex plays a role in perception and is the point at which information enters and leaves the hippocampus [75]. Lesions to the perirhinal cortex and hippocampus can impair recognition memory [77,78].

Rodent models have been established to study the effect of HFD on different behaviours associated with memory function in humans [79]. One commonly used test is the novel object recognition (NOR) test. This test occurs over a period of three days, during which rodents are habituated to the apparatus on day 1, exposed to two similar objects on day 2 and presented with a novel and familiar object on day 3 [80]. The time spent interacting with each object is recorded on day 3, and provides insight into recognition memory [80]. Recognition memory relies on information about the object including its location, appearance and previous interaction with it

[77]. The NOR test relies on the innate ability of the animal (without any external rewards or motivation) to prefer or explore the novel object [81]. This suggests that the animal has an intact memory of the familiar object, and hence will express curiosity and tendency to explore the novel object [81]. Behaviour on the NOR apparatus is influenced by lesions in the hippocampus and surrounding cortices [82]. Specifically, lesions in the perirhinal cortex results in impaired object recognition memory in rats [83]. A diet high in fat can also impair spatial learning and memory in CD-1 male mice [84]. However, little is known about the effect of HFD-induced obesity on memory and learning in female mice during or after pregnancy and, thus, requires further investigation.

Figure 1.6

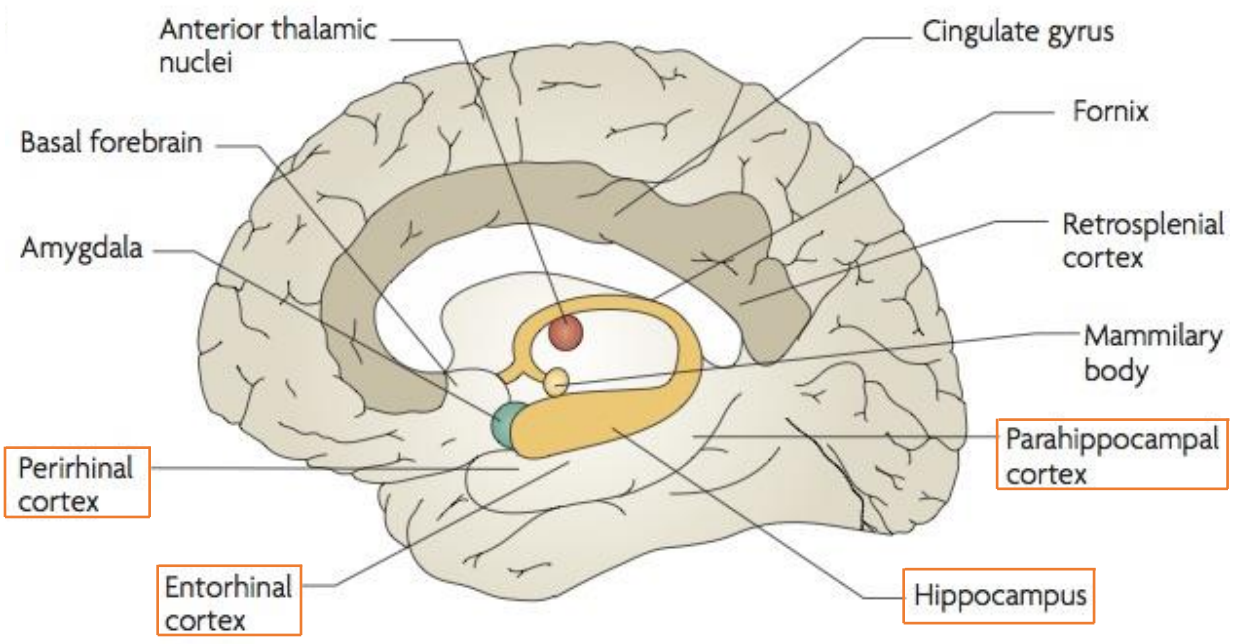

Figure 1.6. The hippocampus and it's cortical inputs. The hippocampus is located in the medial temporal lobe of the brain. The hippocampus is surrounded by and receives cortical inputs from the perirhinal, parahippocampal and entorhinal cortices. Figure 1.6 is reproduced with permission from [75] and has been adapted for this thesis.

1.7 Relationship between HFD-induced obesity and depression onset

Major depression is a type of mood disorder, that's characterized by feelings of sadness, loss of interest in pleasurable activities (anhedonia), changes in appetite or sleep, excessive fatigue, trouble concentrating and suicidal thoughts [85,86]. Many studies have established an association between obesity and depression in humans. In fact, obesity has been found to increase risk of depression, while depression increases the risk of developing obesity [4]. The prevalence of major depressive disorder is almost two times greater in women (21.3%) than men (12.7%), and this difference continues to the time when women are of child-bearing age [87].

Approximately 25% to 35% of women experience depressive symptoms during pregnancy, which resemble changes brought on by pregnancy making it difficult to diagnose [88]. This includes fatigue, changes in appetite or sleep and irritability [87]. However, history of depression, age, lack of social support and marital problems are few additional factors that can exacerbate depressive symptoms in the peripartum period [86]. Pre-pregnancy obesity in women has been associated with increased risk of antenatal depression and postpartum depression within 4 weeks of giving birth [10,89]. Hence, additional mental health screening measures should be in place for women with high pre-pregnancy weight, to allow for early intervention and treatment. Currently, effective, fast-acting and affordable therapies to treat depression are lacking, and this can be attributed to the limited understanding of the underlying mechanisms involved in this disorder [90].

Research suggests that impairments in brain regions regulating mood and emotions may underlie the pathophysiology of depression [85]. However, there is no consensus on one single brain region that may be directly associated with depression. This suggests a role of many brain regions, including those of the limbic system, in mediating depression symptoms [91]. Brain imaging studies and post-mortem autopsies in humans independently suggest altered blood flow and abnormalities in the hippocampus, hypothalamus, thalamus, amygdala, prefrontal cortex and

striatum [91,92]. The hippocampus may play a role in onset of impaired memory, feelings of worthlessness or hopelessness and suicidal tendencies in depression [85]. Indeed, a reduction in hippocampal volume has been noted in depressed individuals [93]. Furthermore, dysregulation of the neurotransmitters, dopamine and serotonin, have also been associated with increased risk of depression [94,95].

Depression is a multifactorial disorder and is difficult to study in humans due to its heterogeneity. Hence, rodent models of depression have been established to study different endophenotypes of depression such as behavioural despair and anhedonia [90]. Behavioural tests, such as the forced swimming test and tail suspension test assess immobility, which reflects behavioural despair in mice [96]. Anhedonia, is a major symptom of depression in humans and is characterized by loss of interest or pleasure in activities [85]. In rodents, a sucrose preference test can be employed to assess anhedonia [96]. In this test, mice are given a choice to drink sucrose water or tap water [90]. Mice experiencing chronic stress have a reduced preference for sucrose water [97] and this is assumed to reflect an anhedonia-like state observed in humans [96]. In adult male Sprague-Dawley rats, a state of anhedonia was brought on by diet-induced obesity [98]. Similarly, male mice fed a HFD for 4 weeks experienced anhedonia [99]. However, little is known about the effect of HFD-induced obesity in pregnancy and postpartum on anhedonia-like behaviour in female mice.

1.8 Brain-derived neurotrophic factor: a biomarker of mental health disorders

Many studies have provided evidence to support the role of brain-derived neurotrophic factor (BDNF), which is a member of the neurotrophin family of growth factors, in the onset of psychiatric disorders, including major depression in humans [100,101]. BDNF is expressed throughout the body, including in many brain regions such as the hippocampus, amygdala,

hypothalamus and neocortex [101]. BDNF is synthesized from its precursor, preproBDNF (Figure 1.8) [100]. After the signal peptide is cleaved, it produces proBDNF (~32 kDa), which is further processed to produce mature BDNF (mBDNF; ~13 kDa) polypeptide and BDNF propeptide (~17 kDa) (Figure 1.8) [100]. The proBDNF binds to p75 neurotrophin receptor, and can prevent dendritic complexity, reduce hippocampal synaptic transmission and increase neuronal apoptosis [101]. Conversely, mBDNF binds to tropomyosin-related kinase receptor type B (TrkB) and supports neuronal differentiation, survival and plasticity [101]. The opposing effects of proBDNF and mBDNF on neurotransmission may underlie the onset of mood disorders, including anxiety and depression [102].

In humans, plasma and serum BDNF levels are significantly reduced in individuals with depression [103,104]. Similarly, humans with obesity have lower serum and plasma BDNF levels [105,106]. During pregnancy, serum BDNF levels decline from the first to the third trimester [107]. However, an additional reduction of serum BDNF levels in late pregnancy has been associated with depressive symptoms in the postpartum period [107]. In fact, 9 out of 13 studies have reported a decrease in serum BDNF levels during and after pregnancy in mothers with depression [102]. Currently, the effect of obesity on BDNF levels in pregnant women is unclear and requires preliminary investigations in animal models.

Rodent models have been established to study the effects of various stressors and antidepressant medications on BDNF levels in different brain regions [108]. A study in rats has shown that stress induced through a forced swimming test reduced BDNF messenger ribonucleic acid (mRNA) levels in the hippocampus [109]. However, treatment with antidepressants increased BDNF mRNA levels in the hippocampus, and made the rats more resilient as assessed by the forced swimming test [109]. A HFD in male BDNF mutant mice induced obesity, hyperphagia and a reduction in satiety [110]. BDNF levels are also reduced in hippocampal brain homogenate

of mice on a HFD [111]. Studies in rodents suggest that blood and plasma BDNF levels reflect homogenized brain BDNF levels [112]. Although these observations in non-pregnant rodents is suggestive, the effect of pregnancy on BDNF levels in mice is unclear. The effect of HFD-induced obesity on BDNF levels in mice prepartum and postpartum is unknown and requires further investigation.

Figure 1.8

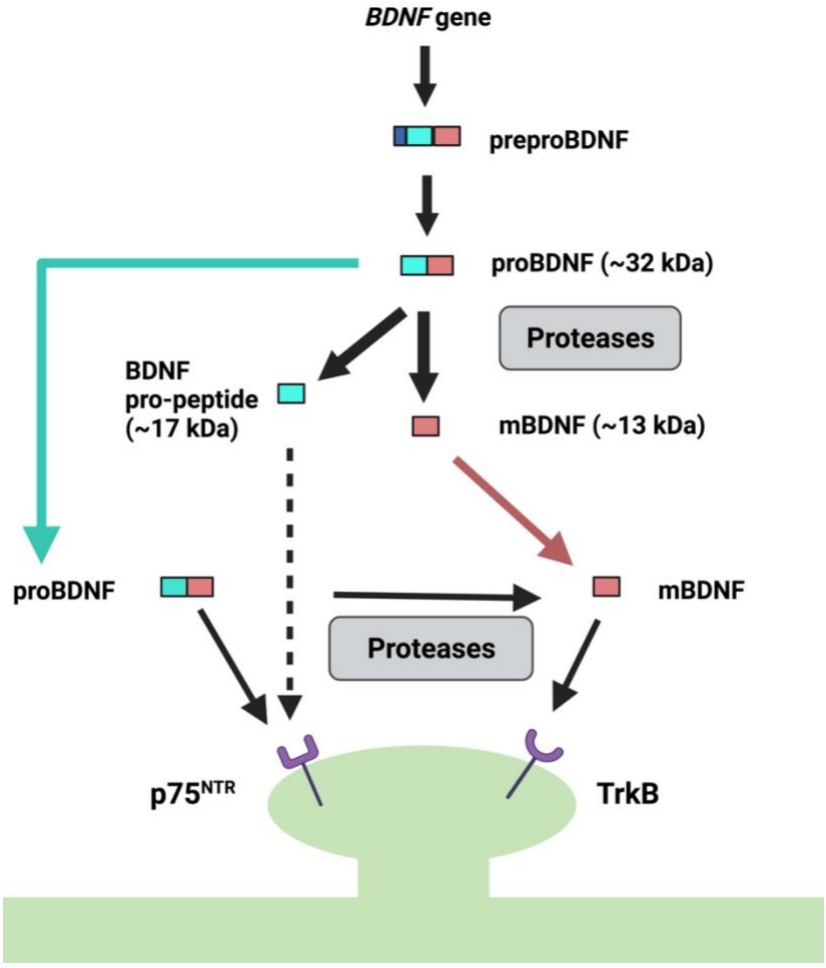

Figure 1.8. A schematic representation of the synthesis of BDNF from its precursor, preproBDNF. After the signal peptide is cleaved, it produces proBDNF (~32 kDa), which is further processed to produce the mature form of BDNF (mBDNF; ~13 kDa) and BDNF pro-peptide (~17 kDa). The proBDNF binds to p75 neurotrophin receptor (NTR), whereas the mature form of BDNF binds to tropomyosin-related kinase receptor type B (TrkB). Figure 1.8 is based on [100]. This figure was made with a paid subscription from biorender.com.

1.9 Animal model used in this study

To study the effect of HFD-induced obesity in pregnancy on maternal behaviour outcome, an acute wild type CD-1[WT] and transgenic CD-1[171hGH/CS] mouse model of obesity was used to compare the effects of a HFD versus regular chow diet (RCD). The CD-1[171hGH/CS] mouse is partially humanized, and differs from the CD-1[WT] mouse through the introduction of one copy of the hGH/PL gene locus in a 171 kilobase fragment of human chromosome 17 that was integrated on mouse chromosome 14 at band 14A1 (see Figure 1 in [113]). Thus, the 171hGH/CS transgene includes the pituitary and placental growth hormone genes (*hGH-N* and *hGH-V*) and both genes (*hPL-A* or *CSH1* and *hPL-B* or *CSH2*) that independently code for hPL [25]. Production of hPL by the placenta during pregnancy in the CD-1[171hGH/CS] mouse has been described in [114]. This mouse line was initially reported in 2009 and has been backcrossed two times over the last 12 years to the CD-1[WT] mice to maintain their genetic background [114]. Occasionally, these mice are inbred to maintain homozygosity of the transgene, but then backcrossed to the CD-1[WT] to maintain their genetic background.

Physiologically, the CD-1[WT] and CD-1[171hGH/CS] mice are similar. Both mice increase in weight when fed a HFD *versus* RCD, are able to breed, have impaired glucose clearance, and give birth to a standard litter size [115]. Moreover, CD-1[171hGH/CS] mice provide a unique opportunity to study the effect of a HFD *versus* RCD on a mouse in which hPL is being produced. Specifically, the CD-1[171hGH/CS] mouse provides the opportunity to gain insight into whether the reported decrease in hPL RNA levels seen with a HFD [115] is associated with anticipated negative effects of a HFD on maternal behaviours, as indicated in human pregnancies [11]. Alternatively, this mouse may provide some insight into possible positive effects of exogenous hPL on maternal behaviour, even with some HFD-related reduction in hPL gene expression [115]. Thus, in both mouse models, maternal behaviour (includes nest building, pup retrieval and nursing),

anxiety-like behaviour, working memory and anhedonia was assessed. A pilot study was also conducted to assess BDNF levels in whole brain homogenate in the CD-1[WT] and CD-1[171hGH/CS] mice prepartum and postpartum. Where appropriate, the effects of a HFD *versus* RCD on the behaviour of pregnant mice were compared to age-matched non-pregnant female CD-1[WT] or CD-1[171hGH/CS] mice.

1.10 Overview of research objectives and hypothesis

A negative correlation exists between overweight or obese status and the onset of mood disorders in humans. Women with pre-pregnancy overweight status and obesity are at a higher risk of developing mental health problems, including PPD [9]. PPD may result from the drastic hormonal changes during pregnancy and at birth [116]. A 44% decrease in hPL RNA levels is associated with clinical depression [11]. Furthermore, maternal obesity is associated with a 40% decrease in hPL RNA levels, which is consistent with overweight status and a link to increased risk for PPD [8]. One other possible biomarker of psychiatric diseases, including depression, is BDNF [117]. In humans, a reduction in serum BDNF levels in early pregnancy is associated with depression during pregnancy [107]. Furthermore, reduced serum BDNF levels in the third trimester (late pregnancy) is also associated with increased depressive symptoms [107]. However, the effect of obesity in pregnancy on serum and brain BDNF levels is unknown and will be pursued in the present study. Currently, PPD is difficult to diagnose (due to diverse mood phenotypes) and treat. Hence, it is important to understand the relationship between overweight/obesity status and maternal mental health disorders to help facilitate the identification of novel biomarkers and treatment options.

It is difficult to study the effect of acute HFD-induced obesity in pregnancy in humans due to the ethical implications on both the mother and the fetus. This includes assessing whole brain

BDNF levels, and maternal behaviours prepartum and postpartum. Furthermore, the consequence of introducing exogenous human PL in pregnant women is unknown and requires preliminary investigation in an animal model system. Thus, a mouse model system (CD-1[WT] and CD-1[171hGH/CS]) was proposed here to study the effects of HFD/overweight/obesity status on maternal behavioural outcome. CD-1[171hGH/CS] mice contain the hPL genes, and the specific expression of hPL in pregnancy is reduced significantly in mice on a HFD [115]. Thus, use of the CD-1[171hGH/CS] mouse presents an opportunity to begin to study the potential use of hPL as a marker of maternal behaviour and the effect of hPL in pregnancy on maternal behavioural outcome. The CD-1 mouse was selected as a background strain as it is (1) outbred, (2) has large genetic diversity, (3) excellent maternal traits and (4) gives birth to large litters.

PPD encompasses a variety of depressive phenotypes and it is difficult to study mood disorders, such as depression, directly in a mouse. Hence, a variety of behavioural phenotypes in mice that may be associated with mental health disorders in humans will be studied. This includes maternal behaviour, anxiety, working memory and anhedonia. Based on previous studies in rodents, the following well-documented maternal behaviour assays were selected as they can be translated to the human condition: nest building (preparing for the arrival of newborns), pup-retrieval (bringing the newborns back to safety when they have been displaced), and nursing (feeding of the newborns).

In rodents, anxiety, working memory, and anhedonia/depressive state cannot be assessed using self-diagnostic tools like questionnaires or surveys as done in humans. Instead, complex assays have been designed and validated to study each of these behaviours in mice. Hence, the elevated-plus maze (EPM) will be used to assess anxiety-like behaviour and novel object recognition (NOR) assay to evaluate working memory. A sucrose preference test will be employed to assess anhedonia and provide some indirect insight into the presence or absence of a depressive-

like state in these mice. In addition, for the first time, the effect of acute HFD-induced obesity on total brain BDNF levels before, during and after pregnancy will be assessed.

Hypotheses

It is ***hypothesized*** that HFD-induced obesity in pregnancy increases risk of impaired maternal behaviour. This will include an anxiety-like phenotype, compromised working memory and anhedonia that is expected to be associated with a reduction in total brain BDNF levels in CD-1 [WT] and [171hGH/CS] mice. It was further ***hypothesized*** that increasing placental lactogen availability will reduce risk of impaired maternal behaviour. These hypotheses were pursued through five research objectives:

Objectives

1. To establish a model system of overeating or HFD-induced obesity, in CD-1[WT] mice (Chapter 3) and CD-1[171hGH/CS] mice (Chapter 4).

Approach A: To determine if mice on HFD gain significantly more weight than mice on RCD

i) All mice will be weighed weekly to assess the amount of weight gained on HFD *versus* RCD. Non-pregnant and pregnant mice on the RCD or HFD will be compared.

Approach B: To assess whether mice on HFD have developed a state of insulin resistance, often a consequence of acute/chronic HFD consumption associated with overweight or obese status, a glucose tolerance test will be performed.

i) A glucose tolerance test will be conducted in mice before and during pregnancy.

2. To investigate whether maternal behaviour is impaired in CD-1[WT] mice (Chapter 3)

and rescued in CD-1[171hGH/CS] mice (Chapter 4) both prepartum and postpartum.

Approach A: Nest building will be assessed prepartum.

i) The criteria to score nest quality will be developed, based on existing literature and initial observations made in a pilot study.

Approach B: To confirm whether maternal behaviour is intact postpartum, nursing behaviour will be assessed.

i) Based on the quality of the recorded videos, assessment parameters for nursing behaviour will be determined.

Approach C: To assess whether pup-retrieval behaviour is intact in dams postpartum.

i) The first day of the assay will reflect immediate maternal response to displaced pups.
ii) Pup-retrieval behaviour, as assessed over a period of three days, to see if the dam's behaviour changes.

3. To determine whether CD-1[WT] mice (Chapter 3) and CD-1[171hGH/CS] mice (Chapter 4) exhibit anxiety-like behaviour, impaired working memory and anhedonia.

Approach A: Anxiety-like behaviour will be assessed and postpartum on the EPM.

i) During the test, the first day assesses anxiety-like behaviour, whereas the subsequent days provide insight into learned behaviour.
ii) Assessment parameters will be determined, and a template will be created using ANYMaze software. Inclusion and exclusion criteria will be defined.

Approach B: Working memory will be evaluated postpartum using the NOR test.

i) Mice will be habituated to the apparatus on day 1, familiarized to two identical objects on day 2, and introduced to a novel and familiar object on day 3.

ii) Assessment parameters will be determined, and a template will be created using ANYMaze software. The inclusion and exclusion criteria will also be defined.

Approach C: Anhedonia will be assessed postpartum using a sucrose preference test pre-weaning and post-weaning.

i) Based on the literature, a sucrose preference formula, as well as inclusion and exclusion criteria will be determined.

4. To determine BDNF RNA levels in CD-1[WT] mice (Chapter 3) in pregnancy.

i) The assay will be optimized first to assess BDNF RNA levels in non-pregnant mice.

ii) RT-PCR will be performed for quantitation of BDNF. Relative RNA levels will be compared amongst all mouse groups.

5. To determine BDNF protein levels in whole brain of CD-1[WT] mice (Chapter 3) and CD-1[71hGH/CS] mice (Chapter 4) before, during and after pregnancy.

i) Whole brain homogenization using a RIPA buffer will be optimized.

ii) ELISA will be used to assess BDNF protein levels in whole brain supernatant. The ELISA will be optimized to determine optimal protein loading amount.

The observations made will be discussed in the context of the effect of HFD-induced obesity or overweight status in CD-1 mice, and whether if the presence of hPL in the CD-1[171hGH/CS] modifies the effects of HFD-induced obesity on behaviour.

CHAPTER 2

Materials and Methods

2.1 Animal model

Procedures that involved animals/tissue were in accordance with the "Guide for the Care and Use of Laboratory Animals published by the Canadian Council on Animal Care" [118]. Before starting the study, all procedures were approved by "Animal Protocol Management and Review Committee at the University of Manitoba" [118] (Protocol #19-018 and #20-012). A resource equation was used to determine the number of mice (n) required for the various experimental tests [119]. Approximately four-week-old female CD-1 mice, either wild-type CD-1[WT] or CD-1[171hGH/CS], were fed a high-fat diet (HFD; Research Diets, D12492; fat=60 kcal%; carbohydrate=20 kcal%; protein=20 kcal%) and regular-chow diet (RCD; Pro-lab, RMH3000 5P00; fat=14 kcal%; carbohydrate=60 kcal%; protein=26 kcal%) for five weeks. Before breeding, mice were separated into the following four groups: pregnant-HFD, pregnant-RCD, non-pregnant-HFD and non-pregnant-RCD [118]. In the prepartum or gestation period, the 'pregnant mice' are pregnant. However, in the postpartum period, 'pregnant mice' refers to mice who recently gave birth or had undergone the pregnancy experience. All mice were housed in pairs prior to breeding, and individually after becoming pregnant, with *ad libitum* access to water and food in a temperature-controlled room, on a 12-hour light/dark cycle (lights on at 6 a.m. and lights off at 6 p.m.) [118]. CD-1[WT] mice underwent a series of behavioural tests (Figure 2.1 A), of which the majority were also conducted in the CD-1[171hGH/CS] mice (Figure 2.1 B). These behavioural tests occurred in the prepartum (during pregnancy) and postpartum period (after parturition).

Figure 2.1

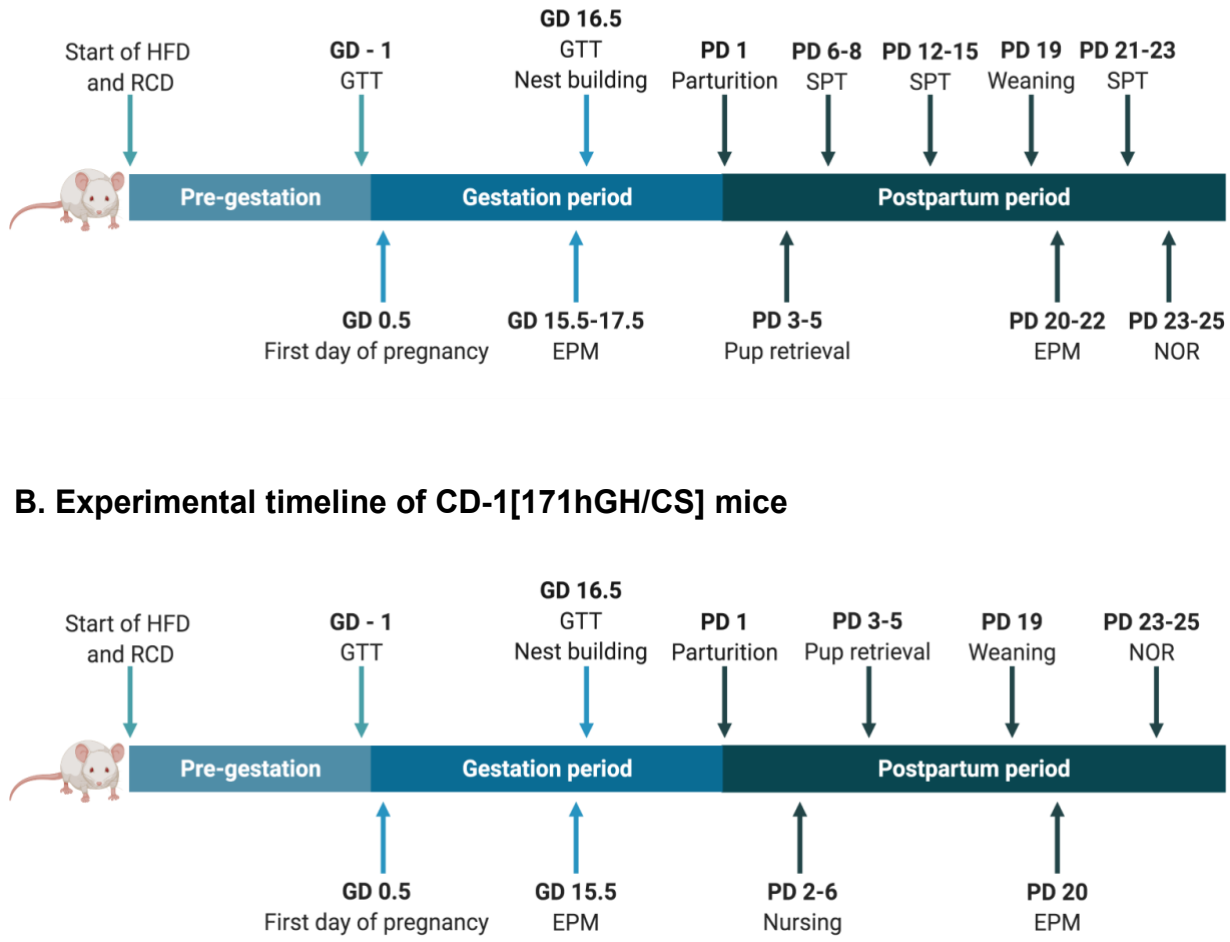

Figure 2.1. Timeline of the behavioural tests conducted in **(A)** CD-1[WT] and **(B)** CD-1[171hGH/CS] mice. Mice were placed on a high-fat diet (HFD) or regular chow diet (RCD) for five weeks. Mice underwent a glucose tolerance test (GTT) before breeding. Following this, mice were bred, and pregnancy was confirmed on gestation day (GD) 0.5. During pregnancy (gestation or prepartum) and after pregnancy (postpartum), CD-1[WT] and CD-1[171hGH/CS] mice underwent a series of similar behavioural tests. In general, during the gestation period, mice underwent GTT, nest building and elevated plus maze (EPM) assessment. After parturition, mice underwent further behavioural testing from postpartum days (PD) 2-25. This included nursing, pup retrieval, a sucrose preference test (SPT), EPM, and a novel object recognition test (NOR). This figure was made with a paid subscription from biorender.com.

2.1.1 Breeding

After five weeks on their respective diets, age-matched male CD-1[WT] and CD-1[171hGH/CS] mice on a RCD were introduced into the female cages at 3 p.m. and allowed to breed overnight in a 1:2 (male:female) ratio [118]. The next morning at 9 a.m., the females were checked for the presence of a vaginal mucous plug and, if present, were presumed to be pregnant considered to be at gestation day (GD) 0.5. The plug positive females were then housed individually in a new room in the animal facility. Mice were monitored on a regular basis for evidence of grooming, feeding, injury and activity, and were weighed weekly. Mice were allowed to continue the HFD or RCD from the beginning till the end of the study. Pregnant and non-pregnant females were tested. The day the females gave birth was considered as postpartum day (PD) 1. The litters of each dam were counted staff in the animal facility, and in some cases, also culled to 8 pups. All pups were euthanized by CO_2 overdose by the animal facility staff postweaning. All adult female mice were euthanized by decapitation.

2.2 Glucose tolerance test (GTT)

A GTT was performed on CD-1[WT] and CD-1[171hGH/CS] mice on RCD and HFD at GD -1 (before mating/pregnancy) and at GD 16.5 as described previously [115]. Briefly, at 5:30 p.m., all mice were weighed, and food was removed from the cages. Mice were then allowed to fast for approximately 16 hours overnight with only water available. The following morning at 9 a.m., a GTT was performed using 2 g/kg of i.p. glucose (Sigma, G7258) dissolved in double-distilled water. Blood was collected from a vein in the tail and glucose level was assessed at 0 minutes, which was before the glucose injection was administered. After the glucose injection, glucose levels were monitored at 15, 30, 60, 90, and 120 minutes using a glucose meter (OneTouch Ultra2 Glucose Monitoring System, Johnson & Johnson, Lifescan, Inc) [115,118], which provides

you with the blood glucose level in millimolar (mM).

2.3 Nest building

Nest building was used to evaluate maternal behaviour prepartum at GD 16.5 and was assessed only in the pregnant groups in CD-1[WT] and CD-1[171hGH/CS] mice. On GD 16.5, at approximately 3 p.m., pregnant mice were given 3 g of nesting material (Ancare Corporation, NES3600) and evaluated for nest building at 9 a.m. on the following day [118]. Based on a pilot study, the nest building scoring criteria were modified from [28] to include: amount of shredded nesting material (scored out of 2), quality of the nest (height and shape) (scored out of 5), and usage of the nesting material for the nest (scored out of 2) (Table 1) [118]. The highest score possible was 9, which indicates a "perfect nest" (Figure 2.3 A) and the lowest score possible was 2 or less which represents "a poor quality or non-existing nest" (Figure 2.3 B) [118].

Table 1. Nest building scoring criteria. This chart has been reproduced with permission from [118] and adapted for the presentation of this thesis.

Nestlet Shredding						
	<50%		50% to 90%		>90%	
Score:	0		1		2	
Nest Quality						
	No nest	Full but flat nest occupying at least ¼ of the cage	Half flat and half raised (walls are half of the mouse is body height when the mouse is curled up on its side)	Fully raised nest (greater than the mouse's body height when the mouse is curled up on its side)	Half raised and half covered (walls are higher than mouse's body height when the mouse is curled up on its side)	Fully covered
Score:	0	1	2	3	4	5
Usage of Nest Material						
	None	~25% used	~50% used	~75% used	~100% used	
Score:	0	0.5	1	1.5	2	

Figure 2.3

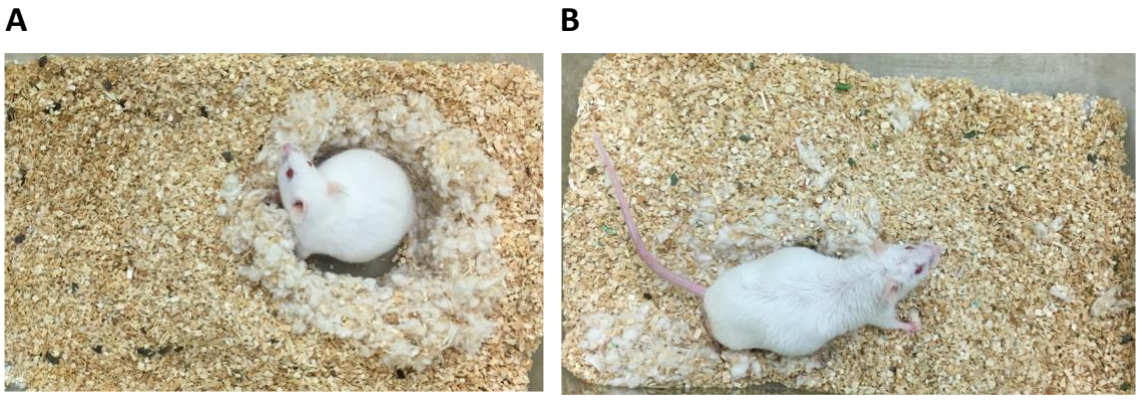

Figure 2.3. A pictorial representation of nest quality in CD-1 mice. **(A)** Representation of a perfect nest, which is given the highest score possible of 9, *versus* **(B)** a poor quality or non-existent nest, which received a score of 2 or less.

2.4 Nursing-related behaviour

To evaluate postpartum maternal or pup-directed behaviour, post-natal nursing was assessed in the CD-1[171hGH/CS] mice from PD 2-6. All pregnant mice were single housed in clear cages in a room with controlled lighting and temperature. Maternal behaviour was recorded for two 30 minute sessions in the light phase, between 10-11 a.m. and 2-3 p.m. daily [120,121]. As the videos were filmed from one side of the cage, the only parameter that could be accurately assessed was the position of the mother in the cage every minute during the observation period. If the mother was in the nest, and appeared to be engaging in any aspect of maternal behaviour, such as nursing, anogenital licking, body licking, nest building or in physical contact with the pups, it was cumulatively assigned to be 'contact rest' with pups [31]. However, if the mother was anywhere outside of the nest, and engaging in non-maternal behaviours such as self-grooming, eating, or drinking, this was assigned as 'non-contact rest' [31].

2.5 Pup retrieval

Pup retrieval was used to assess maternal behaviour postpartum in the CD-1[WT] and CD-1[171hGH/CS] pregnant-RCD and HFD dams from PD 3-5. Before beginning the test, the dam was removed from the home cage (containing the pups) and placed into a temporary cage alone [118]. The home cage, with the pups, was relocated to a testing so that the dam was unable to hear the vocalization of the pups [118]. Four pups were selected randomly and placed facing away from the nest site equidistant apart [118]. Small pits were created within the bedding to place the pups in [118], preventing the pups from crawling back to the nest [118]. After 5 minutes of separation, the dam was introduced back into the home cage [118]. Retrieval behaviour was recorded for 6 minutes each day and the time in seconds to retrieve each individual pup was logged manually. A completion score of 1 was given to dams that successfully retrieved all four pups during the 6 minutes of testing time, while a score of 0 was given to dams that did not retrieve all four pups [118].

2.6 Elevated plus maze (EPM)

To assess anxiety-like behaviour and exploratory drive in CD-1 mice, EPM was used. CD-1[WT] pregnant and non-pregnant mouse groups were tested over a period of three days from GD 15.5-17.5 and PD 20-22. Anxiety-like behaviour was also assessed in CD-1[171hGH/CS] mice at GD 15.5 and PD 20. EPM is plus-shaped apparatus, that consists of a central square-shaped platform, with two open (absence of walls) and two closed (presence of walls) arms of equal size (as described in [118,122]) and raised 70 cm from the ground (Figure 2.6). Prior to the start of the test, female mice were moved to the testing room and allowed to adapt to the new environment for 30-45 minutes [118]. To begin the test, a mouse was positioned in the central maze region, with the head facing an open arm [118]. Mouse activity on the EPM was recorded for 6 minutes using

a mounted web video recorder and 2x Webcam Recorder 1.0.0.1 software [118]. After completing the test, the mouse was returned back to the home cage [118]. The apparatus was wiped clean with 10% (v/v) ethanol in double distilled water to eliminate any olfactory cues [123]. The video recordings were analyzed using ANYMaze software (Version 6.18, Stoelting/ANYmaze, 68000) and the following parameters were calculated (as described previously in [118]): (1) time in seconds spent on the open arms, closed arms and centre region, (2) number of entries in the open arms, closed arms and centre region, (3) total distance travelled in meters, and (4) mean speed in meters per second. If a mouse fell off the maze or displayed signs of freezing or immobility for more than 30% of the total time spent on the open arms, then they were excluded from the analysis [118,122].

Figure 2.6

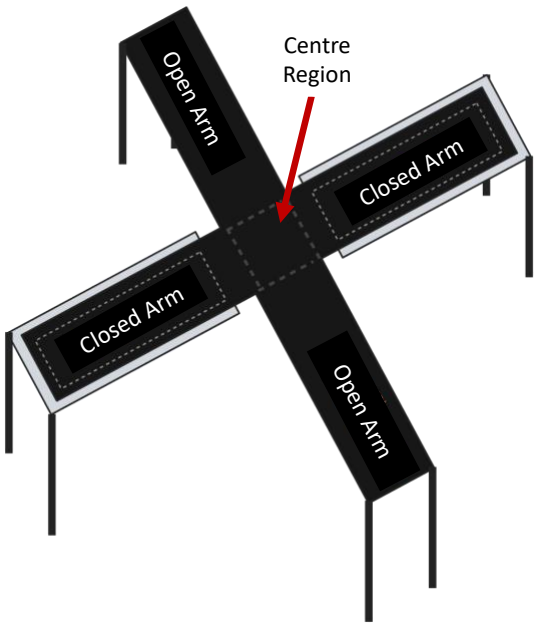

Figure 2.6. Schematic representation of the elevated plus maze (EPM) used to assess anxiety-like behaviour in mice. This apparatus consists of two open arms (absence of walls) and two closed arms (presence of walls), with a centre (square-shaped) region. The EPM apparatus is elevated approximately 70 cm above the ground. More time spent on the closed arms and less time spent on the open arms is considered to be expression of anxiety-like behaviour in mice. This figure was made with a paid subscription from biorender.com.

2.7 Novel object recognition (NOR)

NOR was used to assess working memory in CD-1[WT] and CD-1[171hGH/CS] pregnant and non-pregnant mice over a period of three days beginning at PD 23-25 [80]. The NOR apparatus is described as a square-shaped box, with tall, black colored walls on all sides and an open field in the centre [118] (Figure 2.7). Single housed female mice were moved to the testing room and given 30-45 minutes to adapt to the new environment before the start of the test [118]. On day 1 (PD 23), mice were habituated to the apparatus for 5 minutes [118]. On day 2 (PD 24), mice were familiarized to two similar objects for six minutes [118]. On day 3 (PD 25), the response of the mice to a novel object alongside a familiar object was assessed for 6 minutes [118]. All mouse activity on the NOR apparatus were recorded on day 2 and 3 of the test. After each test, the apparatus and all objects were cleaned with 10% (v/v) ethanol in double distilled water, and the position of the objects were switched to avoid placement preference [118]. A 2x Webcam Recorder 1.0.0.1 software was used to record all videos [118]. All videos were analyzed with ANYMaze software (Version 6.18, Stoelting/ANYmaze, 68000) [118].

For objects places on the right or left side of the NOR apparatus, the preference index score was determined, as described previously by [124], as "the ratio of the time spent in seconds exploring the two similar objects in the familiarization phase, over the total time spent exploring both objects" [118]. Similarly, a discrimination index score was determined as described by [124], "for a novel *versus* familiar object, the difference between the time spent exploring the familiar and novel objects over the total time spent exploring both objects" [118]. A positive discrimination index score indicates greater amount of time spent exploring the novel object, whereas a negative score suggests greater amount of time exploring the familiar object, and a score of 0 implies no evidence of discerning between the familiar and novel objects [124]. On day 2, which is the familiarization stage, any mice that failed to explore both familiar objects for more than 20 seconds

of the total test time, were excluded [80].

On day 3 (PD 25), rearing, which refers to the position during which a mouse stands upright on its hind legs, was counted manually [3]. Rearing is a type of exploratory behaviour observed in mice and has been proposed to be an indicator of anxiety-like behaviour in mice [3]. It's also one other possible measure of assessing learning and memory in mice, as this behaviour is also regulated by the hippocampus [125].

Figure 2.7

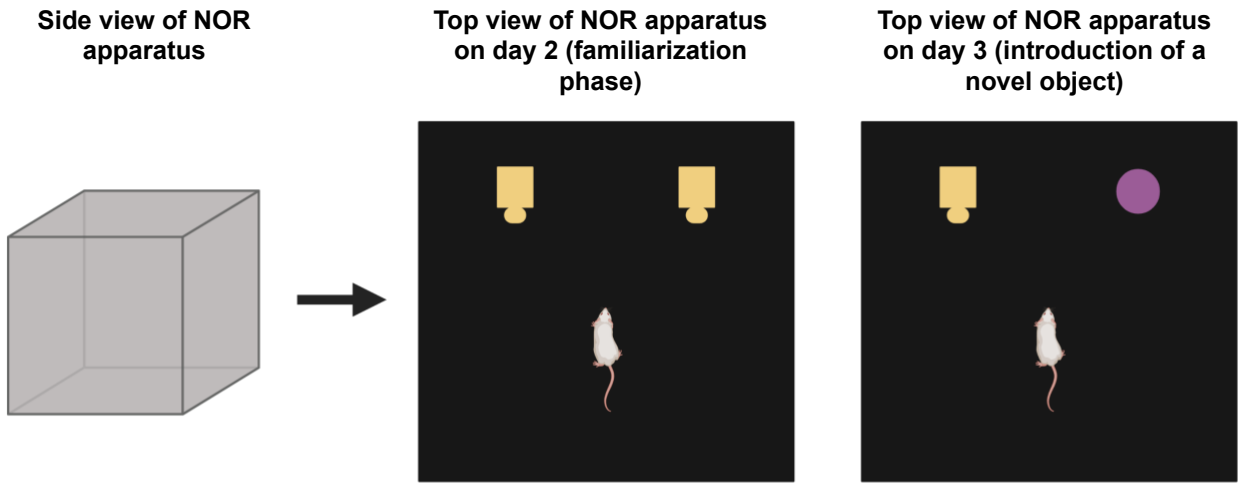

Figure 2.7. Schematic of the novel object recognition (NOR) apparatus. The NOR apparatus is a black, square-shaped box with high walls. On the first day of the test, mice are allowed to habituate to the NOR apparatus for 5 minutes. On day 2 (PD 24), two identical objects are introduced (familiarization phase) and on day 3 (PD 25), one of the familiar objects is replaced with a novel object. The preference index and the discrimination index (DI) are determined on day 2 and 3 respectively. The preference index is based on the ratio of the time (in seconds) spent exploring two similar objects. The DI is calculated from the difference in time spent exploring the novel and familiar object, over the time spent exploring both objects. This figure was made with a paid subscription from biorender.com.

2.8 Sucrose preference test

A sucrose preference test was used to assess anhedonia (inability to feel pleasure) in CD-1[WT] pregnant mice pre-weaning (PD 6-8 and 12-15) and post-weaning (PD 21-23), as well as in age-matched non-pregnant mice [118]. Please refer to Figure 2.8 for a schematic representation of the sucrose preference test setup. Before starting the test, two 50 ml conical tubes (Corning, Cat# 0553855), containing 35-40 ml of tap with spouts were placed into each cage for up to 4 days, to help the dams adapt to the new drinking bottle [118]. To avoid placement preference, the position of the bottles were switched daily [118]. On PD 6, the conical tubes were replaced by two new tubes, of which one contained tap water and the other contained 2% (w/v) sucrose (Sigma, Cat# S9378) in tap water solution [118] (Figure 2.8). The position of the bottles were switched daily to avoid placement preference [118]. The bottles were also weighed and refilled daily [118]. To determine sucrose preference, the percentage of sucrose intake was divided by total fluid intake "(sucrose preference = volume (sucrose solution) / [volume (sucrose solution) + volume (water)] x 100%)" [118]. Sucrose preference was then averaged over all of the days of testing [118]. If a tube containing either sucrose solution or tap water leaked, that mouse was removed from the analysis [118].

Figure 2.8

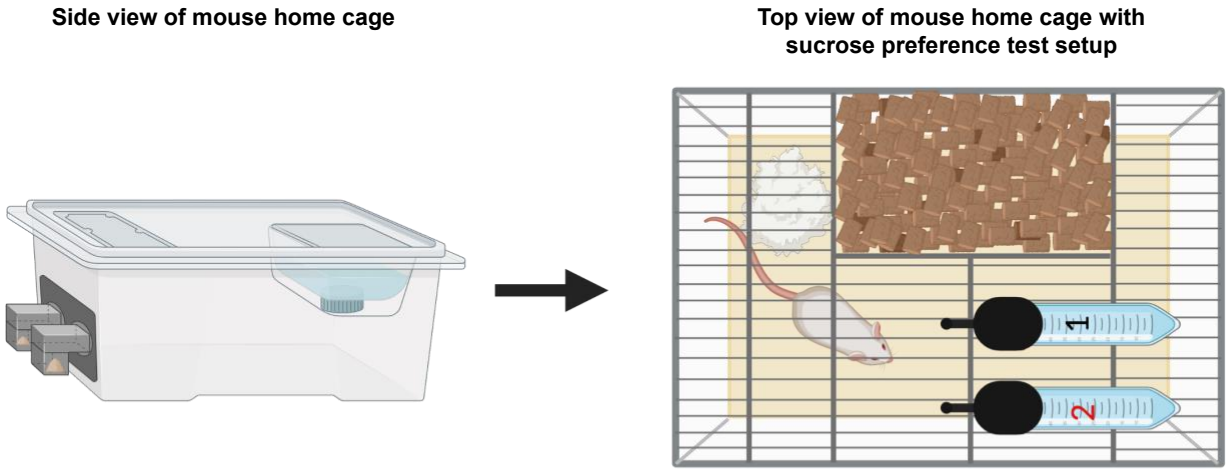

Figure 2.8. Schematic representation of the sucrose preference test setup used in the home cage of CD-1[WT] mice. This test was completed in pregnant mice pre-weaning (postpartum day (PD) 6-8 and 12-15) and post-weaning (PD 21-23), as well as in age-matched non-pregnant mice. First, the original drinking bottle was substituted with new drinking bottles, composed of two 50 ml conical tubes (labelled number 1 and 2 respectively) with spouts. Tube 1 contained 35-40 ml of tap water, whereas tube 2 contained 35-40 ml of 2% sucrose solution. The bottles were weighed and refilled daily. The position of the bottles was also switched every day to avoid placement preference. This figure was made with a paid subscription from biorender.com.

2.9 RNA extraction and analysis

Total RNA was extracted from half brain of CD-1[WT] mice at GD 6, 10.5 and 18.5, using a RNeasy Plus Mini Kit (Qiagen, ON, Canada, Cat#74136) and QIAshredder (Qiagen, Cat#79656). For reverse transcription (RT) polymerase chain reaction (PCR), the QuantiTect Reverse Transcription kit (Qiagen, Cat# 205314) was used to reverse transcribe 2 μg of total RNA, following the instructions from the manufacturer. For quantitative real-time RT-PCR (qPCR), mixtures were prepared using specific primers for mouse BDNF [126] and one reference gene, β-actin (Table 2). These primers are expected to detect BDNF I, IIA-C, III, IV, V, VI, VII, VIII, and IXA [127]. The samples were incubated at 42ºC for 45 minutes, followed by 5 minutes at 95ºC.

The reaction was then stopped and complimentary (c) DNA was stored at 4ºC for qPCR.

RT-PCR was performed to assess relative brain-derived neurotrophic factor (BDNF) RNA levels using the Power SYBR green PCR master mix kit (10ul; Invitrogen, Cat# 4368702), template cDNA (final concentration of 0.02 μg/μl) and 10 μM of the forward and reverse primers (Table 2; Integrated DNA Technologies) in 7500 Real-time PCR system, (Applied Biosystems). 7500 PCR software V2.0.5 was run for 40 cycles, which consisted of three stages: 1) melting of the cDNA strands to two separate strands (denaturation), 2) annealing of the primers to the DNA strands and 3) extension and amplification of the to produce a double stranded DNA. The melt curve was used to identify single-peak amplifications. The expression level of each gene within each sample was extrapolated from its own standard curve. Target RNA transcript levels were then normalized to the CD-1 mouse brain β-actin RNA levels.

Table 2. Primer Sequences used for qPCR

Target RNA	*Primer Sequence*
Mouse BDNF	Forward: 5' – ATGACCATCCTTTTCCTTACT – 3' Reverse: 5' – GCGCCGAACCCTCATAGAC – 3'
Mouse β-Actin	Forward: 5' – GAGACCTTCAACACCCCAGCC – 3' Reverse: 5' – GGAGAGCATAGCCCTCGTAG – 3'

2.10 BDNF protein extraction and analysis

Whole mouse brain tissues were homogenized on ice using a radioimmunoprecipitation assay (RIPA) buffer that includes: 50 mM Hepes, 150 mM sodium chloride (NaCl), 1 mM ethylenediaminetetraacetic acid (EDTA), 2.5 mM ethylene glycol tetraacetic acid (EGTA), 10% (v/v) glycerol, 1% NP-40, 1% deoxycholate (DOC), 0.1% (w/v) sodium dodecyl sulphate (SDS), protease inhibitor cocktail (Sigma, Cat #11836170001), and 2 mM sodium orthovanadate (Sigma,

Cat# S6508), brought to a final pH of 7.5. 1000 µl of RIPA buffer was added to each brain tissue sample. Scissors were initially used to cut the tissue into small pieces. A microfuge tube pestle was used to homogenize the tissue completely. The samples were then transferred to the Q800 Sonicator (QSONICA Sonicator) for the sonification process. All samples were sonicated at 4 degrees Celsius (°C) for 30 seconds (6 seconds pulse on and 5 seconds pulse off) at an amplitude of 60%. Samples were transferred to a cooled centrifuge for centrifugation at 4°C (10,000 g for 13 minutes). The supernatant was aliquoted and stored at -80°C for later use.

The protein concentration was measured using the Bradford protein assay [128]. The standards were prepared using bovine serum albumin (BSA; Sigma, Cat#A7906), sterile distilled water (sdH_2O) and Protein Assay Dye Reagent Concentrate (Biorad #500-0006). For sample preparation, 5 µl of the supernatant was first diluted in 20 µl of sdH_2O. Then, 1 µl of the diluted supernatant was added to 799 µl of sdH_2O, followed by 200 µl of dye. Standards and samples were vortexed and allowed to sit at room temperature for 10 minutes.

The spectrophotometer (Hitachi, U-1100) was used to determine the concentration of the samples and standards. The optical density (OD), which is a measure of absorbance, was at a wavelength of 595 nanometers (nm) and the 'blank' standard was set to 0. Plastic transfer pipettes (Fisher Scientific, Cat #137116M) were used to transfer all of the standards and samples individually and the OD was recorded. The OD values of the standard curve were than graphed in Microsoft Excel. The linear equation ($y = mx + b$) was used to calculate the total protein concentration of the samples.

To determine the total BDNF protein concentration in whole brain supernatant, enzyme-linked immunosorbent assay (ELISA) was used (Human/Mouse BDNF Elisa Kit, R&D Systems, DY248) at room temperature as indicated by the manufacturer. Briefly, each well in a 96 well

microplate (Fisher Scientific, Cat #0720090X) was coated with 100 µl of the BDNF capture antibody and sealed with parafilm and left overnight. The next day, the wells were washed with 400 µl of wash buffer (0.05% Tween-20, (Biorad, Cat#170-6531) in 1x Dulbucco's Phosphate Buffered Saline (PBS; Invitrogen, Cat# 21300058) three times, followed by addition of 300 µl of reagent diluent (1% BSA in PBS, pH 7.2-7.4) to each well for up to 1 hour. The washing step was repeated, and 100 µl of sample or the standards were added to each well and allowed to incubate at room temperature for 2 hours. The wells were washed, and 100 µl of the detection antibody was added to each well and incubated for 2 hours. The wells were washed and 100 µl of streptavidin horseradish peroxidase (S-HRP) was added to each well. After 20 minutes, the wells were washed again and 100 µl of the substrate solution (1:1 mixture of colour reagent A (hydrogen peroxide) and B (tetramethylbenzidine)) was added to each well. After 20 minutes, 50 µl of the stop solution (sulphuric acid) was added to each well. The OD of the samples was immediately read using a Microplate Reader (Megalla, F50 program) set at a wavelength of 450 nm. Previous research in mice suggests that BDNF cannot be detected in serum with an ELISA, but can be detected in brain supernatant [112]. As such, mouse serum samples from CD-1[WT] and [171hGH/CS] were also assessed for BDNF.

2.11 Protein (western) immunoblot analysis

A mortar and pestle were used to powder the brain tissue that was frozen with liquid nitrogen and then stored at a temperature of -80°C. To prepare lysate, a small amount of the powder was transferred to a new 1.5 ml microfuge tube with a buffer containing the following: 10% glycerol, 50mM Tris-HCl, pH 6.8, 1% SDS, 60 mM β-glycerophosphate, 5 mM EDTA, 5mM EGTA, 2mM sodium orthovanadate with protein inhibitor cocktail (Sigma, Cat #P8340), and

phosphatase inhibitor cocktails 1 and 2 (Sigma, Cat #P2850;PPIC2; Sigma, Cat #P5726) at dilutions of 1:100. After homogenization was completed using a pestle, the samples were boiled for 5 minutes, and sonicated at 40 Hertz 3 times for 5 seconds. The samples were centrifuged at 21,000 g for 15 minutes at a temperature of 4°C in order to remove any insoluble materials. The bicinchoninic acid (BCA) protein assay was used to determine the protein concentration.

The brain lysates were analysed in polyacrylamide gels (10% or 12.5%) using kaleidoscope molecular weight markers (10.0-250 kDa). After electrophoresis was completed, the protein was transferred to a polyvinylidene difluoride membrane at a voltage of 100, for 1 hour at 7°C. To prevent non-specific binding, the blots were blocked with 10% (w/v) skim milk in Tris-buffered saline with 0.05% Tween-20 for 1 hour. This was followed by incubation with a primary antibody for BDNF (1:1000 dilution; Abcam, Cat #ab108319) in 1% milk in Tris-buffered saline with 0.05% Tween-20 overnight at 4°C. The following day, the blots were incubated for an additional 1 hour. The membranes were washed for 15 minutes, and then washed 3 times for 5 minutes after with 1% milk in in Tris-buffered saline with 0.05% Tween-20 between primary and secondary antibodies. This was followed by incubation of the membranes with the donkey anti-rabbit-HRP secondary antibodies (Jackson immune research, Cat #711-035-152) for 1 hour. The membranes were then washed. Clarity (BioRad) and exposure to Kodak X-omat film were used to detect antigen-antibody complexes via chemiluminescence. The BioRad Model GS-800 densitometer with molecular analyst software was used to measure the intensity of the bands on the immunoblot. This assay was pursued as a collaboration with the laboratory of Dr. E. Kardami (Human Anatomy & Cell Science, University of Manitoba/Institute of Cardiovascular Sciences, St Boniface Hospital Albrechtsen Research Centre).

2.12 Statistical analysis

For all statistical analysis, Prism 8 software (version 8.4.3) was used. Video recordings were assigned numbers randomly before starting the analysis in order to allow for a blinded assessment [118]. While effort was made to minimize any observer bias, this was limited by the ability to identify diet through its colour and dams through the litters. For single comparisons, a non-parametric unpaired t-test was used [118]. Where appropriate, the *t* statistic with degrees of freedom in parentheses and the significance level (*p*-value) are reported [118]. In addition, a two-way ANOVA was used for multiple comparisons and this was followed by a Tukey's *post hoc* test where appropriate [118]. The *F*-statistic (interactive or individually) and its associated degrees of freedom and the corresponding *p*-value are stated for ANOVA results [118]. A p-value of $p<0.05$ was considered statistically significant. In the figure, the significance is represented as follows: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. All error bars represent the standard error of the mean.

CHAPTER 3

Effect of a high-fat diet in pregnancy on maternal CD-1[WT] mouse behaviour

3.1 Characterization of the CD-1[WT] RCD *versus* HFD mouse model

3.1.1 Total body weights of non-pregnant and pregnant CD-1[WT] mice

Throughout the study, all CD-1[WT] mice were weighed weekly at approximately the same time of day, to allow for comparison between the RCD and HFD pregnant and non-pregnant mice total body weights. Diet had a significant effect on weight gain over the 10 weeks of study in the non-pregnant female mice on HFD when compared to RCD (main effect of diet: $F(1,37)=86.80$; $p<0.001$; main effect of time in weeks: $F(1.233,32.61)=134.6$; $p<0.001$; Figure 3.1.1 A) [118][1]. Similarly, the interaction between diet and the duration of the diet also had a significant effect on weight gain in non-pregnant mice (interaction between diet and time in weeks: $F(9,238)=36.42$; $p<0.001$; Figure 3.1.1 A) [118].

In pregnant female mice fed a HFD, a significant increase in weight gain was detected in weeks 5 and 6 (main effect of time in weeks: $F(3, 68)=34.09$; $p<0.001$; interaction between diet and time in weeks: $F(3,68)=5.636$; $p=0.002$; Figure 3.1.1 B) [118]. These mice were bred in week 5 and continued on the RCD or HFD during pregnancy [118]. At parturition (in week 8), no significant effect of diet on weight gain was seen in the same mice (main effect of diet: $F(1,68)=3.498$; $p<0.066$; Figure 3.1.1 B) [118]. It was also observed that after mice gave birth (weeks 9 and 19), although mice were allowed access to their respective diets' *ad libitum*, the food

[1] Please note all statistics presented in Chapter 3, Sections 3.1-3.4 were previously reported in Moazzam, S *et al* , *Psychoneuroendocrinology* (2021) [117].

tray did not need to be filled as frequently for these mice [118]. Based on observation, this could be a result of the dam spending more time nursing or looking after her newborn litter.

Figure 3.1.1

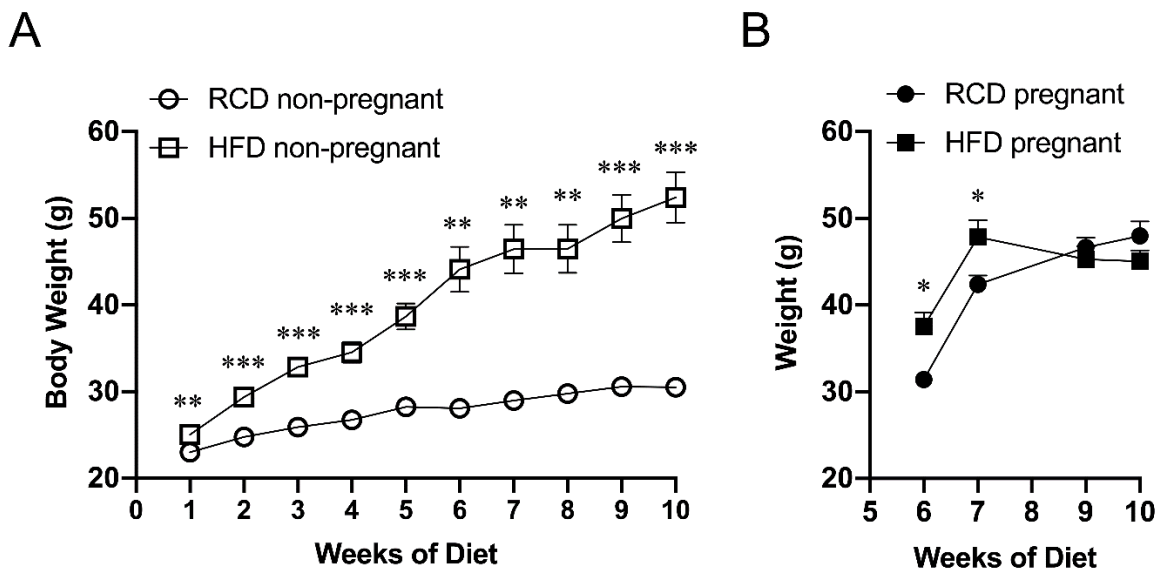

Figure 3.1.1. Total body weights of non-pregnant and pregnant CD-1[WT] mice maintained on either a regular chow diet (RCD) or high-fat diet (HFD) during the course of the study (10 weeks). Total body weight of **(A)** non-pregnant mice from week 1-10, and **(B)** pregnant mice on an RCD or HFD from gestation day (GD) 0-18.5 (weeks 6 and 7) and after giving birth (weeks 9 and 10). Values are expressed as mean ± standard error of the mean and were determined and analyzed by two-way ANOVA. Sample size (n) is 9-10. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. This figure is reproduced with permission from [118] and has been adapted for presentation for this thesis.

3.1.2 Litter size of pregnant CD-1[WT] mice

To evaluate if diet has any influence on the number of pups a dam gives birth to, litter size of CD-1[WT] dams was determined immediately at parturition (prior to culling). The average litter size of dams fed a RCD *versus* HFD was 12.50 and 13.11, respectively, and these values were not significantly different [118]. Based on this, there was no effect of the diets used on litter size ($t(17)=0.852$; $p=0.406$; Figure 3.1.2 C) [118].

Figure 3.1.2

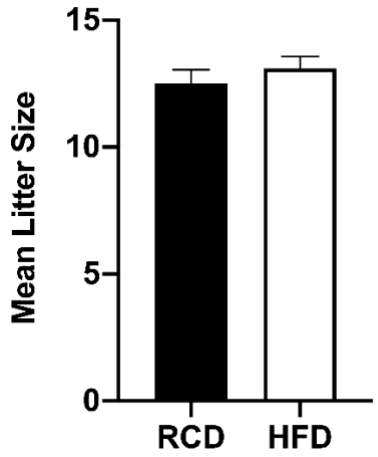

Figure 3.1.2. Mean litter size of pregnant CD-1[WT] mice fed either a regular chow diet (RCD) or high-fat diet (HFD) assessed at parturition (prior to culling). Values are expressed as mean ± standard error of the mean and were determined and analyzed by unpaired t-test. Sample size (n) is 9-10. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. This figure is reproduced with permission from [118] and has been adapted for presentation for this thesis.

3.1.3 Glucose clearance in pregnant CD-1[WT] mice

A glucose tolerance test (GTT), which measures the body's response to glucose or ability to clear glucose, was performed on mice after they had been on their respective HFD and RCD for 5 weeks at GD -1 (before pregnancy) and at 7 weeks (in pregnancy at GD 16.5). After 5 weeks on the HFD, non-pregnant mice displayed significantly impaired glucose clearance at GD -1 (main effect of diet: $F(1,70)=56.68$; $p<0.001$; main effect of time in minutes: (6,70)=80.55, $p<0.001$; interaction between diet and time in minutes: $F(6,70)=3.926$, $p=0.002$; Figure 3.1.3 A) [118]. Similarly, after 7 weeks on their respective diets, pregnant mice fed a HFD also experienced impaired glucose clearance when tested at GD 16.5, compared to mice on a RCD (main effect of diet: $F(1,63)=27.23$; $p<0.001$; main effect of time in minutes: $F(6,63)=15.12$, $p<0.001$; interaction between diet and time in minutes: $F(6,63)=1.192$, $p=0.322$; Figure 3.1.3 B) [118].

Figure 3.1.3

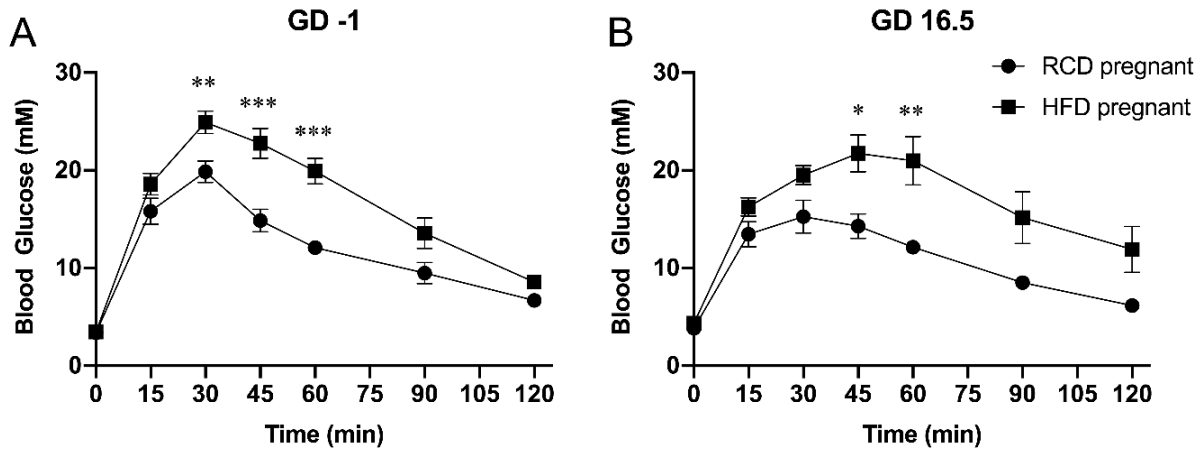

Figure 3.1.3. Glucose clearance was assessed by glucose tolerance test (GTT) in CD-1[WT] mice maintained on either a regular chow diet (RCD) or high-fat diet (HFD). **(A)** Glucose clearance over a period of 120 minutes in non-pregnant CD-1[WT] mice after 5 weeks of being on the diets at gestation day (GD) -1 (before pregnancy) and at **(B)** GD 16.5 in pregnant CD-1[WT] mice. Values are expressed as mean ± standard error of the mean and were determined and analyzed by two-way ANOVA. Sample size (n) are 6 and 4-7, respectively. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. This figure is reproduced with permission from [118] and has been adapted for presentation for this thesis.

3.2 Maternal behaviour in CD-1[WT] mice

3.2.1 Nest building behaviour prepartum in CD-1[WT] mice

To evaluate maternal behaviour prepartum (GD 16.), nest building was assessed using criteria that included amount of nesting material shredded (scored out of 2), and used (scored out of 2), as well as height and shape of the nest (scored out of 5) (Table 1) [118]. Nest building behaviour was assessed prepartum at GD 16.5 [118]. Mice fed a HFD had a significantly lower mean score of 4.68 on the nest building assay when compared to 7.33 for mice on a RCD ($t(18)=3.645$; $p=0.002$; Figure 3.2.1) [118].

Figure 3.2.1

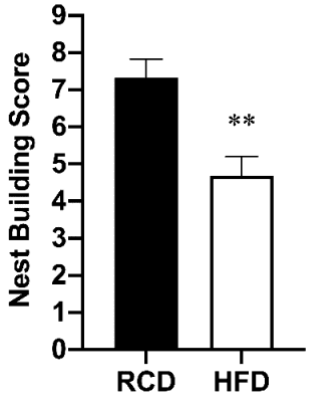

Figure 3.2.1. Maternal behaviour was assessed prepartum via nest building, and average nest building scores were recorded at gestation day (GD) 16.5 in pregnant mice on regular chow diet (RCD) or high-fat diet (HFD). Values are expressed as mean ± standard error of the mean and were determined and analyzed by unpaired t-test. Sample size n=9-10. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. This figure is reproduced with permission from [118] and has been adapted for presentation for this thesis.

3.2.2 Postpartum pup retrieval behaviour in CD-1[WT] mice

Pup retrieval behaviour was assessed in mice from PD 3-5 [118]. At PD 3, HFD-induced obesity had a significant effect on pup retrieval behaviour. Specifically, mice fed HFD had a significantly higher mean score for latency to retrieve pups (288.8) *versus* RCD (121.3) (main effect of diet: $F(1,16)=12.06$; $p=0.003$; main effect of pup retrieval time: $F(3,48)=12.56$; $p=0.001$; interaction between diet and pup retrieval time: $F(3,48)=1.058$; $p=0.375$; Figure 3.2.2 A) [118]. Furthermore, mice fed a HFD had a significantly lower completion rate (2/9 mice; 22%). Based on observations, it seemed that the dams fed HFD were distracted by their surroundings, rather than trying to retrieve the displaced pups [118].

On the second day of the test, which was PD 4, the latency to retrieve each pup by the HFD dams was lower, and almost similar to the dams on the RCD. This suggests some degree of improvement in the mice on the HFD, such that they were able to retrieve the pups faster on the

second day of the test (main effect of diet: $F(1,67)=1.996$; $p=0.162$; main effect of pup retrieval time: $F(3,67)=0.78$; $p=0.51$; interaction between diet and pup retrieval time: $F(3,67)=0.012$; $p=0.998$; Figure 3.2.2 B) [118].

Similar to PD 4, by PD 5, HFD mice had an improved completion rate (7/9 mice; 77.78%) and a lower latency to retrieve each pup (main effect of diet: $F(1,68)=2.438$; $p=0.123$; main effect of pup retrieval time: $F(3,68)=1.37$; $p=0.259$; interaction between diet and pup retrieval time: $F(3,68)=0.041$; $p=0.988$; Figure 3.2.2 C) [118].

Figure 3.2.2

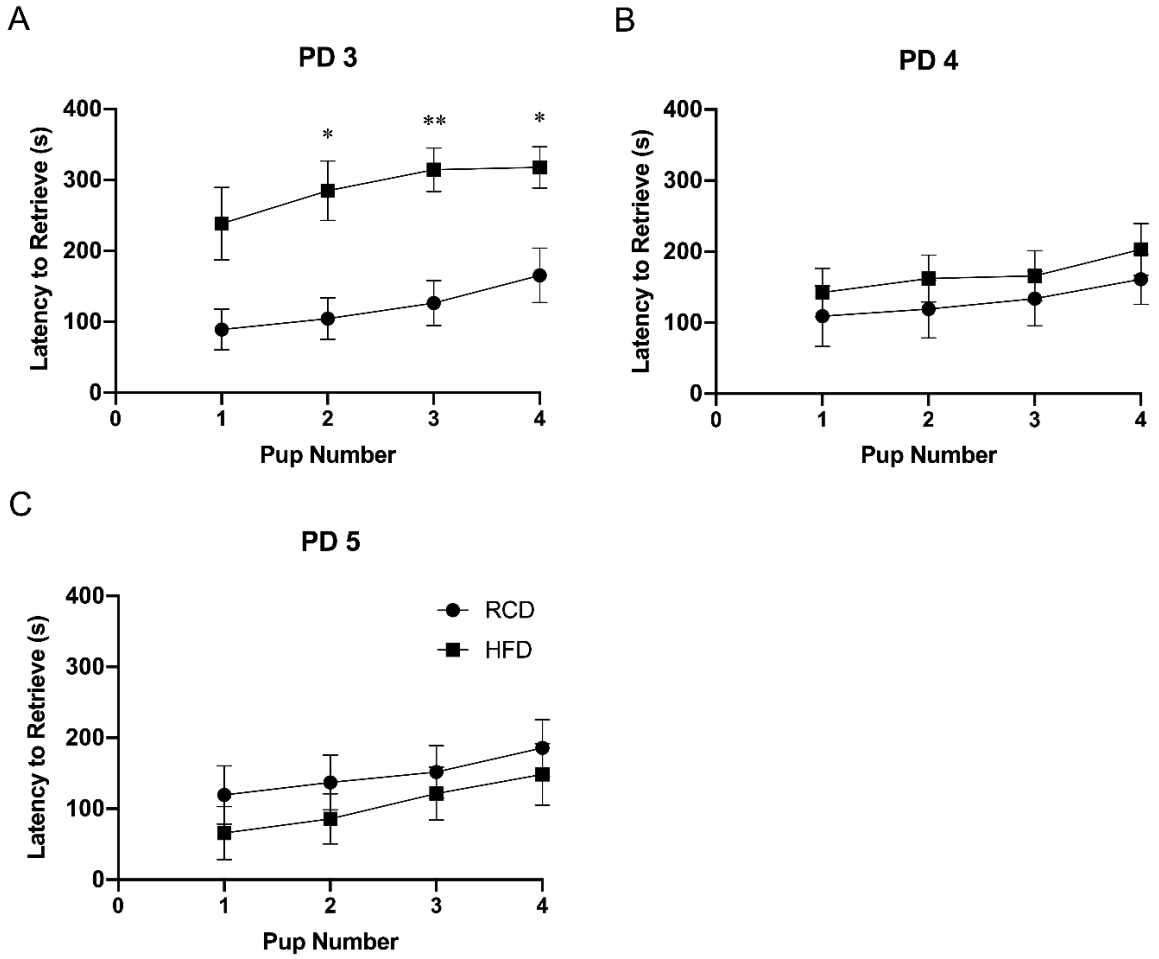

Figure 3.2.2. Maternal behaviour was assessed via pup retrieval assay from postpartum day (PD) 3-5 in mice fed a regular chow diet (RCD) and high-fat diet (HFD). **(A)** Latency to retrieve all four pups within 360 seconds on PD 3, **(B)** PD 4 and **(C)** PD 5. Values are expressed as mean ± standard error of the mean and were determined and analyzed by two-way ANOVA. Sample size n=9-10. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. This figure is reproduced with permission from [118] and has been adapted for presentation for this thesis.

3.3 Anxiety-like behaviour and object recognition memory in in CD-1[WT] mice

3.3.1 Anxiety-like behaviour prepartum and postpartum in CD-1[WT] mice

Anxiety-like behaviour was assessed in pregnant CD-1[WT] mice by an elevated plus maze (EPM) test during the prepartum period, starting at GD 15.5 to GD 17.5, as well as in age and diet-

matched non-pregnant mice [118]. A schematic representation of the EPM test apparatus and the maze regions of assessment are shown in Figure 2.6. In the prepartum period, there were no significant differences in the percentage of time spent on the open arms in all mice regardless of diet or pregnancy-status (main effect of diet: $F(1,34)=0.002$; $p=0.957$; main effect of pregnancy-related status: $F(1.34)=2.607$; $p=0.116$; interaction between diet and pregnancy-related status: $F(1,34)=3.487$; $p=0.071$; Figure 3.3.1 A) [118]. There was also no significant difference between the percentage of time spent on the closed arms in all mice (main effect of diet: $F(1,34)=0.395$; $p=0.534$; main effect of pregnancy-related status: $F(1,34)=2.132$; $p=0.153$; interaction between diet and pregnancy-related status: $F(1,34)=1.282$; $p=0.265$; Figure 3.3.1 B) [118]. Similarly, no difference was seen in percentage of time spent on the centre region in all mice (main effect of diet: $F(1,34)=0.652$; $p=0.425$; main effect of pregnancy-related status: $F(1,34)=0.069$; $p=0.795$; interaction between diet and pregnancy-related status: $F(1,34)=0.136$; $p=0.715$; Figure 3.3.1 C) [118]. The total distance travelled by the pregnant and non-pregnant mice fed HFD was similar but only significantly different between pregnant and non-pregnant mice fed RCD (main effect of diet: $F(1,34)=0.246$; $p=0.623$; main effect of pregnancy-related status: $F(1,34)=9.309$; $p=0.004$; interaction between diet and pregnancy-related status: $F(1,34)=1,871$; $p=1.804$; Figure 3.3.1 D) [118].

In the postpartum period, anxiety-like behaviour was assessed for three days post-weaning, starting at PD 20 to PD 22 in pregnant CD-1[WT] mice and in age and diet matched non-pregnant controls [118]. As this assessment was done in the postpartum period, the terms ‘pregnant’ and ‘pregnancy-status’ refer to mice that recently gave birth or underwent the pregnancy experience. No significant effect of diet or pregnancy-related status was detected on the time spent on the open arms for pregnant and non-pregnant groups (main effect of diet: $F(1,34)=0.168$; $p=0.684$; main effect of pregnancy-related status: $F(1,34)=0.024$; $p=0.877$; interaction between diet and

pregnancy-related status: $F(1,34)=2.06$; $p=0.16$; Figure 3.3.1 E) [118]. However, time spent on the closed arms, in comparison to the pregnant mice fed a RCD, was significantly lower in both non-pregnant mice on RCD and pregnant mice on HFD (main effect of diet: $F(1,34)=1.148$; $p=0.29$; main effect of pregnancy-related status: $F(1,34)=1.374$; $p=0.249$; interaction between diet and pregnancy-related status: $F(1,34)=10.48$; $p=0.003$; Figure 3.3.1 F) [118]. Further *post hoc* analysis indicated that both pregnancy status and HFD independently affect time spent on the closed arms at PD 20 ($p=0.019$ and $p=0.026$; Figure 3.3.1 F) [118].

Time spent in centre region was also not significantly different between all of the groups, and thus was not affected by diet or pregnancy- status in the postpartum period (main effect of diet: $F(1,34)=0.865$; $p=0.359$; main effect of pregnancy-related status: $F(1,34)=1.622$; $p=0.21$; interaction between diet and pregnancy-related status: $F(1,34)=6.933$; $p=0.013$; Figure 3.3.1 G) [118]. Further *post hoc* comparison suggested pregnant mice on the RCD spent significantly less time than non-pregnant mice on RCD in the centre maze region ($p=0.043$; Figure 3.3.1 G) [118]. Finally, an effect of diet ($F(1,34)=6.928$; $p=0.013$), but no effect of pregnancy-related status ($F(1,34)= 0.199$; $p=0.658$) or interaction between diet and pregnancy-related status ($F(1,34)=1.889$; $p=0.178$), was observed on distance travelled in the non-pregnant mice fed a HFD *versus* RCD (Figure 3.3.1 H) [118]. *Post hoc* comparison revealed that non-pregnant mice on HFD travelled significantly less distance on than non-pregnant mice on the RCD ($p=0.031$; Figure 3.3.1 H) [118].

Time spent on the EPM was assessed prepartum (GD 15.5-17.5) and postpartum (PD 20-22) [118]. Overall, the results indicate a trend towards decreased time spent on the open arms and increased time spent on the closed by the RCD and HFD pregnant and non-pregnant groups (Figure 3.3.1.2 A-D) as expected [129]. Thus, first day of the assay allows for 'true' assessment of anxiety, whereas the consecutive days allowed for assessment of possible 'learned behaviour' [118].

Figure 3.3.1

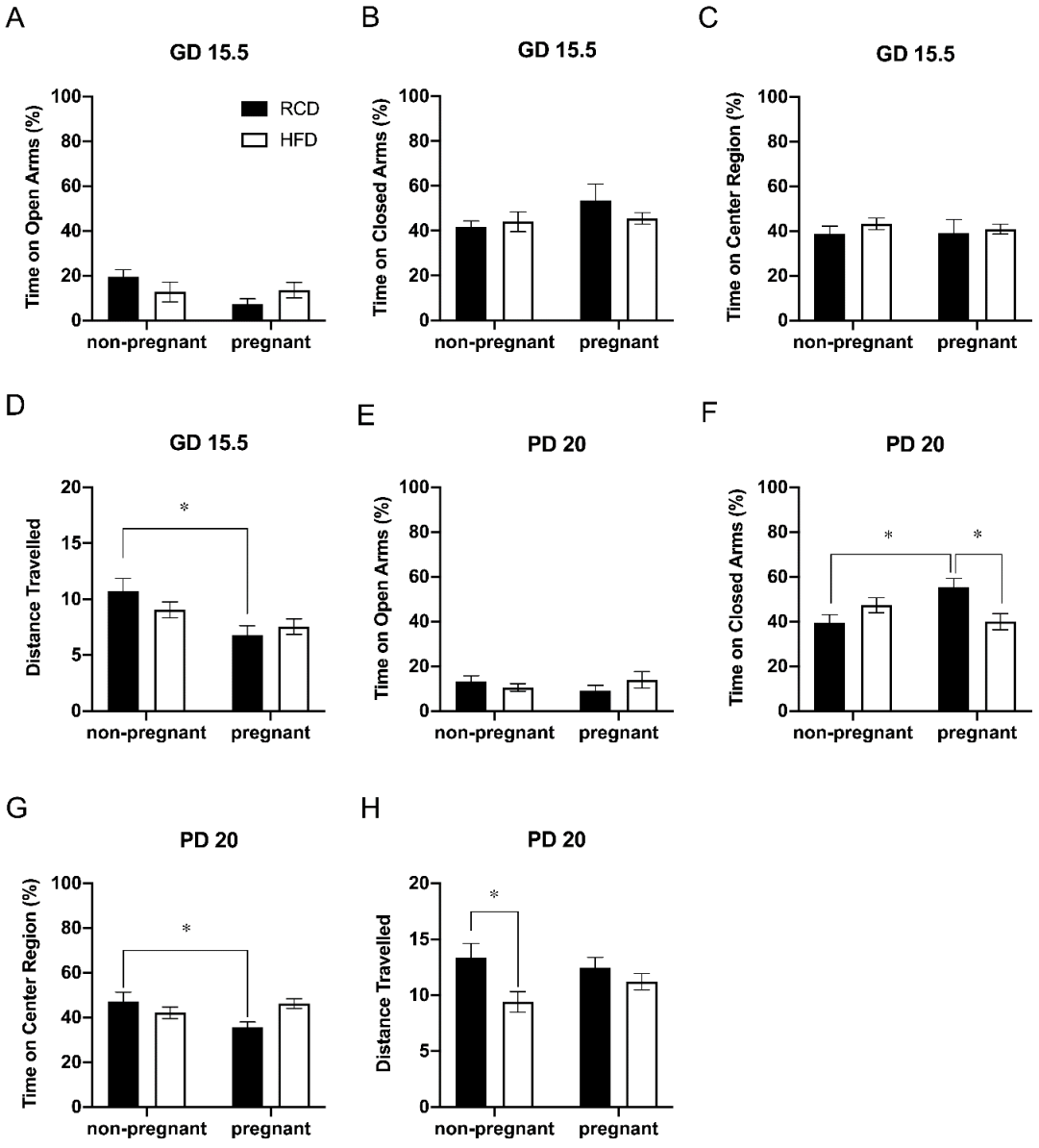

Figure 3.3.1. Effect of regular chow diet (RCD) and high-fat diet (HFD) on anxiety-like behaviour prepartum on gestation day (GD) 15.5 and postpartum day (PD) 20, in pregnant and non-pregnant CD-1[WT] mice. **(A-C)** Percentage of time spent on the closed arms, open arms and centre region of the elevated plus maze (EPM) at GD 15.5. **(D)** Total distance travelled on the EPM at GD 15.5. *(E)* Percentage of time spent on the closed arms at PD 20. **(F-G)** Percentage of time spent on the closed arms, open arms and centre region of the EPM at PD 20. **(H)** Total distance travelled on the EPM at PD 20. One pregnant female on HFD at GD 15.5 fell of the maze and was excluded from the analysis. Values are expressed as mean ± standard error of the mean and were determined and analyzed by two-way ANOVA (panels A-H). Sample size n=9-10. *$p < 0.05$, **$p < 0.01$, ***$p <$ 0.001. This figure is reproduced with permission from [118] and has been adapted for presentation for this thesis.

Figure 3.3.1.2

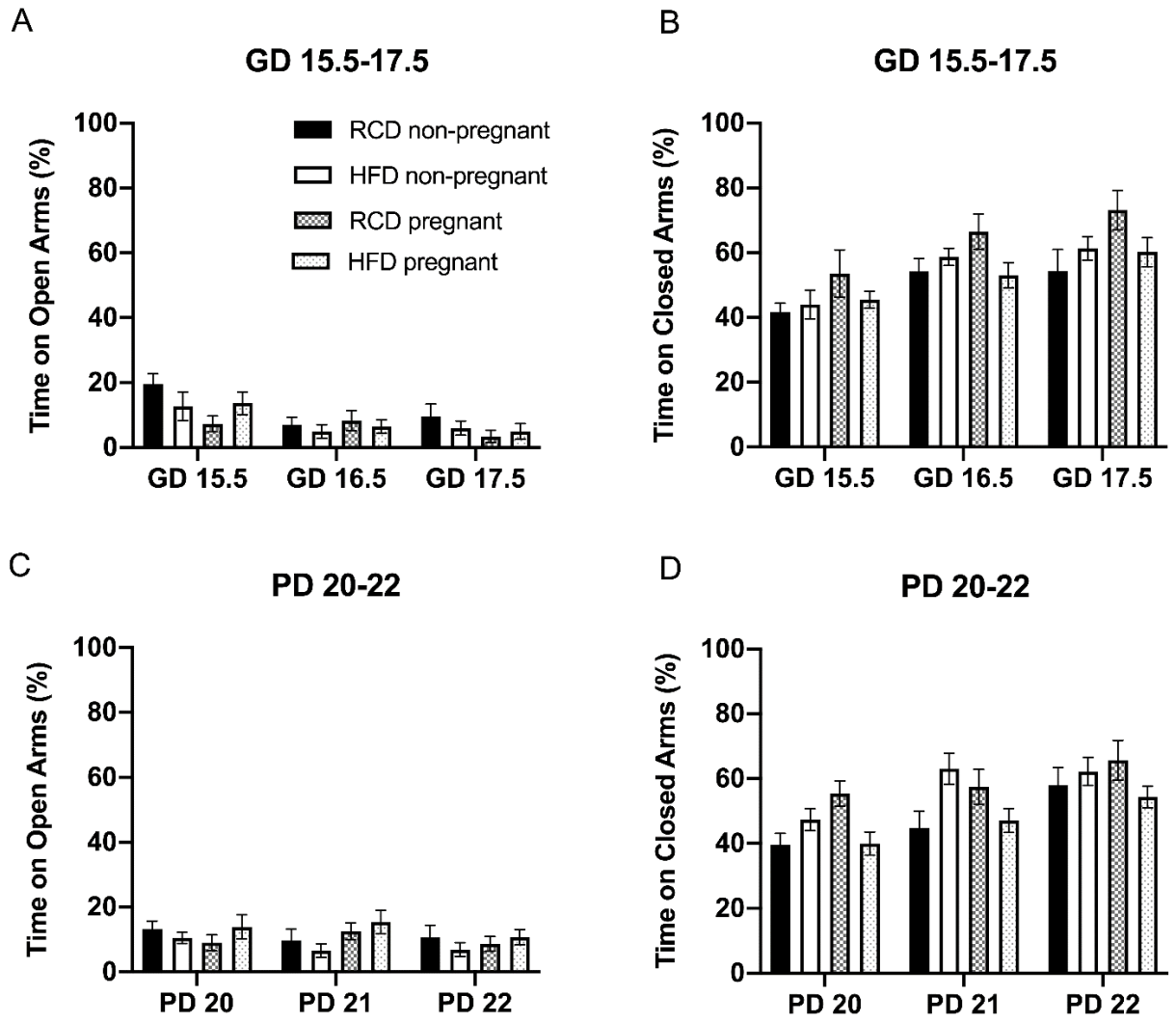

Figure 3.3.1.2 Effect of regular chow diet (RCD) and high-fat diet (HFD) on anxiety-like behaviour prepartum on gestation days (GD) 15.5-17.5 and postpartum day (PD) 20-22, in pregnant and non-pregnant CD-1[WT] mice. **(A-B)** Percentage of time spent on the open arms and closed arms from GD 15.5-17.5 and **(C-D)** PD 20-22 in all mouse groups. Sample size n=9-10.

3.3.2 Object recognition memory assessment in CD-1[WT] mice postpartum

To test cognitive function, a novel object recognition (NOR) test was used to evaluate memory performance in pregnant and non-pregnant mice on HFD *versus* RCD at PD 23-25 [118]. A schematic representation of the NOR assay, including for the familiarization and discrimination

phases, is shown in Figure 2.7. The preference index score was based on the ratio of the time spent exploring the two similar objects (in seconds) in the familiarization phase, over the total time spent exploring both objects [81]. The preference index scores were not significantly different during the familiarization phase amongst all mouse groups (main effect of diet: $F(1,35)=2.29e\text{-}005$; $p=0.996$; main effect of pregnancy-related status: $F(1,35)=1.827$; $p=0.185$; interaction between diet and pregnancy-related status: $F(1,35)=0.009$; $p=0.923$; Figure 3.3.2 A) [118]. This suggests that mice did not have a preference for the familiar objects placed right or left side of the apparatus based on diet or pregnancy status [118].

The discrimination index was calculated for the first 1-minute and 5-minutes of the test on PD 25 post-weaning. The discrimination index was based on the difference between the time spent interacting with the novel *versus* familiar over the total time spent interacting with both objects [81]. For the first 1-minute of the test, no significant differences were found between pregnant and non-pregnant groups of mice on HFD *versus* RCD (main effect of diet: $F(1,35)=0.002$; $p=0.966$; main effect of pregnancy-related status: $F(1,35)=0.331$; $p=0.569$; interaction between diet and pregnancy-related status: $F(1,35)=2.899$; $p=0.096$; Figure 3.3.2 B) [118].

The discrimination index is significantly different for pregnant mice on HFD *versus* RCD for 5-minutes into the test (main effect of diet: $F(1,35)=0.322$; $p=0.574$; main effect of pregnancy-related status: $F(1,35)=1.551$; $p=0.221$; interaction between diet and pregnancy-related status: $F(1,35)=13.03$; $p<0.001$) [118]. *Post hoc* analysis insinuates that even though pregnant mice on the HFD spent almost similar amount of time exploring the familiar and novel objects, mice fed a RCD spent significantly more time exploring the familiar object during the 5-minute testing period ($p=0.03$; Figure 3.3.2 C) [118]. By contrast, in the first 1-minute of the test, diet did not significantly affect the discrimination index for age matched non-pregnant mice [118]. Our observations suggest that mice on the HFD spent slightly more time interacting with the familiar

object (Table 3) [118].

During the 5-minute testing period, the discrimination index is not statistically different between the pregnant *versus* age-matched non-pregnant mice on the RCD [118]. However, *post hoc* analysis suggests that non-pregnant mice fed RCD spent more time with the novel object, while pregnant mice fed RCD spent more time with the familiar object (p=0.007; Figure 3.3.2 C; Table 3) [118]. The incidence of rearing, a type of exploratory behaviour, was also counted manually in these mice [3]. A high frequency of rearing is associated with increased anxiety-like behaviour [130], whereas a low frequency of rearing is associated with decreased anxiety-like behaviour [131]. Here, the rearing frequency was not significantly different amongst all mice suggesting that there is no difference between exploratory behaviour amongst all groups (Figure 3.3.2 D) [118].

Figure 3.3.2

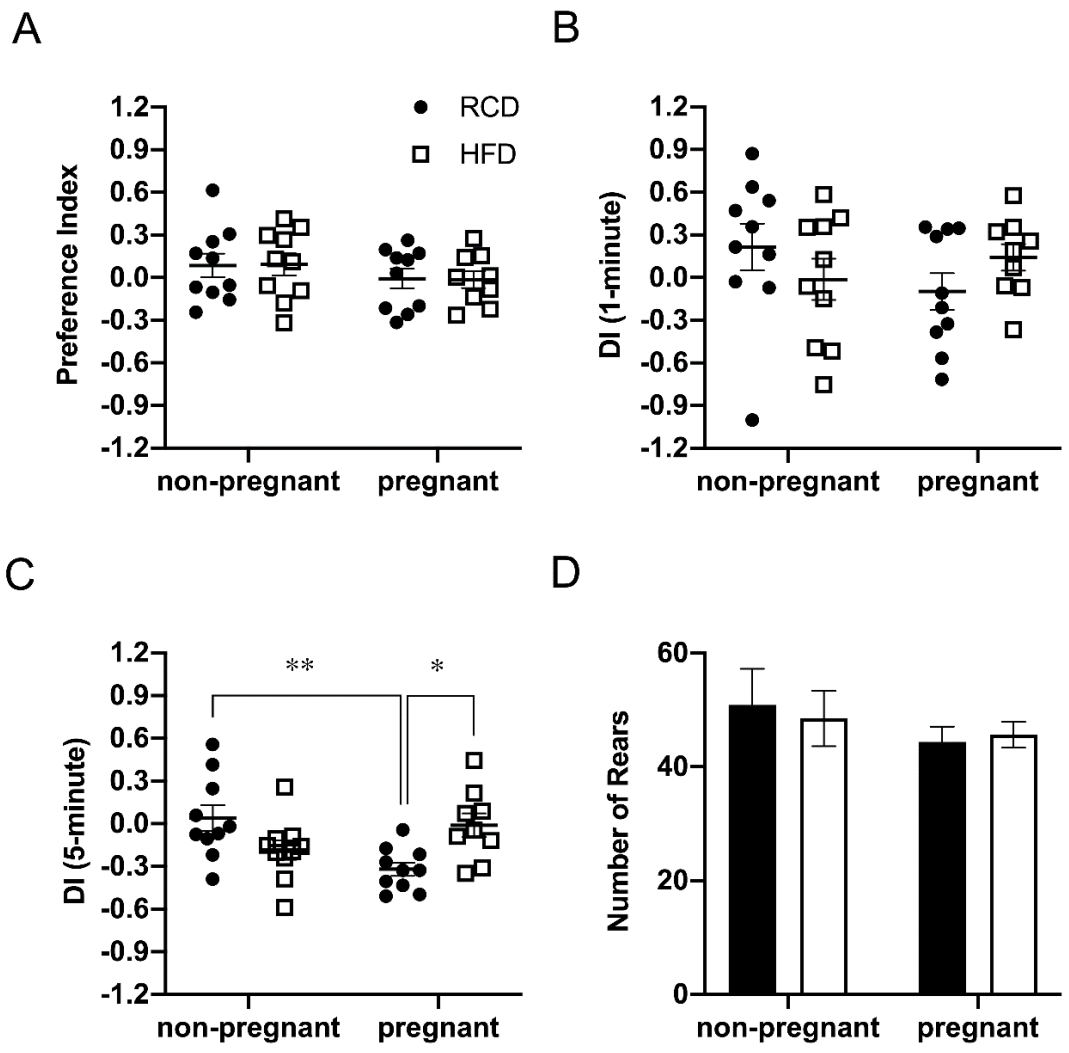

Figure 3.3.2. Effect of regular chow diet (RCD) and high-fat diet (HFD) on cognitive function or interest in novel objects on postpartum day (PD) 24 (familiarization phase) and PD 25 (introduction of a novel object) in pregnant and non-pregnant CD-1[WT] mice. **(A)** Preference index on PD 24. **(B)** Discrimination index (DI) in the first minute at PD 25. **(C)** DI at the 5-minute mark at PD 25. **(D)** Rearing frequency during the novel-object recognition test on PD 25. Values are expressed as mean ± standard error of the mean and were determined and analyzed by two-way ANOVA (panels **A**-**D**). Sample size n=9-10. A score of 0 suggests no evidence of discriminating between the objects, whereas a negative score refers to preference for the familiar object, and a positive score indicates preference for the novel object. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. This figure is reproduced with permission from [118] and has been adapted for presentation for this thesis.

Table 3: Summary of object interaction at the 1- and 5-minute mark on day 3 (introduction of novel object) on PD 25. This table is reproduced from [118] with permission and has been adapted for presentation for this thesis.

	Object Interaction: 1 min	*Object Interaction: 5 min*
RCD non-pregnant	Novel	Novel
HFD non-pregnant	Novel and Familiar	Familiar
RCD pregnant	Familiar	Familiar
HFD pregnant	Novel	Novel and Familiar

3.4 Assessment of anhedonia using a sucrose preference test

A schematic representation of the sucrose preference test is included as Figure 2.8. Sucrose preference was assessed pre-weaning (PD 6-8 and PD 12-15) and post-weaning (PD 21-23) in both RCD and HFD pregnant and non-pregnant age-matched controls [118]. Sucrose preference was calculated using the percentage of the volume of 2% (w/v) sucrose in tap water intake over the total fluid intake [118]. No significant difference between sucrose preference was detected pre-weaning (PD 6-8) amongst all the groups (main effect of diet: $F(1,155)=7.346$; $p=0.008$; main effect of pregnancy-related status: $F(1,155)=0.511$; $p=0.476$; (interaction between diet and pregnancy-related status: $F(1,155)=0.058$; $p=0.811$; Figure 3.4 A) [118].

Conversely, although not statistically significant, pregnant mice on HFD had the lowest mean score for sucrose preference (72.48) and, thus, the lowest preference for sucrose water pre-weaning at PD 12-15 (main effect of diet: $F(1,52)=2.517$; $p=0.119$; main effect of pregnancy-related status: $F(1,52)=0.687$; $p=0.411$; interaction between diet and pregnancy-related status: $F(1,52)=3.407$; $p=0.071$; Figure 3.4 B) [118]. Further *post hoc* analysis found that pregnant mice on HFD had significantly lower sucrose preference when compared to pregnant mice on RCD (95.33; $p<0.002$), or for non-pregnant mice on HFD (90.29) or RCD (88.56) (Figure 3.4 B) [118].

This difference in sucrose preference test values was not detected post-weaning (main effect of diet: $F(1,92)=0.01$; $p=0.919$; main effect of pregnancy-related status: $F(1,92)=0.186$; $p=0.667$; interaction between diet and pregnancy-related status: $F(1,92)=1.970$; $p=0.164$). Comparable mean values were detected in both non-pregnant (88.48 for RCD and 79.09 for HFD) and pregnant mice (75.23 for RCD and 86.10 for HFD) independent of diet (Figure 3.4 C) [118].

Figure 3.4

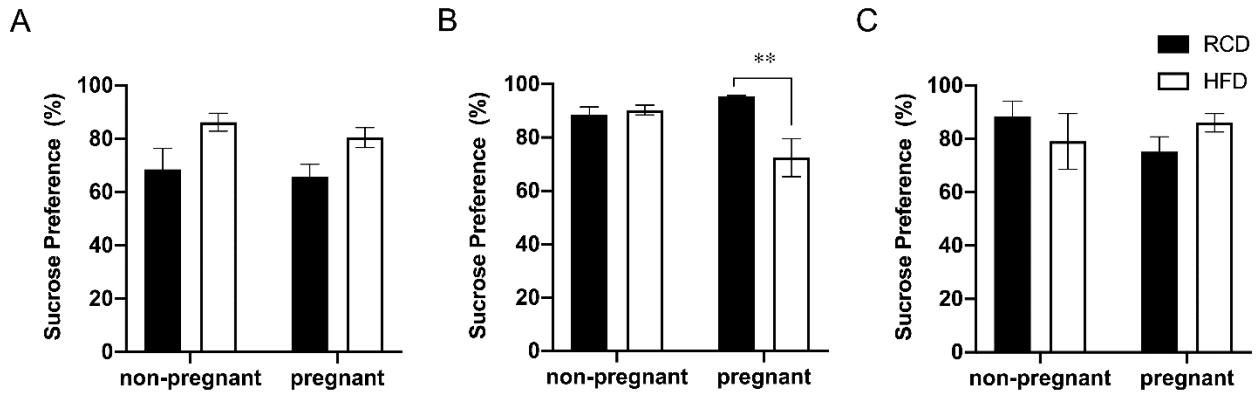

Figure 3.4. Effect of regular chow diet (RCD) and high-fat diet (HFD) on sucrose preference in pregnant and non-pregnant CD-1[WT] mice. **(A-B)** Sucrose preference in the pre-weaning period, postpartum day (PD) 6-8 and 12-15. **(C)** Sucrose preference in the post-weaning period, PD 21-23. Values are expressed as mean ± standard error of the mean and were determined and analyzed by two-way ANOVA (panels **A-C**). Sample size n=10-11 for pregnant groups and n=3-4 for non-pregnant groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. This figure is reproduced with permission from [118] and has been adapted for presentation for this thesis.

3.5 Relative brain-derived neurotrophic factor (BDNF) transcript levels at GD 6, 10.5 and 18.5 in CD-1[WT] mice

BDNF is an important biomarker of depression and other mental health disorders [102]. Brain BDNF RNA transcript levels relative to those of β-actin were determined by RT-qPCR in pregnant and age-matched non-pregnant CD-1[WT] mice at GD 6, 10.5 and 18.5. The mice were

fed either RCD or HFD. No significant effect of diet or pregnancy-related status was observed on relative RNA levels at GD 6, 10.5 and 18.5 (Figure 3.5 A-C).

Figure 3.5

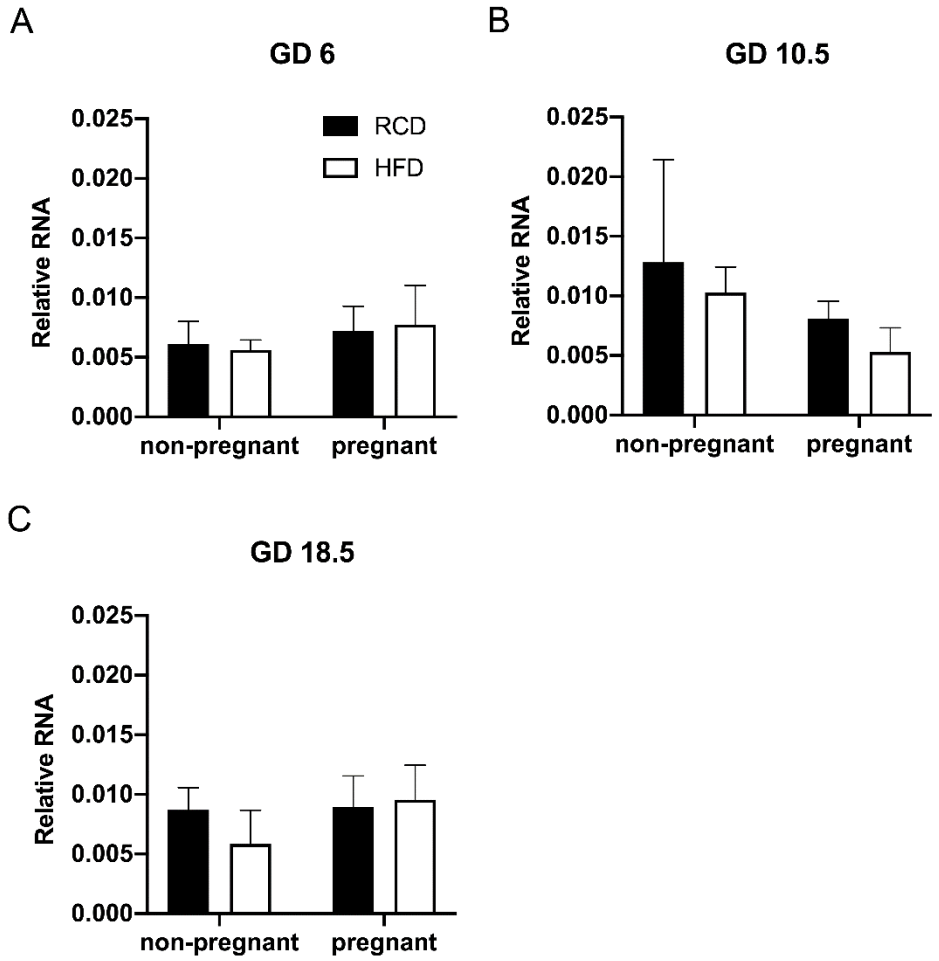

Figure 3.5. Effect of high-fat diet (HFD) and regular chow diet (RCD) on brain-derived neurotrophic factor (BDNF) RNA transcript levels (measured by real-time quantitative polymerase chain reaction (RT-PCR)), normalized to CD-1[WT] brain β-actin RNA levels (shown as relative RNA). This was assessed in non-pregnant and pregnant CD-1[WT] mice at **(A)** gestation day (GD) 6, **(B)**, GD 10.5, and **(C)** GD 18.5. Values are expressed as mean ± standard error of the mean and were determined and analyzed by two-way ANOVA. Sample size n=3-7. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3.6 BDNF protein levels in CD-1[WT] mice at GD 18.5 and PD 30

BDNF is an important biomarker of depression and other mental health disorders [102]. Levels of BDNF were assessed in CD-1[WT] pregnant and non-pregnant mice, fed either HFD or RCD, at GD 18.5 and PD 30. Whole brain samples were obtained and homogenized with a RIPA buffer [112]. The supernatant was collected and processed using an ELISA kit to detect mouse BDNF levels in whole brain samples. At GD 18.5, no significant differences were observed between all the mouse groups (Figure 3.6 A). However, at PD 30, a significant effect of diet ($F(1,28)=35.54$; $p<0.001$), pregnancy-related status ($F(1,28)=6.882$; $p=0.013$) and interaction between diet and pregnancy-related status ($F(1,28)=4.517$; $p=0.0425$) was observed (Figure 3.6 B). Further *post hoc* comparison revealed that non-pregnant mice fed RCD experienced a significant reduction in BDNF levels ($p=0.022$), as compared to mice on HFD. Similarly, *post hoc* analysis showed that pregnant mice on RCD also had lower BDNF levels ($p<0.001$), when compared to mice on HFD.

Figure 3.6

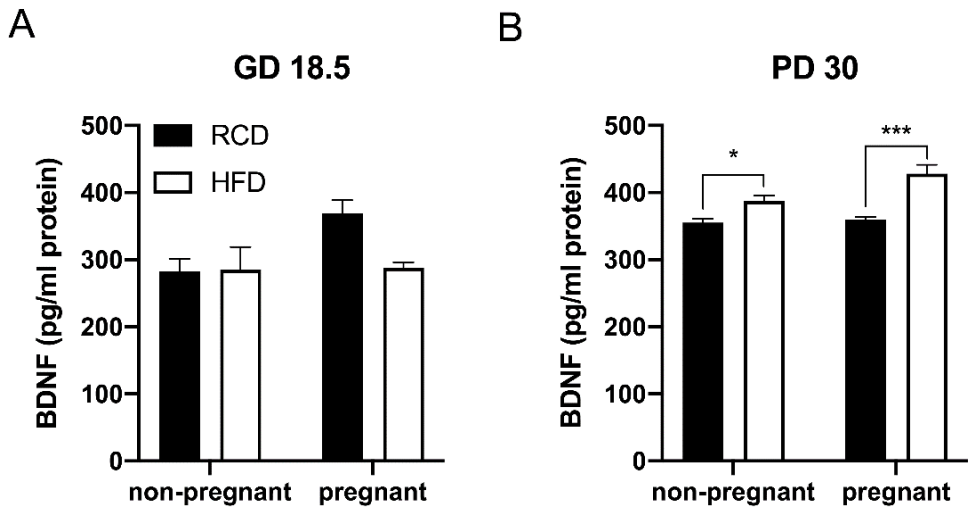

Figure 3.6. Effect of high-fat diet (HFD) and regular chow diet (RCD) on brain-derived neurotrophic factor (BDNF) levels (measured by enzyme-linked immunosorbent assay (ELISA)) in pregnant and non-pregnant CD-1[WT] mice. BDNF levels were assessed at **(A)** GD 18.5, and **(B)** postpartum day (PD) 350. Values are expressed as mean ± standard error of the mean and were determined and analyzed by t-test (A) and two-way ANOVA (B). Sample size n=4, except for pregnant HFD mice, n=2 at GD 18.5. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3.7 Relative levels of proBDNF and mBDNF in CD-1[WT] mice at GD 6, 10.5 and 18.5

Relative levels of the 32 kDa proBDNF and 14 kDa mBDNF were assessed in whole brain tissue of CD-1[WT] pregnant mice on the RCD or HFD at GD 6, 10.5 and 18.5 in collaboration with the laboratory of Dr. E. Kardami (Human Anatomy & Cell Science, University of Manitoba/Institute of Cardiovascular Sciences, St Boniface Hospital Albrechtsen Research Centre). This was pursued using protein (western) immunoblot analysis with post-staining of the transfer membrane with Ponceau Red to normalize loading (Figure 3.7.1). Following densitometry and statistical analysis, no significant differences between relative proBDNF levels were observed between all mouse groups in pregnancy (Figure 3.7.2 A). Similarly, no significant differences were observed in the relative levels of mBDNF regardless of diet-status or gestation day (Figure 3.7.2

B). However, a decreasing trend of mBDNF levels across pregnancy in the mice on the RCD is suggested. This trend is supported by analysis of the effect of RCD on relative mBDNF levels across different gestation days. A significant 34% decrease from GD 6 to GD 18.5 was suggested by one-way ANOVA analysis with multiple comparisons; p=0.012. Similarly, analysis of the effect of HFD on relative mBDNF levels across different gestation days found a significant decrease from GD 6 to GD 10.5 by one-way ANOVA with multiple comparisons; p=0.021. Although not significant, a slight increase in relative mBDNF levels was also suggested at GD 18.5 in the mice on the HFD.

Figure 3.7.1

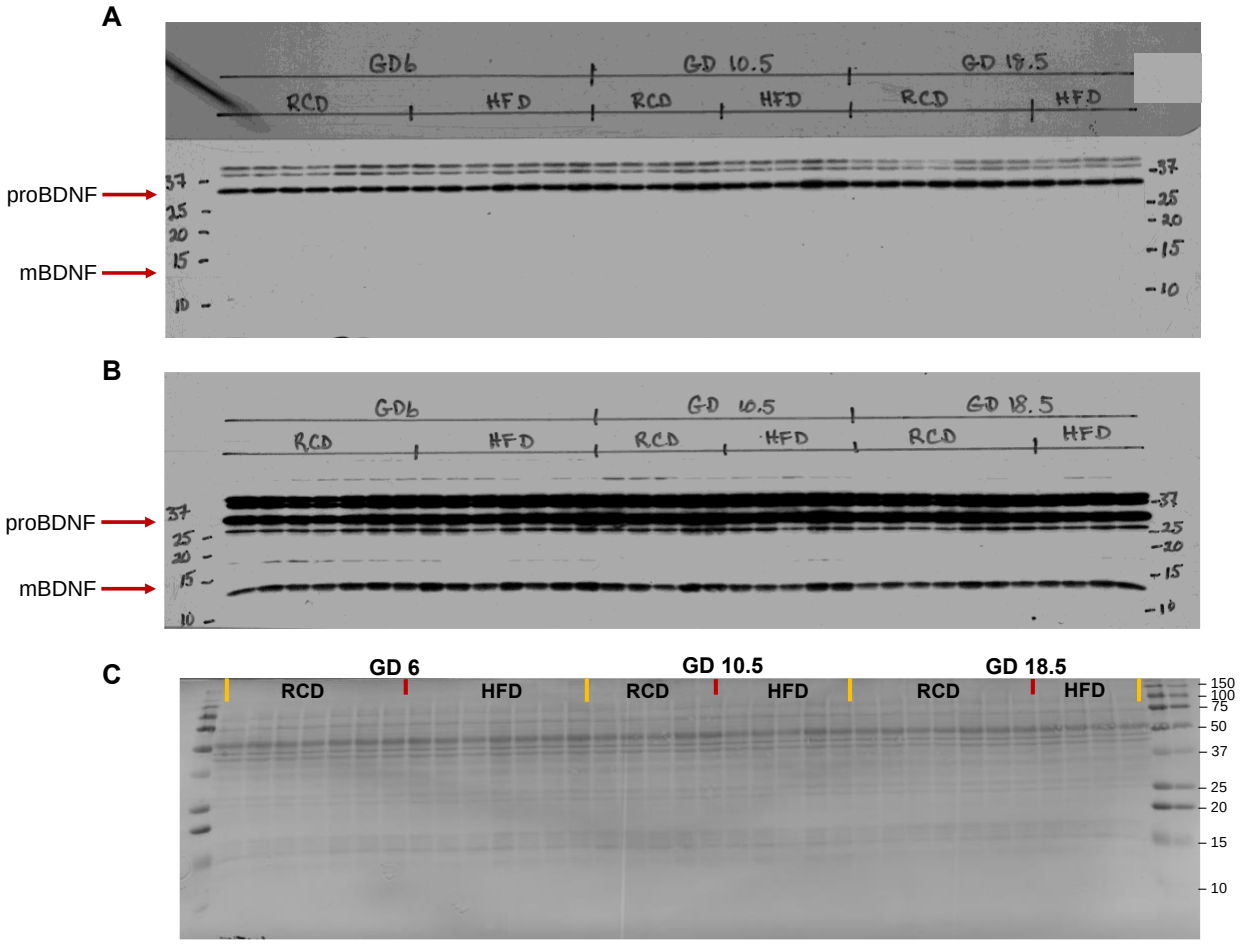

Figure 3.7.1 Effect of high-fat diet (HFD) and regular chow diet (RCD) on pro-brain-derived neurotrophic factor (proBDNF) levels and mature BDNF (mBDNF) levels (measured by protein (western) immunoblot analysis) in pregnant CD-1[WT] mice. This was assessed at gestation day (GD) 6, 10.5 and 18.5. **(A)** Shorter duration of exposure of the immunoblot depicting 32 kilodaltons (kDa) proBDNF band. **(B)** Longer duration of exposure of the same immunoblot showing 32 kDa proBDNF and 14 kDa mBDNF bands. **(C)** Ponceau Red staining of the transfer membrane. In addition, the first lane and the last two lanes of the Ponceau Red image represent marker lanes. The numbers on the side reflect the relative location of the molecular weight markers in kDa. Images provided by the laboratory of Dr. E. Kardami (Human Anatomy & Cell Science, University of Manitoba/Institute of Cardiovascular Sciences, St Boniface Hospital Albrechtsen Research Centre).

Figure 3.7.2

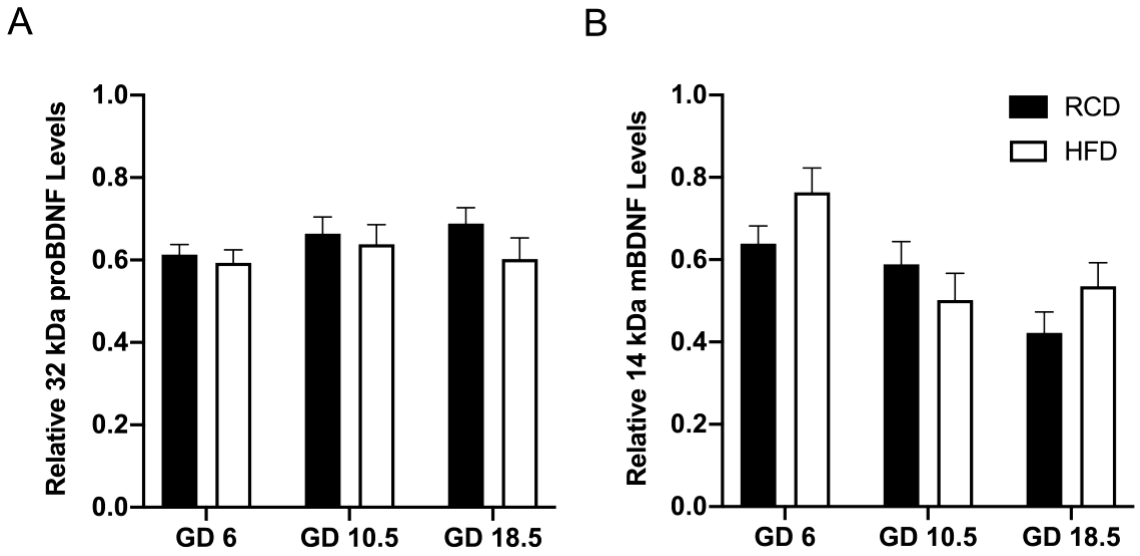

Figure 3.7.2 Effect of high-fat diet (HFD) and regular chow diet (RCD) on pro-brain-derived neurotrophic factor (proBDNF) levels and mature BDNF (mBDNF) levels (measured by protein (western) immunoblot analysis) in pregnant CD-1[WT] mice. **(A)** Relative levels of 32 kDa proBDNF and **(B)** 14 kDa mBDNF at gestation day (GD) 6, 10.5 and 18.5. Values are expressed as mean ± standard error of the mean and were determined and analyzed by two-way ANOVA. Sample size n=4-7. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. This was assessed in collaboration with the laboratory of Dr. E. Kardami (Human Anatomy & Cell Science, University of Manitoba/Institute of Cardiovascular Sciences, St Boniface Hospital Albrechtsen Research Centre).

CHAPTER 4

Effect of a high-fat diet in pregnancy on maternal CD-1[171hGH/CS] mouse behaviour

4.1 Characterization of the CD-1[171hGH/CS] RCD *versus* HFD mouse model

4.1.1 Total body weights of non-pregnant and pregnant CD-1[171hGH/CS] mice

Total body weight of CD-1[171hGH/CS] mice on a HFD or RCD was measured and recorded weekly. A significant weight gain was observed in the non-pregnant female mice on HFD, when compared to mice on RCD (main effect of diet: $F(1,865)=101.8$; $p<0.001$; main effect of time in weeks: $F(10, 865)=169.2$; $p<0.001$; interaction between diet and time in weeks: $F(10,865)=3.522$; $p=0.0001$; Figure 4.1.1 A). Specifically, weight gain was significant in the HFD mice from weeks 3-6 and weeks 10-11 ($p<0.007$).

A significant weight gain was also seen in pregnant female CD-1[171hGH/CS] mice on a HFD *versus* a RCD (main effect of diet: $F(1,173)=51.52$; $p<0.001$; main effect of time in weeks: $F(6,173)=53.73$; $p<0.001$; interaction between diet and time in weeks: $F(6,173)=2.776$; $p=0.013$; Figure 4.1.1 B). These mice were bred towards the end of week 5, and remained pregnant until week 8, when they finally gave birth. Weights were also recorded throughout the postpartum period (weeks 9-11). *Post hoc* comparison detected significant weight gain in HFD mice in weeks 10 ($p<0.001$) and 11 ($p=0.015$) but not in week 9. This can be attributed to the amount of time the dams spent nursing or caring for the newborn litter immediately after parturition. It is known that in the first week after giving birth, dams spend up to 90% of their time with the litter [29]. However, after the first week, dams will start to spend more time outside of the nest [29].

Figure 4.1.1

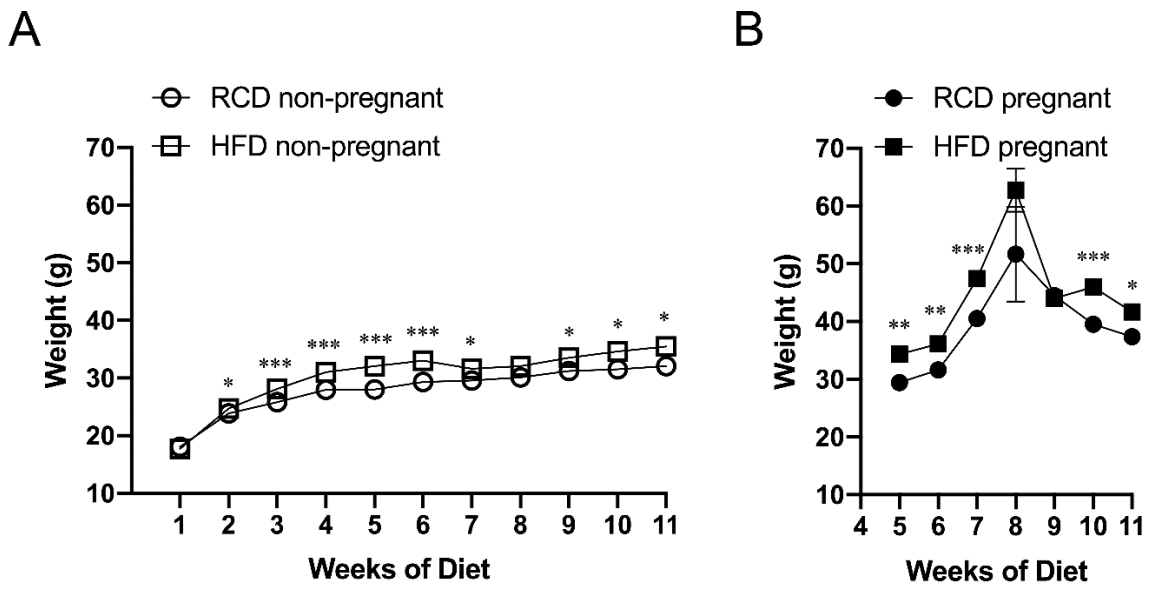

Figure 4.1.1. Effect of high-fat diet (HFD) and regular chow diet (RCD) on body weight (in grams (g)). **(A)** Weights of non-pregnant CD-1[171hGH/CS] mice over the course of the study (11 weeks). **(B)** Weights of pregnant mice during and after pregnancy (weeks 5-11). Values are expressed as mean ± standard error of the mean and were determined and analyzed by two-way ANOVA. Sample size (n) for **(A)** is 38-57 and **(B)** 14-16. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.1.2 Litter size of pregnant CD-1[171hGH/CS] mice

Female CD-1[171hGH/CS] mice on HFD or RCD were bred after being on their respective diets for 5 weeks. Litter size was counted at parturition. The average litter size for dams fed RCD or HFD was 9.64 and 10.42, respectively, and were not significantly different ($t(21)=0.519$; $p=0.609$; Figure 4.1.2). This suggests, that there was no effect of HFD or RCD on litter size, and this is similar to the findings in CD-1[WT] mice.

Figure 4.1.2

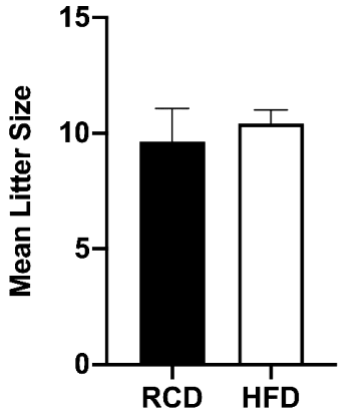

Figure 4.1.2. Mean litter size of pregnant CD-1[171hGH/CS] mice on high-fat diet (HFD) or regular chow diet (RCD) right at parturition. Values are expressed as mean ± standard error of the mean and were determined and analyzed by unpaired t-test. Sample size (n) 14-16. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.1.3 Glucose clearance of pregnant CD-1[171hGH/CS] mice

A glucose tolerance test (GTT) was performed on female CD-1[171hGH/CS] mice that had been on the HFD or RCD diet for 5 weeks at GD -1 (before breeding) and at GD 16.5 (in pregnancy). A HFD for 5 weeks significantly impaired glucose clearance in non-pregnant mice at GD -1 (main effect of diet: $F(1,21)=8.732$; $p=0.0076$; main effect of time in minutes: $F(3.119,65.49)=5$, $p<0.001$; interaction between diet and time in minutes: $F(6,126)=2.748$, $p=0.0152$; Figure 4.1.3 A). Similarly, at GD 16.5, HFD of approximately 7 weeks was associated with significant impairment in glucose clearance in pregnant CD-1[171hGH/CS] female mice (main effect of diet: $F(1,119)=88.07$; $p<0.001$; main effect of time in minutes: $F(6,119)=106.5$, $p<0.001$; interaction between diet and time in minutes: $F(6, 119)=5.376$, $p<0.001$; Figure 4.1.3 B). Hence, the CD-1[171hGH/CS] mice had impaired glucose clearance before and during pregnancy, similar to the CD-1[WT] mice.

Figure 4.1.3

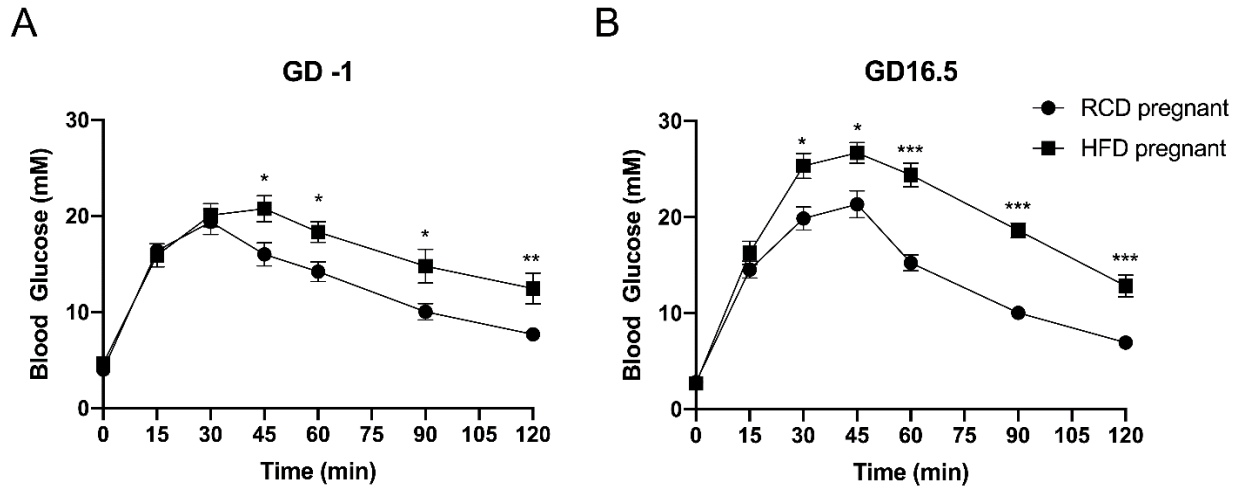

Figure 4.1.3. Glucose clearance assessed by glucose tolerance test (GTT) in CD-1[171hGH/CS] non-pregnant and pregnant female mice on a high-fat diet (HFD) or regular chow diet (RCD). Glucose clearance was assessed over a period of 120 minutes at **(A)** GD -1, after 5 weeks on their respective diets and at **(B)** GD 16.5 (during pregnancy). Values are expressed as mean ± standard error of the mean and were determined and analyzed by two-way ANOVA. Sample size (n) for **(A)** is 8-11 and **(B)** is 11-12. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.2 Maternal behaviour in CD-1[171hGH/CS] mice

4.2.1 Nest building behaviour prepartum in CD-1[171hGH/CS] mice

Maternal behaviour was assessed in pregnant female CD-1[171hGH/CS] mice in the prepartum period at GD 16.5 via nest building. Mice had been on their respective diets, HFD or RCD, for approximately 7 weeks by this time. Mice on an HFD or RCD had an average mean score of 6.115 and 7.05, respectively. These values are not significantly different ($t(21)=1.119$; $p=0.2758$; Figure 4.2.1). Thus, in contrast to the CD-1[WT] mice (Figure 3.2.1), the HFD did not significantly impair prepartum nest building behaviour in these mice.

Figure 4.2.1

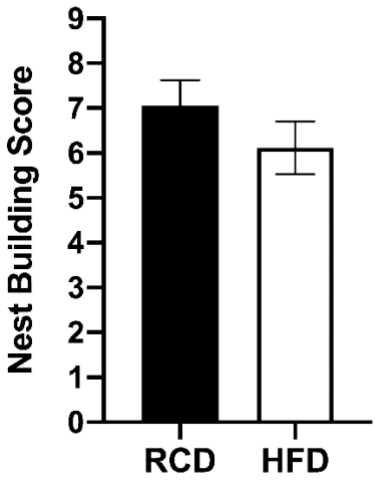

Figure 4.2.1. Average nest building scores in CD-1[171hGH/CS] mice at gestation day (GD) 16.5 in pregnant mice on regular chow diet (RCD) and high-fat diet (HFD). Values are expressed as mean ± standard error of the mean and were determined and analyzed by unpaired t-test. Sample size n=10-13. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.2.2 Postpartum pup retrieval behaviour in CD-1[171hGH/CS] mice

Pup retrieval behaviour was used to assess maternal behaviour postpartum in pregnant CD-1[171hGH/CS] mice fed a regular chow diet (RCD) or high-fat diet (HFD). Pup retrieval behaviour was assessed from PD 3-5. On the first day, at PD 3, the mean score for latency to retrieve was similar for mice on HFD (159.5) and RCD (131.5) (main effect of diet: $F(1,104)=1.798$, $p=0.183$; main effect of pup retrieval time: $F(3, 104)=3.535$, $p=0.0174$; interaction between diet and pup retrieval time: $F(3, 104)=0.044$, $p=0.988$; Figure 4.2.2 A). Further *post hoc* comparison also found no significant difference between retrieval times for each pup. In addition, although, mice on a HFD had a higher completion rate (13/16; 81.25%) than mice on a RCD (9/12; 75%), it was not significantly different.

At PD 4, the mean score for latency to retrieve for mice on HFD (127.5) improved and is similar to the mice on the RCD (115.3). Though mice on an HFD are able to retrieve the pups faster on the second day, there was no significant difference between the retrieval times for the HFD and RCD mice (main effect of diet: $F(1,104)=0.2669$, $p=0.606$; main effect of pup retrieval time: $F(3,104)=1.210$, $p=0.31$; interaction between diet and pup retrieval time: $F(3,104)=0.024$, $p=0.995$; Figure 4.2.2 B).

By the third and last day of the assay, PD 5, HFD-induced obesity significantly impairs pup retrieval behaviour, where the mean score for latency to retrieve is significantly higher in mice on HFD (119.3) compared to RCD (63.94) (main effect of diet: $F(1,104)=8.9226$, $p=0.0035$; main effect of pup retrieval time: $F(3,104)=2.840$, $p=0.0415$; interaction between diet and pup retrieval time: $F(3,104)=0.192$, $p=0.902$; Figure 4.2.2 C). Further *post hoc* comparison found no significant difference between retrieval times for each pup regardless of diet status. Unlike at PD 3, mice on a HFD had a lower completion rate by PD 5 (13/16; 81.25%), when compared to mice on RCD (11/12; 91.67%). Thus, the negative effect of HFD on pup retrieval in CD-1[WT] mice, and specifically on the first day of testing (Figure 3.2.2), was not detected in CD-1[171hGH/CS] mice.

Figure 4.2.2

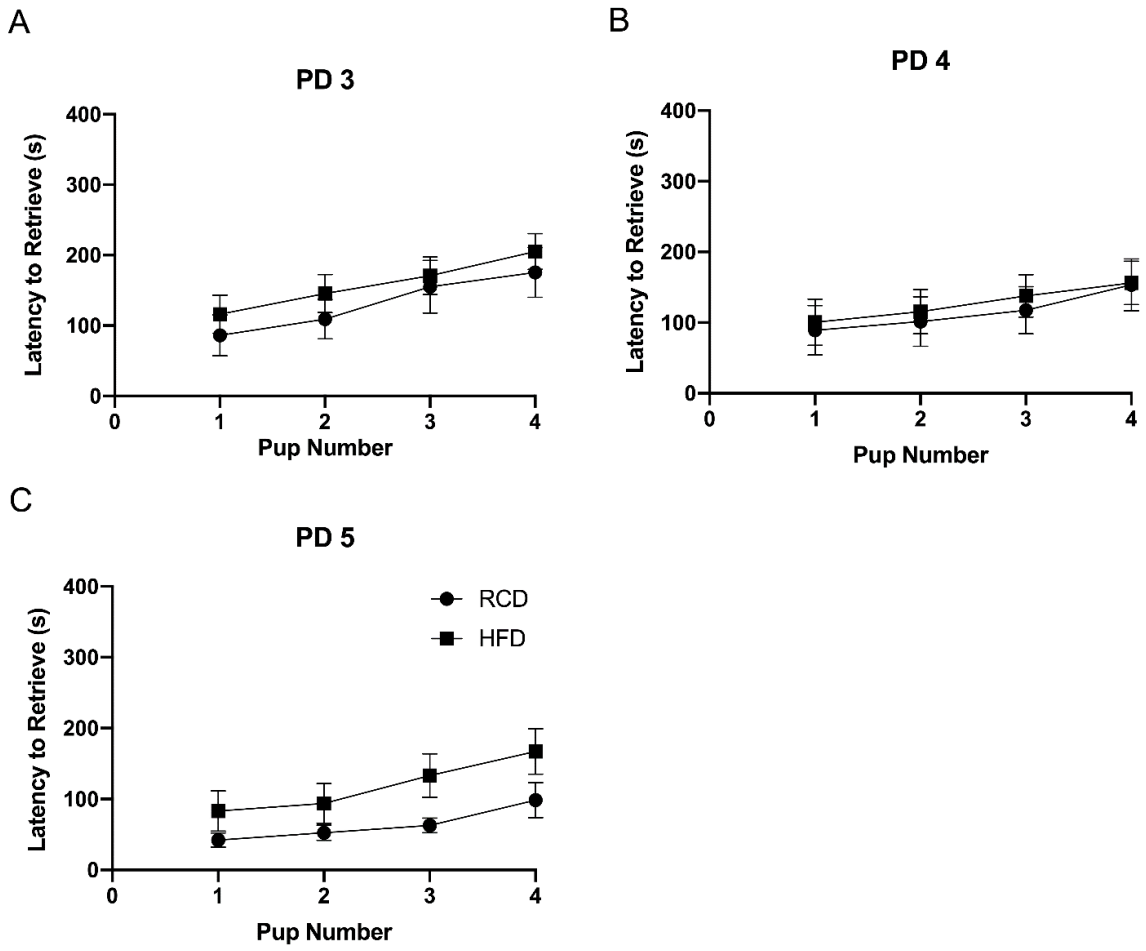

Figure 4.2.2. Pup retrieval behaviour was assessed from postpartum day (PD) 3. Latency to retrieve all four pups within 360 seconds on **(A)** PD 3, **(B)** PD 4 and **(C)** PD 5. Values are expressed as mean ± standard error of the mean and were determined and analyzed by two-way ANOVA. Sample size n=12-16. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.2.3 Postpartum nursing behaviour in CD-1[171hGH/CS] mice

Maternal behaviour was assessed postpartum through nursing behaviour observations in CD-1[171hGH/CS] female mice from PD 2-6. Nursing behaviour was evaluated for 30 minutes in two different periods (10-11 a.m. and 2-3 p.m.) during the light phase [31]. As defined in section

2.4, if the dam was in the nest, and engaging in any aspect of maternal behaviour, such as nursing, anogenital licking, body licking, nest building or in physical contact with the pups, it was cumulatively assigned to be 'contact rest' with pups [31]. However, if the dam was outside of the nest, and engaging in non-maternal behaviours such as self-grooming, eating, or drinking, this was assigned as 'non-contact rest' [31]. There was no significant difference between the contact rest of mice on RCD or HFD, during the morning observation periods from PD 2-6 (main effect of diet: $F(1,70)=1.635$, $p=0.205$; main effect of postpartum day: $F(1,701)=1.86$, $p=0.127$; interaction between diet and postpartum day: $F(4,70)=1.1$, $p=0.364$; Figure 4.2.3 A). Similarly, no significant difference was observed in the frequency of non-contact rest, in RCD or HFD mice groups (main effect of diet: $F(1.,70)=0.811$, $p=0.371$; main effect of postpartum day: $F(4,70)=2.194$, $p=0.078$; interaction between diet and postpartum day: $F(4,70)=2.102$, $p=0.089$; Figure 4.2.3 B).

When contact rest was evaluated in the afternoon, no significant difference was observed in mice fed RCD or HFD (main effect of diet: $F(1,68)=0.496$, $p=0.483$; Figure 4.2.3 C). However, postpartum day appears to have a significant influence on contact rest frequency in the afternoon (main effect of postpartum day: $F(4,68)=2.714$, $p=0.036$; interaction between diet and postpartum day: $F(4,68)=0.789$, $p=0.536$; Figure 4.2.3 C). Further *post hoc* comparison did not reveal any significant difference in contact rest frequency between the individual postpartum days assessed. Regardless, the graph does show a trend of slow but gradual decline in contact rest with pups from PD 2-6 in the afternoon (Figure 4.2.3 C). Similarly, diet did not influence frequency of non-contact rest in the afternoon in the RCD or HFD mice (main effect of diet: $F(1,68)=0.704$, $p=0.404$; Figure 4.2.3 D), but postpartum day does have a significant effect (main effect of postpartum day: $F(4,68)=3.177$, $p=0.018$; interaction between diet and postpartum day: $F(4,68)=0.547$, $p=0.702$; Figure 4.2.3 D). However, *post hoc* comparison did not find any significant difference between

each postpartum day. The graph does show a trend of increasing frequency of non-contact rest with the pups from PD 2-6 for mice on HFD or RCD (Figure 4.2.3 D).

Figure 4.2.3

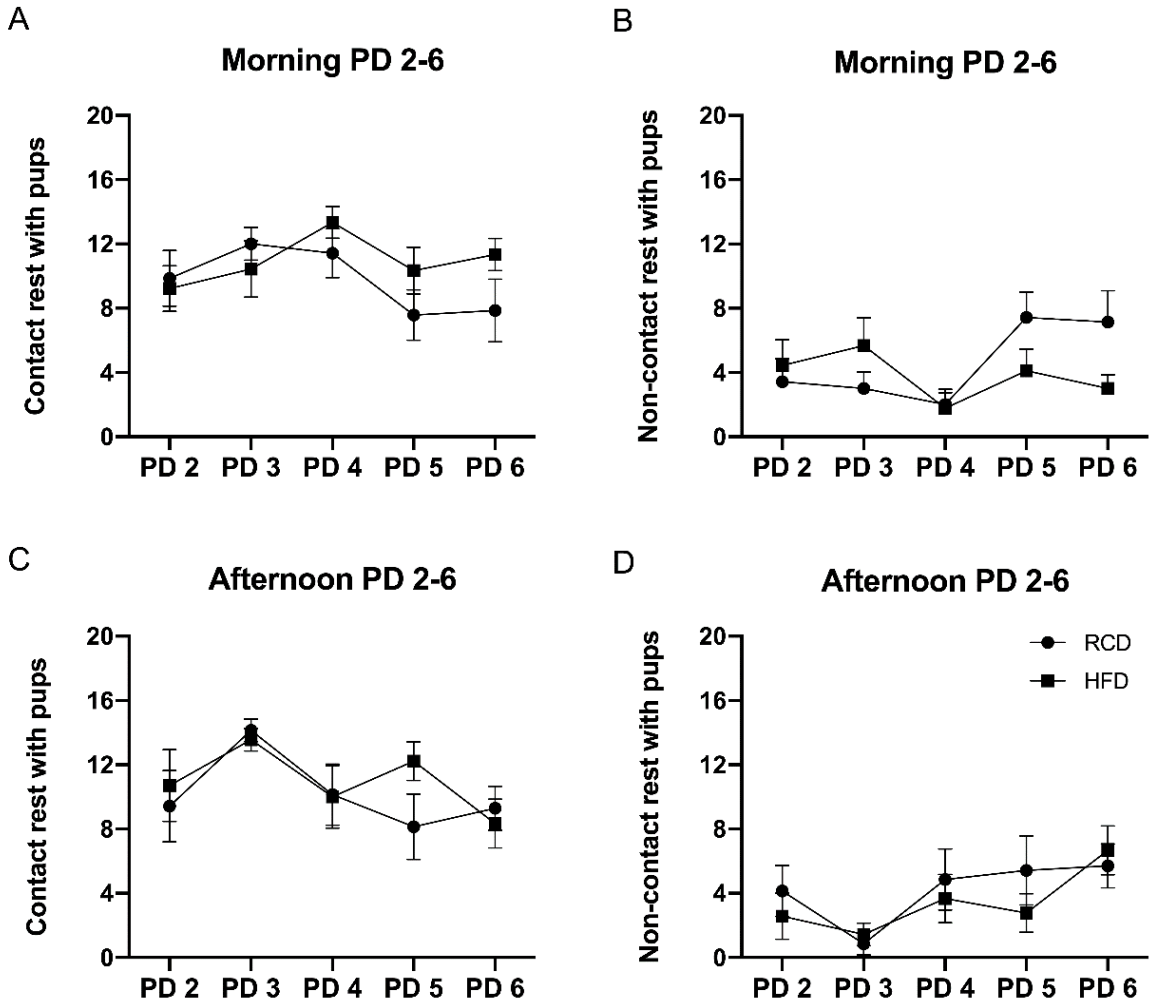

Figure 4.2.3. Maternal behaviour, as represented by contact rest, which measured nursing, anogenital licking, body licking, nest building or physical contact with the pups *versus* non-contact rest, which measured non-maternal behaviour, such as the dam being outside of the nest, and engaging in self-grooming, eating, or drinking. This was assessed from postpartum day (PD) 2-6 in female CD-1[171hGH/CS] mice during two periods in the light phase (morning and afternoon). **(A)** Frequency of contact rest with pups and **(B)** non-contact rest with pups in the morning. **(C)** Frequency of contact rest with pups and **(D)** non-contact rest with pups in the afternoon. Values are expressed as mean ± standard error of the mean and were determined and analyzed by two-way ANOVA. Sample size n=7-9. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.3 Anxiety-like behaviour and object recognition memory in CD-1[171hGH/CS] mice

4.3.1 Anxiety-like behaviour pre- and postpartum in CD-1[171hGH/CS] mice

Anxiety-like behaviour was assessed in CD-1[171hGH/CS] mice prepartum and postpartum at GD 15.5 and PD 20, respectively. This helped provide some insight into the effect of pregnancy-status in the prepartum and postpartum period on anxiety-like behaviour. Pregnant and non-pregnant age-matched mice on RCD or HFD were used. In the prepartum period at GD 15.5, there was no significant difference in the percentage of time spent on the open arms between all groups (main effect of diet: $F(1,56)=0.0132$, $p=0.907$; main effect of pregnancy-related status: $F(1,56)=0.943$, $p=0.336$; interaction between diet and pregnancy-related status: $F(1.56)=2.481$, $p=0.121$; Figure 4.3.1 A). Also, time spent on the closed arms was not significantly different amongst all groups (main effect of diet: $F(1,56)=0.056$, $p=0.814$; main effect of pregnancy-related status: $F(1,56)=0.126$, $p=0.724$; interaction between diet and pregnancy-related status: $F(1,56)=0.676$, $p=0.414$; Figure 4.3.1 B). Furthermore, there was no significant effect of diet ($F(1,56)=0.004$, $p=0.949$) or pregnancy-related status ($F(1,56)=1.885$, $p=0.175$; Figure 4.3.1 C) on the percentage of time spent in the centre region. However, the interaction between diet and pregnancy-related status ($F(1,56)=5.899$, $p=0.018$; Figure 4.3.1 C) led to a significant difference for the percentage of time spent in the centre region in these mice. Further *post hoc* comparison did not reveal any significant differences between the pregnant and non-pregnant mice on RCD or HFD. Also, neither diet ($F(1,56)=2.151$, $p=0.148$) nor pregnancy-related status ($F(1,56)=0.343$, $p=0.56$) had a significant effect on total distance travelled on the EPM on GD 15.5. However, a significant effect of interaction between diet and pregnancy-related status was observed ($F(1,56)=4.198$, $p=0.045$; Figure 4.3.1 D).

Postpartum anxiety-like behaviour was assessed at PD 20 post-weaning, in pregnant and non-pregnant age-matched CD-1[171hGH/CS] female mice on RCD or HFD. There was no

significant difference in the percentage of time spent on the open arms (main effect of diet: $F(1,55)=0.509$, $p=0.478$; main effect of pregnancy-related status: $F(1,55)=1.527$, $p=0.222$; interaction between diet and pregnancy-related status: $F(1,55)=0.509$, $p=0.478$; Figure 4.3.1 E). Similarly, there was no significant difference in the percentage of time spent on the centre region (main effect of diet: $F(1,55)=0.816$, $p=0.37$; main effect of pregnancy-related status: $F(1,55)=0.261$, $p=0.112$; interaction between diet and pregnancy-related status: $F(1,55)=0.036$, $p=0.849$; Figure 4.3.1 G). In addition, diet ($F(1,55)=0.150$, $p=0.747$) and interaction between diet and pregnancy-related status ($F(1,55)=2.261$, $p=0.138$; Figure 4.3.1 F) did not have a significant effect on the percentage of time spent on the closed arms. However, pregnancy-status ($F(1,55)=78.43$, $p<0.001$; Figure 4.3.1 F) had a significant effect on percentage of time spent on the closed arms. *Post hoc* comparison revealed that pregnant mice on HFD spent a significantly lower percentage of time on the closed arms when compared to non-pregnant mice on HFD ($p<0.001$; Figure 4.3.1 F). Similarly, time spent on the closed arms was significantly decreased in the pregnant mice on RCD when compared to non-pregnant mice on RCD ($p<0.001$; Figure 4.3.1 F). Lastly, total distance travelled on the EPM on PD 20 was not significantly different between the pregnant and non-pregnant mice fed RCD or HFD (main effect of diet: $F(1,55)=0.076$, $p=0.784$; main effect of pregnancy-related status: $F(1,55)=0.348$, $p=0.558$; interaction between diet and pregnancy-related status: $F(1,55)=0.112$, $p=0.738$; Figure 4.3.1 H).

Figure 4.3.1

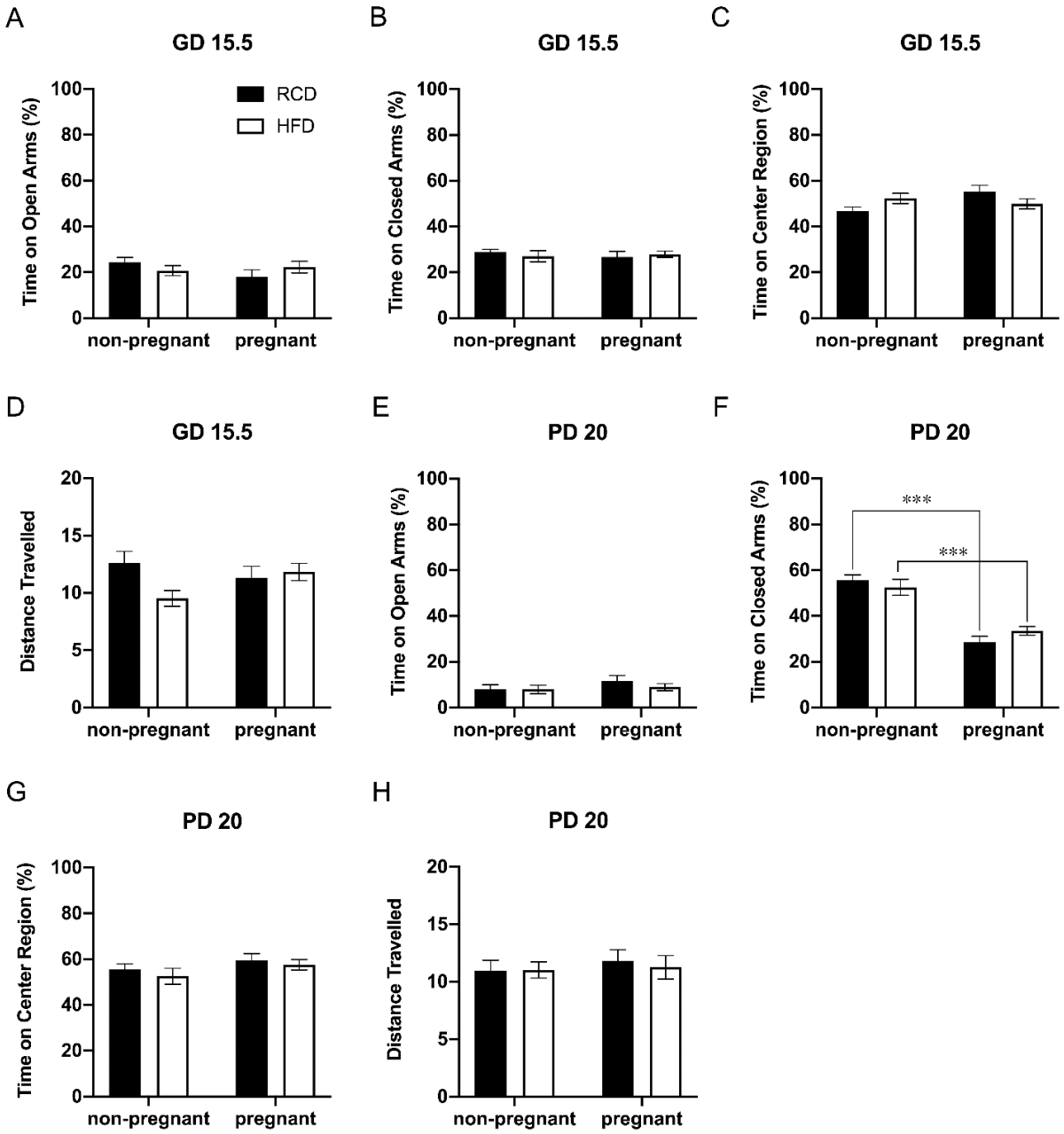

Figure 4.3.1. The effect of high-fat diet (HFD) and regular chow diet (RCD) on anxiety-like behaviour prepartum, as assessed by elevated plus maze (EPM), on **(A-D)** gestation day (GD) 15.5 and **(E-H)** postpartum day (PD) 20, in pregnant and non-pregnant CD-1[171hGH/CS] mice. **(A-C)** Percentage of time spent on the closed arms, open arms and centre region of the EPM at GD 15.5. **(D)** Total distance travelled on the EPM at GD 15.5. **(E-G)** Percentage of time spent on the closed arms, open arms and centre region of the elevated plus maze (EPM) at PD 20. **(H)** Total distance travelled on the EPM at PD 20. Values are expressed as mean ± standard error of the mean and were determined and analyzed by two-way ANOVA (panels **A-H**). Sample size n=12-17. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.3.2 Object recognition memory assessment in CD-1[171hGH/CS] mice postpartum

Object recognition memory or cognitive function was assessed in CD-1[171hGH/CS] mice via novel object recognition (NOR) test post-weaning from PD 23-25 as described in Figure 2.7. Pregnant and non-pregnant age-matched female mice on RCD or HFD were used. Following a habituation period (PD 23), mice underwent the familiarization phase (PD 24) and introduction of a novel object phase (PD 25). The preference index, which is based on the ratio of the time (in seconds) spent exploring two similar objects, was determined for the familiarization phase. The preference index was not significantly different amongst all mouse groups, and hence was not affected by diet or pregnancy-related status (main effect of diet: $F(1,55)=0.58$, $p=0.4495$; main effect of pregnancy-related status: $F(1,55)=0.58$, $p=0.449$; interaction between diet and pregnancy-related status: $F(1,55)=0.237$, $p=0.628$; Figure 4.3.2 A). The preference index suggests that there was no preference for the identical objects placed on the left or right side of the NOR apparatus during the 5-minute testing period.

The discrimination index, which is the difference in time spent exploring the novel and familiar object, over the time spent exploring both objects, was determined for the introduction of a novel object phase. The discrimination index was assessed at the 1- and 5-minute mark of the total test time on PD 25. At the 1-minute mark, diet had a significant effect on discrimination index ($F(1,55)=5.29$, $p=0.025$), unlike pregnancy-related status ($F(1,55)=3.55$, $p=0.065$) and interaction between diet and pregnancy-related status ($F(1,55)=2.07$, $p=0.155$; Figure 4.3.2 B). Further *post hoc* comparison revealed a significant difference between the non-pregnant mice on RCD *versus* HFD ($p=0.045$; Figure 4.3.2 B). Specifically, the majority of non-pregnant mice fed RCD preferred the novel object, whereas the majority of non-pregnant mice fed HFD preferred the familiar object. Although not significant, our data suggest that pregnant mice on RCD or HFD also preferred the familiar object (Table 4).

When the discrimination index was assessed at the 5-minute mark, no significant differences were found amongst all mouse groups (main effect of diet: $F(1,55)=0.808$, $p=0.373$; main effect of pregnancy-related status: $F(1,55)=1.814$, $p=0.184$; interaction between diet and interaction between diet and pregnancy-related status: $F(1,55)=0.661$, $p=0.419$; Figure 4.3.2 C). Although not significant, our data suggest that all mice preferred the familiar object at the 5-minute mark (Table 4). Total distance travelled on the NOR on PD 25 was not significantly different amongst all groups (main effect of diet: $F(1,55)=0.324$, $p=0.571$; main effect of pregnancy-related status: $F(1,55)=0.778$, $p=0.382$; interaction between diet and interaction between diet and pregnancy-related status: $F(1,55)=0.735$, $p=0.395$; Figure 4.3.2 D).

Figure 4.3.2

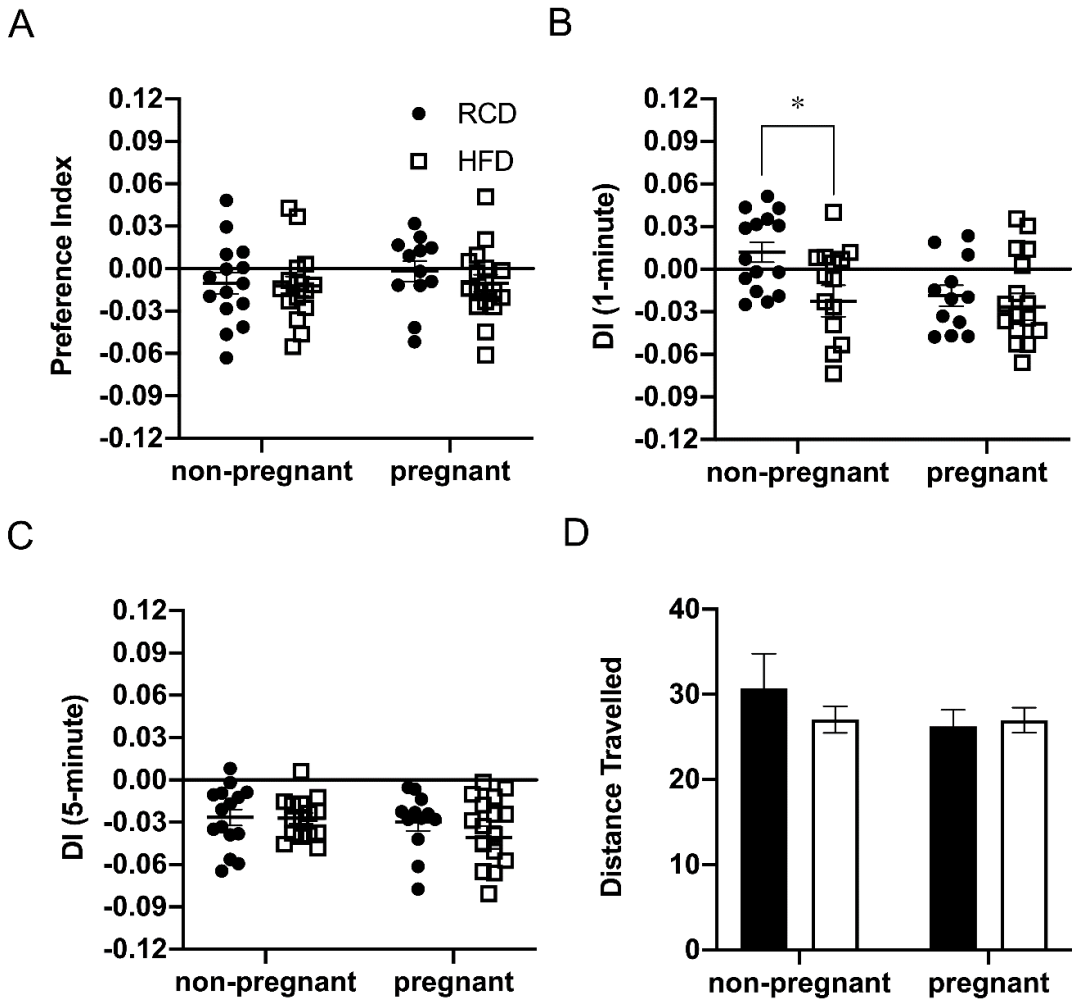

Figure 4.3.2. Effect of high-fat diet (HFD) and regular chow diet (RCD) on cognitive function or object recognition memory on postpartum day (PD) 24 (familiarization phase) and PD 25 (introduction of a novel object) in pregnant and non-pregnant CD-1[171hGH/CS] mice. **(A)** Preference index on PD 24. **(B)** Discrimination index (DI) in the first minute and **(C)** at the 5-minute mark on PD 25. **(D)** Total distance travelled during the 5-minute testing duration on the novel-object recognition test on PD 25. Values are expressed as mean ± standard error of the mean and were determined and analyzed by two-way ANOVA (panels **A-D**). Sample size n=12-17. A score of 0 suggests no evidence of discrimination between the objects, whereas a negative score refers to preference for the familiar object, and a positive score indicates preference for the novel object. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table 4: Summary of object interaction at the 1- and 5-minute mark on day 3 (introduction of novel object) on PD 25

	Object Interaction: 1-minute	*Object Interaction: 5-minute*
RCD non-pregnant	Novel	Familiar
HFD non-pregnant	Familiar	Familiar
RCD pregnant	Familiar	Familiar
HFD pregnant	Familiar	Familiar

4.4 *Assessment of BDNF protein levels in CD-1[171hGH/CS] mice at GD -1, GD 18.5 and PD 26*

Levels of BDNF were assessed in CD-1[171hGH/CS] pregnant and non-pregnant mice on HFD or RCD at GD -1 (one day before pregnancy), GD 18.5 and PD 26, using an enzyme-linked immunosorbent assay. Whole brain samples were collected at the specified times and homogenized using a RIPA buffer [112]. No significant differences were observed in the brain BDNF levels at GD -1 between non-pregnant mice on RCD or HFD (Figure 4.4 A). However, a significant effect of diet ($F(1,25)=117.4$, $p<0.001$), pregnancy-related status ($F(1,26)=39.38$, $p<0.001$) and interaction between diet and pregnancy-related status ($F(1,26)=29.27$, $p<0.001$; Figure 4.4 B) was observed by GD 18.5. Age-matched non-pregnant mice on HFD had significantly lower BDNF levels ($p<0.001$) than mice fed RCD (Figure 4.4 B). BDNF levels were also significantly reduced in the pregnant mice on the HFD ($p=0.0021$), compared to the RCD (Figure 4.4 B). Similar findings were observed on the BDNF levels at PD 26, where a significant effect of diet ($F(1,28)=10.48$, $p=0.003$), pregnancy-related status ($F(1,28)=4.46$, $p=0.044$) and interaction between diet and pregnancy-related status ($F(1,28)=6.71$, $p=0.015$; Figure 4.4 C) was noted. Further *post hoc* comparison did not find a significant difference between the BDNF levels of non-

pregnant mice fed HFD or RCD. Conversely, *post hoc* comparison revealed that pregnant mice on HFD had significantly lower BDNF levels (p=0.0006) when compared to mice on RCD.

Figure 4.4

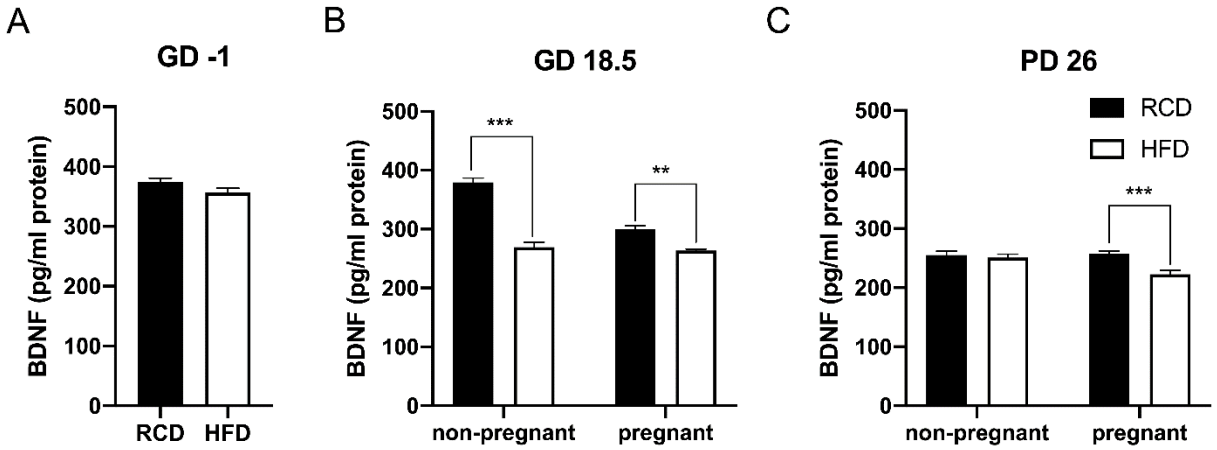

Figure 4.4. Effect of high-fat diet (HFD) and regular chow diet (RCD) on brain-derived neurotrophic factor (BDNF) levels measured by Enzyme-Linked Immunosorbent Assay in pregnant and non-pregnant CD-1[171hGH/CS] mice. BDNF levels were assessed **(A)** before pregnancy at gestation day (GD) -1, **(B)** GD 18.5, and **(C)** postpartum day (PD) 26. Values are expressed as mean ± standard error of the mean and were determined and analyzed by unpaired t-test (panel **A**) and two-way ANOVA (panels **B-C**). Sample size n=3-4. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

CHAPTER 5

Discussion

An attempt was made to study the effects of overeating, in the form of a HFD, and pregnancy-status on prepartum and postpartum behaviour. This was done in age and diet-matched outbred pregnant and non-pregnant female CD-1[WT] and CD-1[171hGH/CS] mice. This study included assessment of maternal behaviour (nest building, nursing and pup retrieval), anxiety (EPM), working memory (NOR), and anhedonia (sucrose preference test). Based on the results, this study provides novel insights into the effects of HFD-induced obesity on behaviour during pregnancy and postpartum in both a wild-type and partially humanized mouse model expressing the genes coding for human placental lactogen (hPL). Also, for the first time, this study presents the effects of HFD-induced obesity and pregnancy-status on whole brain BDNF levels before, during and after pregnancy in mice.

5.1 Distinct responses of diet and pregnancy-status in CD-1[WT] and CD-1[171hGH/CS] mice

Wild-type CD-1[WT] mice and CD-1[171hGH/CS] mice, a transgenic mouse line that expresses members of the human growth hormone gene family and produce hPL during pregnancy [113], were used to study the effects of HFD and RCD on maternal behaviour, anxiety, working memory and anhedonia. The results are summarized below in Table 5. Briefly, HFD consumption over the 10-11 weeks of study led to significant weight gain in both pregnant and non-pregnant CD-1[WT] and CD-1[171hGH/CS] mice. This also resulted in impaired glucose clearance in

pregnant CD-1[WT] and CD-1[171hGH/CS] mice. The mean litter size was not significantly different amongst both mouse models.

Subsequently, maternal behaviour was assessed prepartum by nest building and postpartum by pup retrieval and nursing in one or both CD-1 mouse models. CD-1[WT] mice on HFD *versus* RCD had significantly lower nest building score, whereas no such difference was observed in the CD-1[171hGH/CS] mice. Similarly, CD-1[WT] mice on HFD *versus* RCD had a significantly higher latency to retrieve pups on PD 3, while the CD-1[171hGH/CS] mice on HFD performed comparable to the mice on RCD. Nursing behaviour was only assessed in the CD-1[171hGH/CS] mice and no significant effect of HFD was observed on nursing. As HFD can affect anxiety levels, working memory and anhedonia in non-pregnant rodents [132], the effect in pregnant mice was explored prepartum and postpartum. Both CD-1[WT] and CD-1[171hGH/CS] mice did not display anxiety-like behaviour prepartum. However, HFD alone induced anxiety-like behaviour in non-pregnant CD-1[WT] mice postpartum but not CD-1[171hGH/CS] mice. In contrast, HFD and pregnancy-related status led to a decrease in anxiety like-behaviour in CD-1[WT] mice but did not have an effect in CD-1[171hGH/CS] mice postpartum. Working memory was significantly impaired postpartum in CD-1[WT] non-pregnant and pregnant mice on HFD but was not observed in CD-1[171hGH/CS] mice. CD-1[WT] pregnant mice on HFD had significantly lower sucrose preference, which suggests they are experiencing anhedonia pre-weaning. Due to the effect of HFD on maternal behaviour, anxiety, working memory and anhedonia, BDNF RNA and protein levels were assessed in CD-1[WT] mice. However, no difference in BDNF RNA and protein levels were observed in pregnancy, while total brain BDNF protein levels were significantly higher in mice on HFD at PD 30 as assessed by ELISA . Thus, HFD is associated with increased BDNF levels in CD-1[WT] mice in the postpartum period and, therefore, may possibly affect behavioural outcome during this time. Furthermore, since BDNF levels were not assessed earlier in pregnancy, the

possibility that modified BDNF levels also play a role in mental behaviour prepartum, cannot be ruled out. However, this was not supported by protein (western) immunoblot analysis done as part of a collaborative study with the laboratory of Dr. E. Kardami (Human Anatomy & Cell Science, University of Manitoba/Institute of Cardiovascular Sciences, St Boniface Hospital Albrechtsen Research Centre). Data were provided for use in this thesis on the relative levels of proBDNF and mBDNF in whole brain tissue of pregnant CD-1[WT] mice on the RCD or HFD at GD 6, 10.5 and 18.5. Independent levels of proBDNF and mBDNF were not significantly different in pregnant CD-1[WT] mice at each of these time points, regardless of diet. These findings are discussed later in the context of known effects of BDNF isoforms on hippocampal neurons and behavioural outcome.

Total brain BDNF protein levels were assessed in both CD-1[WT] and [171hGH/CS] mice by ELISA. However, in contrast to CD-1[WT] mice, BDNF protein levels were found to be significantly reduced in non-pregnant and pregnant mice on HFD at GD 18.5 and in pregnant mice on HFD at PD 26, more than 3 weeks postpartum. It was initially hypothesized that a HFD would be associated with reduced BDNF levels, which in turn would impair maternal behaviour. However, despite the decrease in BDNF levels at GD 18.5, maternal behaviour (as assessed by pup retrieval) is maintained in the CD-1[171hGH/CS] but not CD-1[WT] on a HFD. Thus, this decrease in BDNF level in the CD-1[171hGH/CS] mice may be associated with preserving appropriate maternal behaviour or rescuing this behaviour from the negative effects of a HFD. Again, BDNF levels were not assessed earlier in pregnancy by ELISA, and, thus, the possibility that modified BDNF levels also play a role in maintaining mental behaviour prepartum, cannot be ruled out.

Table 5: Summary of observations made in Chapters 3 and 4

Does HFD-induced obesity negatively affect outcome on the following assays:	*CD-1[WT] mice*	*CD-1[171hGH/CS] mice*
Weight	Yes	Yes
Glucose tolerance	Yes	Yes
Nest building	Yes	No
Litter size	No	No
Pup-retrieval	Yes	No
Nursing	Not studied	No
Elevated plus maze prepartum	No	No
Elevated plus maze postpartum	Yes	No
Novel object recognition	Yes	No
Sucrose preference pre-weaning	Yes	Not studied
Sucrose preference post-weaning	No	Not studied
BDNF RNA levels in pregnancy	No	Not studied
BDNF RNA levels postpartum	No	Not studied
BDNF protein levels in pregnancy	No	Yes
BDNF protein levels postpartum	Yes	Yes
proBDNF levels in pregnancy	No	Not studied
mBDNF levels in pregnancy	No	Not studied

A HFD regimen of 10 weeks in the CD-1[WT] mice and 11 weeks in the CD-1[171hGH/CS] mice resulted in significant weight gain in the non-pregnant and pregnant groups on RCD or HFD respectively [115]. In fact, they demonstrated a significant weight gain, which was already observed in CD-1[WT] mice that were maintained on a HFD pre-conception. This is most likely due to an increase in epididymal fat mass [133,134]. Although the degree of weight gain observed in the non-pregnant CD-1[WT] mice on HFD is greater than in CD-1[171hGH/CS] mice, these mice share similar physiological outcomes. The HFD regimen employed in the present study also led to glucose intolerance in the CD-1[WT] and CD-1[171hGH/CS] mice, which was detected by GTT before pregnancy at GD -1 and during pregnancy at GD 16.5. This is indicative of delayed glucose clearance, which is consistent with an increase in insulin resistance during the pregnant state, and thus onset of obesity-like traits due to the HFD [115] in both CD-1[WT] and CD-1[171hGH/CS] mice. This is consistent with a previous report of chronic HFD intake in C57BL/6J mice, which was associated with greater visceral fat mass, resulting in insulin resistance and hyperinsulinemia [135]. The mean litter size was comparable between both CD-1[WT] and CD-1[171hGH/CS] mice on RCD or HFD. Hence, both CD-1[WT] and CD-1[171hGH/CS] mice experienced weight gain on the HFD protocol, suffered glucose intolerance and had similar litter sizes. As the CD-1[WT] and CD-1[171hGH/CS] mice had similar physiological characteristics in terms of weight gain, litter size and glucose clearance, different effects on prepartum and postpartum maternal behaviours due to transgene expression and specifically hPL production in CD-1[171hGH/CS] was not a given.

Appropriate maternal care and behaviour is characterized by nest building prior to parturition and gathering, nursing, licking, grooming, and protecting the pups after birth. These behavioural outcomes in the new dam are driven by pregnancy hormones and changes in the neural circuitry in late pregnancy [21]. Withdrawal of pregnancy hormones at parturition allows for the

activation of these critical postpartum maternal behaviours and neuroendocrine functions important for lactation [21]. Thus, changes related to pregnancy, birth and lactation prime the dams brain to care for the pups and express appropriate maternal response [136]. Here, maternal behaviour was assessed by nest building prepartum and pup retrieval (gathering of pups) and nursing (feeding the pups) postpartum. Pregnant and non-pregnant mice build different quality nests as they serve different purposes. Non-pregnant mice build thermoregulatory, saucer shaped nests, which are often smaller in size, whereas, pregnant mice build large, complex, brood nests with several entrances [29]. This type of nest is critical for keeping the ectothermic pups warm and for increasing their chance of survival [29,137]. Here, nest building was observed in pregnant CD-1[WT] and CD-1[171hGH/CS] mice prior to birth of the pups at GD 16.5. Pregnant CD-1[WT] mice fed a HFD scored significantly lower on the nest building assay, building incomplete, flat and poor-quality nests, as compared to mice on an RCD, which build large, brood nests [29]. Nest building is considered to be a highly motivated behaviour [137]. Thus, the absence of motivation to build an appropriate nest for the pups, suggests that pregnant mice on the HFD exhibit reduced maternal care, which is a direct result of impaired behaviour in the dam. Conversely, CD-1[171hGH/CS] mice on HFD built good-quality nests, which were similar to the nests built by mice on RCD and received similar nest building scores. This raises the possibility that the presence of the hGH/CS transgene may be limiting the negative effects of a HFD seen on maternal behaviour in the CD-1[WT] mouse. Whether this is through a direct effect of the products of the transgene or indirect effect on other factors is unclear but is discussed later.

Similarly, postpartum maternal behaviour was also impaired in the CD-1[WT] mice on HFD, as demonstrated by the pup retrieval assay, which measures active and goal-directed maternal behaviour [138] (Table 5 – Summary of observations). On the first day, pregnant mice on a HFD had a higher latency to retrieve each pup. In fact, majority of dams on the HFD were

unsuccessful at retrieving all four pups within the first 6 minutes of the assay. This is consistent with a study in which dams on a HFD had increased latency to sniff the first pup and displayed disorganized behaviour, which is characterized by increased self-grooming and decreased pup-directed behaviour [49]. In the current study, HFD pregnant dams often attempted to climb out of the cage, instead of retrieving the pups during the test. However, on the consecutive days, the latency to retrieve all four pups was significantly improved in the HFD pregnant group. The ability of the dams to retrieve pups on the second and third day of the assay implies that the HFD did not impair olfaction (ability to smell) or mouthing (ability to hold the pup with the mouth) in these mice [139]. If the mice did have an oral motor or olfactory deficit, they would fail to retrieve all four pups on the second and third day of testing.

The findings in the CD-1[WT] mice on the HFD are consistent with HFD-consumption in female rats, which also has a negative effect on retrieval behaviour of the dam, as well as the ability of their pups to produce appropriate ultrasonic vocalizations [140]. Specifically, dams on HFD retrieved fewer pups, and the pups produced fewer and shorter ultrasonic vocalizations, which may in turn influence the dam's retrieval response [140]. Ultrasonic vocalizations may be important for maintaining blood flow in the pups during colder temperatures [141], as well as provoking maternal care in the dam when the pups are in distress [142,143]. Hence, reduced vocalizations by pups on the first day of the retrieval test is one possible explanation for the impaired retrieval behaviour in our CD-1[WT] mice on the HFD at PD 3. Similarly, improvement in retrieval behaviour at PD 4 and PD 5 in our CD-1[WT] mice on the HFD could also be due to the improved ultrasonic vocalizations by the pups. Although the focus of our study was on the behaviour of the dam, the ability of the pups to influence maternal response cannot be ruled out and requires further investigation.

Pup retrieval behaviour was also assessed in CD-1[171hGH/CS] mice to investigate a possible influence of the hGH/CS transgene on maternal behaviour outcome after parturition. In contrast to CD-1[WT] mice, no difference was noted between the pregnant mice on HFD or RCD on the first, second or third day of the test. In fact, dams on HFD performed just as well as dams on RCD, with relatively similar retrieval latencies and completion rates. Thus, one possible reason for this outcome is that the hPL expressed in the CD-1[hGH/CS] mice may be contributing to priming the brain more efficiently, directly or indirectly, in pregnancy to express maternal care at parturition. This would assume that hPL is expressed in the mouse placenta during pregnancy and is able to enter the maternal circulation, cross the blood brain barrier and affect brain physiology. There is evidence in support of PL crossing the blood brain barrier. In human pregnancy, hPL is present in the cerebrospinal fluid at high concentrations [144], and in rats, PL II is also detected in the cerebrospinal fluid close to the end of pregnancy [145]. In addition, hPL can bind lactogenic receptors in the brain located in the choroid plexus [146] or ependymal cells [21]. Previously, hPL administration directly into the medial preoptic area of the brain in nulliparous rats, where prolactin was inhibited by bromocriptine, was shown to stimulate maternal behaviour, such that the rats began to foster young pups [146]. It is important to note that hPL was able to bind and act through the rat lactogenic receptors, suggesting the possibility, that in the present study with CD-1[171hGH/CS] mice, the endogenous hPL is capable of doing the same. It is also possible that prolactin and/or perhaps levels of one or more other mouse PLs is negatively affected by HFD but the presence of the hGH/CS transgene and specifically expressed hPL is sufficient to rescue this deficit in terms of the behavioural response. Previously, low levels of prolactin in early pregnancy have been implicated in impaired pup retrieval behaviour and increased anxiety in the postpartum period in mice [36]. HFD-induced obesity leads to a prolactin resistant state in C57BL/6 female mice, resulting in reduced maternal behaviour postpartum [48]. Specifically, they have increased

latency to retrieve and group pups during the pup retrieval assessment [48]. Thus, while an ~30% decrease in hPL is expected in CD-1[hGH/CS] mice on a HFD [115], it is possible that the remaining ~70% hPL production is sufficient to compensate for the negative effect induced by HFD on the pup retrieval test.

Nursing behaviour or lactation is also a crucial aspect of maternal care. At parturition, there is a rapid decrease or change in pregnancy hormone levels, specifically removal of PL and prolactin, which triggers lactation [21]. Prolactin secretion is stimulated again by the suckling stimulus, followed by oxytocin secretion from the pituitary gland for milk letdown [21]. In the CD-1[171hGH/CS] mouse model, no significant effect of HFD-induced obesity was observed on nursing behaviour. Normally, diet-induced obesity can lead to a delay in lactogenesis in mice and impair mammary gland development, thereby decreasing milk production and letdown [50], as well as an early termination of breastfeeding in humans [51]. It is of great interest that such behaviour was not observed in these transgenic mice. This raises the possibility that since the CD-1[171hGH/CS] mice produce both hPL and hGH, these hormones mute any negative effect of HFD on lactation, potentially working with endogenous mouse prolactin, PL, and GH in pregnancy to result in sufficient milk secretion in postpartum mice on HFD [147], and is discussed later.

A limitation of this assay is that it was difficult to exclusively determine nursing behaviour alone. Instead, a range of behaviours (as described in Chapter 2, section 2.4) were categorized into maternal behaviours (contact rest which included nursing, anogenital licking, body licking, nest building or physical contact with the pups) and non-maternal behaviours (non-contact rest which included self-grooming, eating, or drinking) [31]. Thus, for nursing, it was difficult to determine from the recording if the dam was engaged in arched-back nursing, blanket nursing, or supine nursing [31]. It is important to distinguish between the different nursing postures because arched-back nursing and blanket nursing requires the dam to be actively nursing the pups by laying over

them, whereas, supine nursing is more passive, as the pups are nursing while the dam is resting either on her back or side [148]. Also, due to the overlapping time points of the different behavioural tests, nursing behaviour was not pursued in CD-1[WT] mice. Despite this, our CD-1[171hGH/CS] nursing data is supported by the nest building and pup retrieval results. In the CD-1[WT] mice, a negative effect of HFD induced obesity is observed on nest building and pup retrieval, whereas a lack of negative effects of HFD are observed in the CD-1[171hGH/CS] mice.

A key to understanding the relationship between HFD-induced obesity and maternal behavioural outcome is to investigate other mental health factors that can affect this relationship. As such, in both CD-1[WT] and CD-1[171hGH/CS] mouse models, anxiety-like behaviour and exploratory drive were assessed prepartum and postpartum via EPM, which is a well validated test of anxiety-like behaviours [149-151]. As described previously, the first day of the EPM test gives insight into true anxiety-like behaviour, as well as the exploratory drive of the mouse [118,149,150]. This assay relies on natural preference of mice for dark and enclosed spaces, reflected by the closed arms, and avoidance of open, bright and elevated spaces, which is reflected by the open arms of the maze [150,151]. The open and closed arms are connected by a central square-shaped region [151]. It is assumed that less time spent on the open arms and more time spent on the closed arms is suggestive of anxiety [150]. However, interpretation of the time spent on the centre region remains ambiguous [123,129]. Anxiety-like behaviour was assessed in pregnant and non-pregnant CD-1[WT] and CD-1[171hGH/CS] mice either on HFD or RCD. Prepartum observations indicate no significant effect of HFD or pregnancy status on anxiety-like behaviour in the CD-1[WT] and CD-1[171hGH/CS] mice.

Postpartum assessment of anxiety indicated decreased anxiety-like behaviour in the non-pregnant CD-1[WT] mice on a RCD, whereas, increased anxiety-like behaviour was suggested in pregnant CD-1[WT] mice on a RCD. The results presented in Figure 3.3.1 are tabulated and

presented in Table 6. Similarly, both non-pregnant and pregnant CD-1[WT] mice on a HFD exhibited increased anxiety-like behaviour. However, within the pregnant groups, CD-1[WT] mice on the RCD experienced higher anxiety-like behaviour than mice on a HFD. The opposite was observed in the CD-1[171hGH/CS] mice, where non-pregnant mice on RCD or HFD had increased anxiety-like behaviour, and the pregnant CD-1[171hGH/CS] mice on RCD or HFD had significantly reduced anxiety-like behaviour. The results presented in Figure 4.3.1 are presented in Table 7. Moreover, no significant differences were observed amongst the individual CD-1[171hGH/CS] non-pregnant and pregnant groups of mice, suggesting diet alone did not affect anxiety-like behaviour.

Table 6. Percentage of time spent on open arms, closed arms, and centre region of the EPM at PD 20 in CD-1[WT] mice

Time spent on:	Non-pregnant mice on RCD	Pregnant mice on HFD	Pregnant mice on RCD	Pregnant mice on HFD
Open arms	13.2%	10.5%	9%	13.8%
Closed arms	39.5% (significantly lower compared to RCD pregnant)	47.3%	55.4%	39.9% (significantly lower compared to RCD pregnant)
Centre region	47.2%	42.1%	35.5% (significantly lower compared to RCD non-pregnant)	46.2%

Table 7. Percentage of time spent on open arms, closed arms, and centre region of the EPM at PD 20 in CD-1[171hGH/CS] mice

Time spent on:	Non-pregnant mice on RCD	Non-pregnant mice on HFD	Pregnant mice on RCD	Pregnant mice on HFD
Open arms	7.9%	7.9%	11.6%	8.9%
Closed arms	55.9%	52.5%	28.7% (significantly lower compared to RCD non-pregnant)	33.5% (significantly lower compared to HFD non-pregnant)
Centre region	55.5%	52.5%	59.5%	57.5%

The anxiety-induced state of normal pregnancy is a protective mechanism aimed at protecting the pregnancy or litter from dangerous stimuli, such as by exhibiting aggression towards predators [137]. This serves as a possible explanation for the increased anxiety-like behaviour observed in CD-1[WT] pregnant mice on RCD. In addition to this, anxiety was assessed 24 hours post-weaning, and the CD-1[WT] pregnant mice on RCD may have been influenced by the removal of their pups. On the other hand, pregnant CD-1[WT] mice on HFD experienced a reduction in anxiety-like behaviour. The inability of the pregnant CD-1[WT] mice on a HFD to express this innate behavioural response postpartum, as observed on the EPM, in addition to the results from the nest building, and pup retrieval assays, suggests that maternal behaviour is impaired. This is consistent with observations made in ovariectomized rats [152], where ovariectomy increased the risk of anxiety-like behaviour on the EPM in rats fed a RCD, while a consumption of HFD overturned the anxiety-like state [152]. A protective effect of HFD has also been described in male C57BL/6J mice exposed to chronic stress, as evaluated by a series of behavioural tests including the light-dark box test and forced swimming test [153]. Previously, a

HFD regimen was shown to promote exploratory activity of rats and attenuated anxiety-like behaviour [154], which is consistent with our observations. Learning acquisition and memory retention were improved in rats on a HFD, as assessed on the Morris water maze, whereas the reference memory was impaired [154]. It has also been suggested that foods that are rich in fat and carbohydrates, can help improve mood, decrease stress and/or anxiety, and stimulate exploratory activity and learning [153-155]. Although unclear, this provides some support for the decrease in anxiety-like behaviour observed in the pregnant CD-1[WT] mice on the HFD. Furthermore, serotonin, which is a crucial hormone for stabilizing mood, can be affected by HFD, and its dysregulation can lead to mood disorders [94,154]. Serotonin action in the brain regions, hypothalamus and hippocampus, can lead to weight gain that is induced by a HFD, alleviate anxiety and regulate cognitive function [154]. Although not assessed, it is possible that HFD in the CD-1[WT] has negatively affected serotonin function.

It is interesting that CD-1[171hGH/CS] pregnant mice on HFD, although not significant, experienced an increase in anxiety-like behaviour when compared to mice on RCD. This could be due to the priming effects of hPL in pregnancy, which might have prevented the anxiolytic effects of the interaction between HFD and pregnancy-related status, as observed in the CD-1[WT] mice. In humans, low serum concentrations of hPL at term are associated with maternal anxiety and depression [156]. Therefore, reduced lactogenic signalling in pregnancy can increase the risk of perinatal mood disorders [156]. Hence, the CD-1[171hGH/CS] mice may have higher levels of PL available to counter a negative effect of HFD on anxiety levels as a result of the presence of hPL in addition to mouse PLs.

The differences in anxiety levels prepartum and postpartum could be a consequence of the duration of the HFD. A bi-directional effect of HFD on anxiety has been described, where a HFD of five weeks decreased anxiety levels, but a HFD of 15 weeks increased anxiety in male and

female mice [157]. Although the effects of HFD in non-pregnant mice has been studied, the effect in pregnant and postpartum mice is unclear. Similar to our findings, no changes in anxiety levels, as assessed by EPM, were found in pregnant C57BL/6J female mice fed either HFD or control diet at GD 16 [49].

The total distance travelled, which represents the locomotion or the ability of the mouse to move, was also assessed on the EPM test prepartum and postpartum. The locomotor ability of the mice can be affected by weight and pregnancy status. In the prepartum period at GD 15.5, pregnancy status significantly decreased distance travelled by the CD-1[WT] RCD pregnant mice. However, non-pregnant and pregnant CD-1[WT] mice on HFD travelled almost similar distance during the test, implying that the total distance travelled may also indicate the exploratory drive of these mice. Moreover, CD-1[WT] pregnant mice on RCD presented a desire to spend the majority of their time on the closed arms when compared to the non-pregnant CD-1[WT] RCD mice. This suggests that pregnancy state may be reducing the exploratory drive of these mice. Whether this serves as a means of protecting the unborn litter is unclear. Conversely, the pregnant CD-1[WT] mice on HFD spent less time on the closed arm at GD 15.5, suggesting that this normal maternal drive is reduced. Factors such as dominance status, hunger and breeding can affect exploratory drive, which in turn can affect locomotor activity of mice in behavioural tests [158]. Although the role of these factors in our mouse model may be negligible, the possibility of pregnancy being a protective state that affects exploratory drive in the prepartum period remains.

The differences in distance travelled detected in CD-1[WT] mice were not observed in the CD-1[171hGH/CS] mice prepartum, regardless of diet and pregnancy status. Again, this can be attributed to the presence of hPL which may be muting the negative effect of HFD in pregnancy, either directly or indirectly, and allowing for exploratory drive to remain intact.

The results of the postpartum EPM test also revealed that non-pregnant CD-1[WT] mice

on HFD travel significantly less distance, when compared to the non-pregnant CD-1[WT] mice on RCD. It is possible that a HFD regimen of approximately 9-10 weeks led to increased weight gain, which in turn has decreased the locomotor ability of these mice. However, the pregnant CD-1[WT] mice travel a similar distance during the test. This further supports the notion that exploratory drive may be affected, and possibly muted, by pregnancy status at GD 15.5. These effects of pregnancy status are not observed in the postpartum period at PD 20. Similarly, all CD-1[171hGH/CS] mouse groups travel similar distance at PD 20. Here, the negative effects of HFD on exploratory drive are not observed in the postpartum period.

HFD consumption has been associated with many health problems, including impaired cognitive function, which can result in neurodegenerative diseases [111,159]. Cognitive function involves a broad range of mental processes, such as attention, learning and memory. In rodents, there are many behavioural tests that can be used to investigate learning and memory [159]. Here, the NOR test was implemented to study working memory, which is also referred to as recognition memory. In this task, the mouse needs to recognize and have an intact memory of the object that was encountered previously [160]. This test does not require external motivation and relies solely on the innate exploratory drive of the mouse [161]. In both the CD-1[WT] and CD-1[171hGH/CS] mice, the preference index validated the data by demonstrating equal exploration of both objects, on day 2 of the test, by all groups of mice. As there were no significant differences between the preference index scores, it demonstrated that all mice familiarized themselves with both similar objects during the familiarization phase and had no preference for a particular object or side of the apparatus. The discrimination index (DI) provides a measure of whether mice were able to discriminate between the novel and familiar object. As mice are naturally curious animals, it is assumed that they will spend more time exploring a novel object, compared with an object that is familiar to them [81]. Based on studies reported by others, the test phase has lasted anywhere

between 3 to 15 minutes, with 3 and 5 minutes being the most commonly used [81]. In the study reported here, in addition to 5 minutes, a determination of whether 1-minute of object exploration was a more rigorous time for mice to discriminate between novel and familiar objects and provide insight into cognitive function was made.

In the CD-1[WT] mice, there were no significant findings in the first minute, although, non-pregnant mice on the RCD spent more time exploring the novel object and the non-pregnant mice fed HFD spent a similar amount of time with the novel and familiar object. The lack of preference for novel or familiar object in the HFD non-pregnant CD-1[WT] mice suggests that immediate memory recall may be compromised causing both objects to appear similarly novel. Similarly, non-pregnant CD-1[171hGH/CS] mice on RCD spent significantly more time exploring the novel object, compared to HFD non-pregnant mice. The CD-1[WT] pregnant mice on RCD spent more time with the familiar object. This implies a possible role of the protective effects of pregnancy continuing into the postpartum period and may dominate the naturally curious behaviour seen in the non-pregnant mice on RCD. This could be a consequence of the physiological changes in the brain during pregnancy, that allows the dam to evolve from indulging in self-directed behaviour to caring for the needs of the newborn pups [162]. Taking this into account, one possibility is that the dam perceives the novel object as a potential threat and refrains from exploring it. This can be related to our findings on the EPM in the postpartum period, where the pregnant mice fed a RCD spent more time on the closed arms, seemingly reflecting a site of greater safety by the dam [162]. In contrast, CD-1[WT] pregnant mice on HFD displayed a trend towards more time spent with the novel object, raising the possibility that normal maternal behaviour is modified by the HFD. Specifically, the HFD appears to be associated with a reduction in anxiety-like-behaviour during pregnancy and protective behaviour postpartum. In the CD-1[171hGH/CS] mice, pregnant mice, regardless of diet, spent more time with the familiar object.

When memory recall was assessed at the 5-minute mark in the NOR test, non-pregnant CD-1[WT] mice on HFD spent more time exploring the familiar object, while the pregnant mice on HFD were unable to differentiate between the familiar and novel object. This suggests disinterest or inability to declare a preference for the novel or familiar object as a result of cognitive dysfunction in the pregnant CD-1[WT] mice on HFD. One possibility for the differences observed in the non-pregnant and pregnant CD-1[WT] mice on HFD is their anxiety levels. Based on the data from the EPM test at PD 20, HFD induced anxiety-like behaviour in non-pregnant mice but decreased it in pregnant mice (based on the amount of time that was spent on the closed arms). Hence, the state of anxiety induced by HFD in the non-pregnant CD-1[WT] mice might be overridden by the interaction between HFD and pregnancy state in the pregnant mice.

At the 5-minute mark, all CD-1[171hGH/CS] mice spent more time exploring the familiar object. Hence, both diet and pregnancy-status appear to have similar effects on memory recall in these mice, although, pregnancy experience results in a slightly higher preference for the familiar object. It has been noted that the effects of HFD on learning and memory are affected by many different factors including animal strain, age, duration and type of diet, and behavioural test used to assess cognition [159]. Hence, this has resulted in a mixture of studies with varying results regarding the relationship between consumption of HFD and cognitive outcome [159]. In a study with female APP/PS1 transgenic mice, a commonly used Alzheimer disease animal model, HFD (45% calories from fat) did not have a detrimental effect on memory/cognition, when compared to mice fed a control diet [163]. Here, the presence of hGH/CS transgene in CD-1[171hGH/CS] mice may modify behaviour outcome in the NOR test, although the mechanism(s) underlying this are unclear. Studies in male Sprague Dawley rats fed a high-fat (38% calories from fat) and high refined sugar diet found that memory was impaired on the NOR test [164], whereas no effect on memory impairment was seen in male Long-Evans rats after being on a HFD (60% calories from

fat) for 12 weeks [165]. Unlike other cognitive behavioural tests, the relationship between weight gain due to diet and performance on the NOR test, does not appear to be strongly correlated [159]. Alternatively, the impairment in working memory in postpartum CD-1[171hGH/CS] mice may be influenced by a slightly greater degree of insulin resistance in these mice, due to the presence of hGH [166]. Although glucose clearance was not tested in the postpartum period, the endogenous hGH, in addition to mouse GH in the CD-1[171hGH/CS] mice may lead to a greater insulin resistance state. Poor control of glucose has been associated with inability to perform specific tasks, especially those that involve learning and memory [167]. However, levels of serum hGH levels are relatively low compared mouse GH in CD-1[171hGH/CS] mice [133] and its functional relevance remains unclear.

One of the most important brain regions involved in learning and memory is the hippocampus [81] (Figure 1.6). This structure is a crucial component of the limbic system, which regulates motivation, memory, reward and emotion [168]. Appropriate hippocampal function is critical for acquisition of the familiar object and the experience associated with it, in order to recall it on the last day of the test [81]. Hence, impaired hippocampal function may lead to deficits in memory and lead to poor behavioural outcome on the NOR test in CD-1 mice. Rodent studies specify the importance of the parahippocampal regions, which are cortical regions surrounding the hippocampus [76]. This includes the perirhinal, entorhinal and inferior temporal cortices, which play an important role in object recognition memory [76] (Figure 1.6) . The perirhinal cortex has been explicitly implicated in recognition memory formation for short term [169], and is essential for coding information that is important for object discrimination [81]. The function of the hippocampus, however, is critical for long-term object recognition [82]. Hence, lesions to the perirhinal cortex or the hippocampus can affect behaviour on the NOR.

Several studies have also suggested that there is pregnancy-related neuroplasticity occurs by way of neurogenesis [36,170,171]. Brain-derived neurotrophic factor (BDNF) plays an important role in neuronal plasticity by assisting with the growth, maturation and maintenance/survival of new neurons, and has been implicated in both learning and memory [172]. A HFD and high-sucrose diet has been shown to decrease hippocampal BDNF levels, resulting in decreased neuronal plasticity and impaired learning ability in non-pregnant rats [172]. Furthermore, although the mechanism underlying pregnancy-induced neurogenesis remains unclear, BDNF may play a crucial role in the vitality and function of new neurons, presumably in the hippocampus. Consistent with our observations, a reduction in hippocampal-dependent memory was observed in rodents when the HFD was started at four weeks of age [167,173]. Thus, it is possible that HFD-induced obesity in our CD-1 mice may be resulting in a similar decrease in hippocampal BDNF, thereby affecting object recognition memory. This can also be translated to humans, where a HFD has also been shown to decrease hippocampus volume, impair memory and cognitive function, which in turn increases their risk for developing anxiety and depression [15].

Pre-pregnancy obesity in women has also been associated with a 33% increased risk of developing depressive symptoms in pregnancy [10]. These symptoms include changes in appetite, trouble sleeping, lack of energy, feelings of worthlessness or hopelessness, and anhedonia (loss of interest or pleasure in activities previously considered pleasurable) [14]. If left untreated, this can result in postpartum depression, which has negative implications for both the mother and the baby [14].

Due to the heterogenous nature of depression, it is difficult to diagnose and study in humans. Hence, rodent models have been established to study specific symptoms. To assess hopelessness or behavioural despair in mice, a forced swimming test and tail suspension test are often used [96], while anhedonia is often assessed by sucrose preference test and intracranial self-stimulation [96].

Anhedonia is an endophenotype of depression that can be modelled and measured in laboratory animals in a reliable manner [96]. In the present study, anhedonia was assessed in postpartum CD-1[WT] mice, pre-weaning and post-weaning, using a sucrose preference test, as it was considered to be the least invasive. Mice were given a choice to consume either sucrose or tap water, with the assumption that mice would normally prefer the more pleasurable experience of drinking sucrose water. However, if a mouse is experiencing anhedonia, it will show reduced interest in the sucrose water, resulting in lower preference. Although this was not observed pre-weaning at PD 6-8 in the pregnant CD-1[WT] mice on a HFD, pregnant mice on a HFD had approximately 23% lower mean preference for sucrose water, when compared to the pregnant RCD mice. One possible explanation is that during PD 6-8, dams are engaged in nursing the pups which are completely dependent on the mother for their nutritional needs, and hence spend the majority of their time in the nest [29]. However, by PD 12-15, nursing demand decreases as the pups are further developed and beginning to eat and drink on their own, allowing the dam to move around freely [29]. This suggests that although the dam was able to leave the nest site, the dam had a reduced preference for sucrose water, which would normally be a pleasurable experience. Hence, the ability of the pregnant dam on HFD to feel pleasure from previously pleasurable activities may not have been observed at PD 6-8 but was diminished or lost at PD 12-15. In a previous study, a HFD for six weeks in mice resulted in sucrose and hypothalamic pituitary adrenal axis hypersensitivity to stress [174]. This is consistent with rodent models of HFD, in which increased corticosterone levels and inflammation have been noted when rodents were fed a HFD [175,176]. These changes caused by HFD have also been observed in animal models of depression, as well as in humans [177-179]. This includes increased corticosterone levels and neuron atrophy, as well as reduction in hippocampal neurogenesis and neurotrophic factors [177,178]. Due to the similar physiological outcomes

associated with consumption of HFD and depression, it is possible that HFD may play a role in the onset of anhedonia-like-state in the CD-1[WT] mice pre-weaning.

In the post-weaning period, at PD 21-23, the sucrose preference was relatively similar across all groups. A possible explanation is that the dams were ready to have their pups weaned, and hence spent more time engaged in self-directed behaviours (eating, drinking and grooming) outside of the nest, resulting in increased preference for sucrose water across all mouse groups. Normally, at approximately 3-4 weeks of age, pups become nutritionally independent and are ready to be weaned [137]. Although unknown, it is possible that chronic exposure to HFD may lead to attachment problems in the dams with their pups. Therefore, when the pups are removed, the anhedonia-like state is reversed, and in turn, increases sucrose preference. Anhedonic behaviour has also been associated with dysfunction in the mesolimbic dopamine system, which plays an important role in perception of reward [96]. Similarly, HFD-induced obesity has been shown to promote anxiety- and depressive-like behaviours in mice, and decrease tyrosine hydroxylase, a rate-limiting enzyme that is crucial for the synthesis of dopamine [180]. Dopamine plays a role in the neural circuitry that regulates motivation and perception of reward, and HFD induces changes in the reward circuitry leading to the onset of a depressive-like state in rodents [180]. Studies in both human [181,182] and rodent models of obesity [183,184] have indicated a reduction in dopamine signaling in the onset of depression and mood disorders [168]. Thus, although not investigated, it is possible that HFD-induced obesity in the CD-1[WT] mice has impaired dopamine signalling, thereby inducing an anhedonic state pre-weaning.

One possible biomarker of psychiatric disorders, including depression, is BDNF [112]. As described earlier, BDNF is a neurotrophic factor involved in the survival, differentiation and maturation of new neurons, as well as regulation of excitatory and inhibitory synaptic transmission in the brain [117]. Low levels of serum BDNF have been associated with greater risk of developing

major depression [112]. Similarly, lower serum levels of BDNF in the third trimester of pregnant women has been linked with higher risk for depressive symptoms postpartum [107]. Based on this, relative BDNF RNA levels in the brain was investigated in the CD-1[WT] mice at GD 6, 10.5 and 18.5. No significant differences were observed between the pregnant and non-pregnant mice on the RCD or HFD. BDNF protein levels were also assessed in the brain of CD-1[WT] mice at GD 18.5 and PD 30 by ELISA. It was hypothesized that a HFD would be associated with a reduction in BDNF levels in the CD-1 (WT and 171hGH/CS) mice. However, BDNF levels were not significantly different at GD 18.5 and by PD 30 CD-1[WT] mice on the HFD had significantly higher BDNF levels, when compared to the mice on the RCD. As the response to the HFD appeared alike in both pregnant and non-pregnant mice, the ratio of the BDNF levels in the HFD to RCD group was compared at PD 30 and found to be relatively similar in the non-pregnant (1.09) and pregnant (1.19) mice. Hence, the increase in BDNF levels is presumably attributable to the HFD and not the pregnancy-related experience in the CD-1[WT] mice postpartum. Furthermore, the increase in BDNF levels in CD-1[WT] mice on the HFD is associated with impaired maternal behaviour postpartum (for example, pup retrieval) and thus may have played a role. Therefore, this observation of a change in BDNF values (if not a decrease) does support the original hypothesis at least in part. It is important to note that the BDNF levels were not assessed earlier in pregnancy by ELISA in the CD-1[WT] mice. However, protein immunoblotting data suggests that neither proBDNF nor mBDNF levels in total brain were significantly affected by HFD in pregnancy and thus associated with changes in maternal behaviour, including impaired nest building. This does not rule out, however, effects of diet and BDNF in specific regions of the brain on maternal CD-1 mouse behaviour.

Similarly, BDNF protein levels were assessed in the brain of CD-1[171hGH/CS] mice at GD -1, GD 18.5 and PD 26 by ELISA. Although no significant effect of diet was observed at GD

-1, pregnant CD-1[171hGH/CS] mice on the HFD had significantly lower levels of BDNF at GD 18.5, as well as age-matched non-pregnant mice on HFD, when compared to mice fed a RCD. In pregnant women, serum BDNF levels have been found to decrease from the first to the third trimester and increase in the postpartum period [107]. Here, BDNF levels were only investigated in late pregnancy, and hence it is unclear if BDNF levels changed from GD 0.5 to GD 18.5. This pattern of decreased BDNF levels at GD 18.5 in the CD-1[171hGH/CS] mice fed a HFD *versus* a RCD continued into the postpartum period, as assessed at PD 26. Based on the findings in the CD-1[WT] mice, the ratio of BDNF levels in HFD to RCD mice was investigated in the CD-1[171hGH/CS] mice. In contrast to the CD-1[WT] mice, the CD-1[171hGH/CS] non-pregnant (0.71) and pregnant (0.87) mice had lower ratio of BDNF in mice fed HFD to RCD at GD 18.5. The relatively lower ratios observed at GD 18.5 were also seen at PD 26 in the non-pregnant (0.98) and pregnant (0.86) CD-1[171hGH/CS] mice. This, together with the two-way ANOVA analysis of postpartum serum data (Figure 4.4 C), suggests that the changes in BDNF are largely due to the type of diet, and not pregnancy-related experience in these mice. However, unlike the CD-1[WT] mice, the negative effects of HFD on maternal behaviour outcome were not observed in the CD-1[171hGH/CS] mice, and this was associated with a decrease in BDNF levels in pregnancy and postpartum. This further supports a role for a negative effect of increased brain BDNF levels as a result of a HFD on postpartum maternal behaviour as seen in the case of pup retrieval in CD-1[WT] mice. These observations do not rule out the possibility that the decrease in BDNF levels in CD-1[171hGH/CS] mice on a HFD still has behavioural consequences. Thus, the association between the presence of the transgene in the CD-1[171hGH/CS] mice and the ability to compensate for the negative effects of a HFD may include influencing BDNF levels.

Total brain BDNF of the CD-1[WT] and CD-1[171hGH/CS] mice were assessed using a BDNF specific ELISA, which did not distinguish between the proBDNF and mBDNF. This is a

limitation of the assay since proBDNF and mBDNF have opposing effects in the brain [101]. ProBDNF binds to the p75 receptor and sortilin, and plays a role in neuronal apoptosis and inhibits neurogenesis [101,185]. Conversely, mBDNF binds to the TrkB receptor and assists with neuron vitality and plasticity [101]. Together, both proBDNF and mBDNF play important roles in the onset of mood disorders [185]. The protein (western) immunoblot data, provided through collaboration, allows differentiation between proBDNF and mBDNF and this was assessed in pregnant CD-1[WT] mice at GD 6, 10.5 and 18.5. No significant difference was observed in the relative levels of proBDNF and mBDNF in pregnant mice on RCD or HFD across pregnancy. Furthermore, a trend of decreasing mBDNF levels was observed across pregnancy (from GD 6 to GD 18.5) for the mice on the RCD. However, the mice on the HFD not only had higher relative mBDNF levels at GD 6 than the mice on the RCD, but they also had a significant reduction on mBDNF levels from GD 6 to GD 10.5. This suggests that the HFD in early pregnancy led to a greater increase in mBDNF levels by GD 6, and this was associated with a greater decrease in mBDNF levels by GD 10.5.

The decreasing trend observed in the CD-1[WT] mice on the RCD is consistent with what is seen in humans [107]. Studies have shown that proBDNF levels are increased in the hippocampus of rats exhibiting anxiety and depression-like behaviour, while its downregulated in the nucleus accumbens of the basal ganglia of forebrain in rats exhibiting learned helplessness (phenotype of depression) [101]. Therefore, proBDNF levels may be differentially expressed in different brain regions in models of depression [101]. This helps explain why no significant differences were observed between whole brain BDNF levels of mice on RCD or HFD. Additionally, research has shown that HFD-induced obesity in mice impairs hippocampal neurogenesis, and leads to a decrease in mBDNF in the hippocampus [111]. This disruption in normal hippocampal physiology can also interfere with memory and learning. Thus, an

investigation of the independent levels of proBDNF and mBDNF in the hippocampus of pregnant mice with HFD-induced obesity is warranted.

The goal of this study was to model the human condition in a mouse system to further our understanding of the effect of a high-fat diet before, during and after pregnancy, on mental health disorders. As the investigation was in a mouse model, tests that provide insight into mouse behaviour were used, which could be translated to phenotypes associated with mental health disorders in humans were used. Here, both CD-1 (WT and 171hGH/CS) models present different behavioural outcomes in response to overeating/HFD-induced obesity prepartum and postpartum. Although both CD-1[WT] and CD-1[171hGH/CS] mice on HFD exhibit similar physiological characteristics such as significant weight gain, impaired glucose clearance and similar litter sizes, they have contrasting behavioural outcomes. CD-1[WT] mice on HFD display a decrease in maternal behaviour, reduced anxiety-like behaviour, and impaired working memory in pregnant mice postpartum, as well as an onset of anhedonia-like state pre-weaning. Conversely, the CD-1[171hGH/CS] mice on HFD express normal maternal response, anxiety-like behaviour and working memory, which is similar to mice on RCD. Thus, the absence of negative effects of HFD observed in the CD-1[171hGH/CS] but not the CD-1[WT] mice, are most likely attributable to the presence of hGH/CS transgene (for example, site of integration) and/or direct and indirect effects of transgene expression.

The hGH/CS transgene in the CD-1[171hGH/CS] mice contains genes for the pituitary GH (*hGH-N*/*GH1*), placental GH (*hGH-V*/*GH2*), hPL/CS (*hCS-A*/*CSH1*) and *hCS-B*/*CSH2*) and a pseudogene (*hCS-L*/*CSHL1*) [25]. As mentioned in Section 1.9, the hGH/CS transgene is located on mouse chromosome 14 at band 14AI (Figure 1.9) [113]. The genes, *hCS-A* and *hCS-B,* each independently code for hPL and are expressed by trophoblastic cells in the mouse placental labyrinth in pregnancy [114]. In CD-1[171hGH/CS] mice, hCS-A RNA expression has been

detected as early as GD 11.5 in the placenta, and encompasses approximately 90% of all hCS RNA expression in pregnancy [114]. In addition, hPL is detected in the placental labyrinth at GD 18.5 by immunohistochemistry [114] and is consistent with potential release of hPL into the maternal circulation. Pilot data from Noshin Noorjahan in Dr. Cattini's laboratory (University of Manitoba) has confirmed the presence of hPL in the maternal circulation of pregnant CD-1[171hGH/CS] by ELISA (Noorjahan and Cattini, unpublished observation).

The integration of the transgene on mouse chromosome 14 in the CD-1[171hGH/CS] mice also presents one possibility for the absence of the negative effect of HFD on maternal behaviour. However, this is unlikely to be the case. The hGH/CS transgene locus is flanked by insulator or boundary elements. This effectively insulates the hGH/CS gene locus such that the pituitary and placental genes are, to all intents and purposes [186]. Thus, the hGH/CS transgene displays copy number dependence in transgenic mice and the transgene RNA products will be proportional to its copy number [187]. In addition, the CD-1[171hGH/CS] mice are backcrossed to the CD-1[WT] mice to reduce genetic drift [113], which is loss of infrequently occurring alleles in small populations [188]. Based on this, it is unlikely that the insertion of the transgene is influencing the lack of negative effects of HFD in the CD-1[171hGH/CS] mice.

A direct or indirect effect of hGH/CS transgene expression on lactogenic signalling is the most likely reason for the absence of a negative effect of HFD on maternal behaviour in the CD-1[17hGH/CS] mice . This includes pituitary GH and placental GH that bind the GH receptor with high affinity and the prolactin receptor with relatively low affinity [24,189]. In contrast, hPL is the most likely candidate, as it binds the prolactin receptor with 10 times higher affinity than prolactin itself, and has a very low affinity and fails to dimerize the GH receptor [24,190]. Moreover, hPL RNA levels are detected as early as GD 11.5 in CD-1[hGH/CS] mouse pregnancy, and hPL RNA levels are more than 10 times greater than for placental GH until term [114]. Thus, the early

detection of hPL RNA at GD 11.5 is consistent with a potential positive role in supporting nest building behaviour in CD-1[hGH/CS] *versus* CD-1[WT] mice at GD 16.5. Although not tested, it is possible that hPL may be present in the placenta prior to GD 11.5, thus, playing a role in maternal behaviour outcome from the beginning of pregnancy. In addition, the presence of hPL alongside mouse lactogens may also help prime the dam's brain more efficiently in pregnancy, allowing for initiation of appropriate maternal care at parturition. Moreover, even though it has been previously reported that HFD leads to an approximately 30% decrease in hCS-A RNA levels in the CD-1[171hGH/CS] mice in pregnancy, the majority of production (~70%) would still be present [115]. If so, hPL would be available to influence CD-1[171hGH/CS] mice *versus* CD-1[WT] mouse pregnancy and by extension maternal mouse behaviour. Indeed, despite the previously observed decrease in hCS-A RNA levels, the present study shows that this is not associated with a negative effect on maternal behaviour outcome in the CD-1[171hGH/CD] mice prepartum and postpartum. In fact, it maintains normal maternal behaviour in the CD-1[171hGH/CS] mice even in the presence of negative effects exerted by the consumption of a HFD.

Here, possible mechanisms for the positive effect of hPL on maternal behaviour including the apparent rescue of HFD-induced negative effects in CD-1[171hGH/CS] *versus* CD-1[WT] mice are considered. One possibility is that although the levels of hPL may be reduced in the CD-1[171hGH/CS] mice fed a HFD, they are still sufficient to compensate for deficits in other endogenous lactogens, such as mouse PL(s) during pregnancy. As stated earlier, hPL is present in the maternal circulation of CD-1[171hGH/CS] mice and is assumed to be entering the brain by crossing the blood brain barrier through lactogenic receptors located at the choroid plexus [146] or ependymal cells [21]. In humans and rats, hPL and rat PLII respectively, are detected in the cerebrospinal fluid at high concentrations close to parturition [144,145]. Moreover, the ability of hPL to initiate maternal behaviours has been demonstrated in nulliparous rats [146]. Specifically,

administration of hPL directly into the medial preoptic area of the hypothalamus, where prolactin secretion is inhibited by bromocriptine, initiated a normal maternal response in rats [146]. Hence, it is possible that the hPL in the CD-1[171hGH/CS] mice system is playing a critical role in initiating normal maternal behaviour prepartum and postpartum.

Another possible reason for the effects observed in the CD-1[171hGH/CS] mice could be due to the products of the transgene that may have an indirect effect on other factors or hormones, which in turn, promote normal maternal behaviour. In the CD-1[171hGH/CS] mice, hPL and mouse PLs are produced by the trophoblastic giant cells of the placenta, as suggested by immunohistochemical analysis and *in situ* histohybridization of the mouse placenta [114,191]. Thus, the presence of hPL might result in a stimulation or maintenance of sufficient lactogenic signaling to support maternal behaviour, even in mice on a HFD. Furthermore, human PL is also known to stimulate pancreatic beta cell mass and increase the capacity for insulin production under conditions of increased insulin resistance that occurs during pregnancy [190]. Thus, it is possible that hPL, similarly, stimulates proliferation or survival of PL-producing trophoblasts, or endogenous mouse PL production itself, resulting in sufficient lactogenic signaling to compensate for the negative effects exerted by HFD.

In conclusion, the findings from this study suggest that both mouse models present unique avenues to study the effect of HFD-induced obesity prepartum and postpartum. The CD-1[WT] mice illustrate the human condition more closely in terms of the maternal behaviours. In contrast, the highly related CD-1[171hGH/CS] mice, with the hGH/CS transgene, are able to stimulate appropriate maternal behaviours in response to a HFD. Specifically, HFD-induced obesity had a negative effect on maternal behaviour in CD-1[WT] but not in CD-1[171hGH/CS] mice. Furthermore, the negative effects of HFD on pup-retrieval as observed in the CD-1[WT] mice, was associated with increased total brain BDNF levels in the postpartum period. However, in the

CD-1[171hGH/CS] mice, this increase in BDNF levels was not observed, along with a negative effect of HFD on pup-retrieval. Together, they provide mouse models to further investigate a possible role for hPL in mediating a normal maternal response, as well as attenuating the negative effects induced by HFD. Thus, in addition to hPL serving as a potential marker for maternal mental health disorders during the prepartum and postpartum period, it may be possible to understand the mechanism by which (if any) hPL may affect the onset of mental health disorders, including depression.

CHAPTER 6

Future Directions

The experimental studies conducted in this thesis provide an understanding into the effect of HFD-induced obesity on maternal behaviour, anxiety-like behaviour, working memory and anhedonia in CD-1[WT] and CD-1[171hGH/CS] mice prepartum and postpartum. In addition, this thesis also provides novel insight into whole brain homogenate brain-derived neurotrophic factor (BDNF) levels in mice during pregnancy and postpartum. These findings suggest a negative effect of HFD in CD-1[WT] mice but not in the CD-1[171hGH/CS] mice. This suggests a beneficial effect of the products of the transgene, however, the mechanisms underlying these outcomes remains unclear. Hence, although a number of studies can be pursued, a few require preliminary investigation and are discussed below.

6.1 *Hormonal profile of mouse PLs and hPL in CD-1[171hGH/CS] mice*

While it is acknowledged that other hormones play a role in determining maternal behaviour, including steroid hormones, a role of lactogenic signaling was supported in this study. Specifically, CD-1[171hGH/CS] mice were used to investigate the effect of endogenous hPL on maternal behaviour in a HFD-induced obesity mouse system. Indeed, a positive effect of hPL was observed in mice on a HFD in the prepartum period on nest building, and postpartum on pup retrieval and nursing behaviour. However, the extent of production and secretion of hPL in the maternal circulation in these mice was unclear. Hence, hPL specific ELISA was implemented to determine the presence of hPL in serum by Noshin Noorjahan in Dr. Cattini's laboratory (University of Manitoba). This helped confirm the presence of hPL in the maternal circulation of

pregnant CD-1[171hGH/CS] (Noorjahan and Cattini, unpublished observation) at GD 10.5 and 18.5. However, the relative concentrations of mouse PLI and PLII in the CD-1[171hGH/CS] mice during pregnancy remains unknown. To gain a better understanding of the lactogen profile of the CD-1[171hGH/CS] mice, specific ELISAs to assess mouse PL(s) and determine the relative concentrations of PLI and PLII at GD 6, 10.5 and 18.5 in serum would be investigated.

Furthermore, it is unknown whether hPL in the CD-1[171hGH/CS] mice is able to enter the brain via cerebrospinal fluid. Hence, the ability for hPL protein, in addition to mouse PLs, to enter the cerebrospinal fluid in pregnancy at GD 6, 10.5 and 18.5, would be examined. This can be tested by collecting cerebrospinal fluid, which has been done previously in mice [192]. The concentration of the individual hormones can then be detected by a hormone-specific ELISA, as done previously for ghrelin detection in mice [193]. This will help determine the possible route of entry of hPL into the brain, and whether it crosses the blood-brain barrier.

6.2 *Competitive binding assay for prolactin receptor*

It is hypothesized that the maintenance of normal maternal behaviour in the CD-1[171hGH/CS] mice in the presence of negative effects induced by HFD, may be due to the hPL in these mice. Whether the effects are actually due to the presence of the endogenous hPL in this mouse system was not investigated in this study. Hence, this is one limitation of this thesis, and addressing this can further our understanding of how hPL may be directly or indirectly involved in maternal behaviour outcome in the CD-1[171hGH/CS] mice.

One possible way of determining a direct beneficial effect of hPL on maternal behaviour in a pregnant CD-1[WT] mouse on the HFD, is by administering hPL through a minipump during mouse pregnancy, as done previously in Sprague-Dawley rats [194,195]. CD-1[WT] mice on a HFD and treated with hPL in pregnancy are expected to show improvements in maternal behaviour

relative to CD-1[WT] mice on the HFD alone. Furthermore, these mice may perform as well as CD-1[WT] mice on a RCD and CD-1[171hGH/CS] mice on a HFD. The minipump delivering hPL would be surgically implanted into the subcutaneous tissue in the back of the neck in the first week of pregnancy [194,195]. The minipump, which will be active until term, will be loaded with a range of hPL concentrations based on the literature and taking into account average mouse body weight in pregnancy [195], to determine and deliver an appropriate daily dose. This can be followed by nest building assessment in pregnancy, and nursing and pup retrieval behaviour postpartum.

Furthermore, it is known that hPL and prolactin both bind and signal via the prolactin receptor in pregnancy [33]. However, in human pregnancy PL has ten times greater affinity for the prolactin receptor than prolactin itself [33]. In the CD-1[171hGH/CS], it is unclear which lactogenic hormones, whether it's prolactin, hPL or mouse PL(s), are driving maternal behaviour outcome. Additionally, the affinity of prolactin, hPL and mouse PL(s) for the prolactin receptor in these mice is unknown. It is known that hPL in rats is able to bind to and signal from the rat prolactin receptor in the brain [146], suggesting the possibility that hPL in CD-1[171hGH/CS] mice is capable of doing the same. Hence, a prolactin receptor competitive-binding assay can be used to test the affinity of each ligand (hPL, prolactin and mouse PLs(s)) to prolactin receptor, as done previously for the estrogen receptor [196]. This will provide insight into the lactogen that's driving maternal behaviour during pregnancy in the CD-1[171hGH/CS] mice.

6.3 *Levels of proBDNF versus mBDNF in the hippocampus*

In this study, total BDNF levels were assessed in whole brain homogenate to acquire a general understanding of the levels during pregnancy and in the postpartum period, and whether these levels are affected by HFD-induced obesity. As discussed previously, BDNF levels are

differentially expressed in the brain [101]. BDNF expression is high in the hippocampus, a region that plays an important role in learning and memory, as well as symptoms related to mood disorder, such as depression [101]. In addition, BDNF is expressed in different forms, which includes proBDNF and mBDNF [100]. Both of these play opposing roles in the brain [185]. A follow-up investigation using protein (western) immunoblot analysis was initiated, in collaboration with the laboratory of Dr. E. Kardami (Human Anatomy & Cell Science, University of Manitoba/Institute of Cardiovascular Sciences, St Boniface Hospital Albrechtsen Research Centre), to differentiate between proBDNF and mBDNF in whole brain tissue during pregnancy.

Pilot total brain protein immunoblot data was provided for inclusion in this thesis. However, the hippocampus appears to be the most important brain region in which BDNF expression is the highest and most involved in learning, memory and depression-related symptoms. Hence, to gain a better understanding of proBDNF *versus* mBDNF levels in the hippocampus, a hippocampal homogenate would be generated as described by others to assess BDNF levels [111]. In brief, mouse hippocampus would be isolated following brain dissection, homogenized and assessed by protein (western) immunoblot analysis to determine the relative levels and ratio of mBDNF to proBDNF [197]. A high ratio of mBDNF to proBDNF has previously been associated with major depression disorder in humans [197]. However, the ratio of mBDNF to proBDNF remains unclear in mouse models of HFD-induced obesity in pregnancy.

CHAPTER 7

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